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Spore germination-enzyme inhibition assay for rapid detection of Pesticide residue in milk

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Research

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ABSTRACT

In current investigation an attempt was made to develop a bacterial enzyme inhibition-based assay for rapid detection of pesticides. In this regard different enzymes of *Bacillus megaterium* strain were assessed for their potential for biosensor development for pesticide detection. Among the targeted ten enzymes, eight enzymes namely ß-glucosidase, α-glucosidase, α-galactosidase, α-amylase, protease, alkaline phosphatase, peroxidase, and esterase were found expressed in used strain, however, expression time/enzyme activity was found varied among different enzymes. All expressed enzymes were screened for their activity inhibition by twenty-four pesticides of different groups using a microtiter plate assay. The inhibition of β-D-glucosidase, α-D-glucosidase, α-D-galactosidase, protease, peroxidase, and esterase was observed at pesticide concentrations of 200 ppm, 100 ppb, 10 ppb, 100 ppm, 100 ppm, and 10 ppb respectively for different pesticides. It was found that there is not a single pesticide that can be used as a model pesticide for the development of enzymatic inhibitions-based biosensors for another pesticide. The esterase was selected for further its inhibition potential due to its better reaction time i.e. 15 minutes. With a further optimized protocol, the esterase enzyme showed the inhibition at 1 ppb concentration of fenitrothion, monocrotophos, tetrachlorovinphos, paraoxon methyl, amisulbrom, ametoctradin, carbendazim, maneb, zineb, and asulam. The optimized enzyme inhibition assay offered an excellent sensitivity (limit of detection) of 0.1 ppb for captan pesticide.

Keywords: enzymes inhibition, pesticides detection, chromogenic assay

1. INTRODUCTION

Pesticides are synthetic or biological agents utilized widely in agricultural production and public and livestock health to prevent or reduce harmful impacts from pests. In modern agriculture, pesticides have significantly increased productivity worldwide but the threat of their toxicity has also increased (Alengebawy et al. 2021; Yang et al. 2020; Ishaq et al. 2018; Korrapati et al. 2018). Detection methods for pesticide contamination need high sensitivity and accuracy because they may be present at trace levels (Mishra et al. 2012). Usually, pesticides detection techniques like Liquid Chromatography-Mass Spectrometry (LC-MS), Gas Chromatography-Mass Spectrometry (GC-MS), or High-Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) (Liu et al. 2012) are used. Though these methods are sensitive, efficient, and reliable, but need costly infrastructure establishment, complicated sample preparation steps, and are time-consuming and laborious. Consequently, there is much demand for the development of cheap, specific, and fast screening methods to allow high throughput analysis.

The inhibition of enzyme activity in the presence of a target analyte is a well-known concept. However, it has fundamentally been employed to identify pesticide residues followed by heavy metals and other inhibitors (Amine et al. 2006). The working principle of interference-based sensors generally exercises the measurement of enzyme activity in the presence and absence of pesticides. The activity of the enzyme decreases in the presence of pesticide residues and this drop in the activity can be further compared with the concentration of pesticides in the sample under analysis. Inhibition of enzyme activity in the presence of pesticide can be determined using the following formula:

$$\mathbf{I\% = (A_o\text{-}A_i/A_o) \times 100} \qquad \textbf{(i)}$$

Where I %- the percent inhibition, A_o- the activity of the enzyme in the absence of pesticide, A_i- the activity of the enzyme in presence of pesticide.

The above formula (i) is used for reversible or irreversible types of enzyme inhibition (Arduini et al., 2010). Different enzymes like esterase, tyrosinase, ascorbate oxidase, alkaline phosphatase, acid phosphatase, peroxidase, acetolactatesynthetase, aldehyde dehydrogenase have been used for chemical contaminants detection (Bravo et al. 2019; Dong et al. 2020; Liang et al. 2013; Marty et al. 1993; Mazzei et al. 1996; Nguyen and Jang 2021; Rekha et al. 2000; Seki et al. 1996; Vidal et al. 2008; Yang et al. 2018).

Bacterial cells are a simple, easy-to-use, and inexpensive source for biosensor development for various analytes (Kylilis et al. 2019, Gaudin V. 2017, Raut et al. 2012). The unique advantages of bacterial cells include the cost-effectiveness compared to pure enzyme preparation, effortless storage, and transport. The current study aimed to develop an enzyme inhibition-based assay for monitoring pesticide residues.

2. MATERIALS AND METHODS

2.1. Materials

All the chemicals such as pesticides, chromogenic substrates, and organic solvents (LCMS grade) were purchased from Sigma Aldrich USA and Sodium hydroxide (NaOH) crystals were purchased from Himedia. Milli-Q water was obtained from a Milli-Q water purification system (Millipore, USA), a multimode plate reader (TECAN Infinite M200 PRO), and a *Bacillus megaterium* strain (IP status: Indian patent reg. no. 3819/DEL/2015).

2.2. Methodology

2.2.1. Revival, Maintenance, and activation of bacterial cells

The freeze-dried form of *Bacillus megaterium* strain (IP status: Indian patent reg. no. 3819/DEL/2015) was transferred in the tube having 5.0 mL of nutrient broth and incubated at 37°C for

24.0±2.0 hours to regain viability. Following incubation, a loopful of revived culture was streaked on a nutrient agar medium and incubated at 37˚C for 16.0±2.0 h. The purity of culture was examined microscopically by Gram and spore staining. The *B. megaterium* culture was maintained as glycerol stocks at -20 °C in the ultra-low deep freezer, until further use. For this, the culture was propagated in 50 mL of nutrient broth at 37 °C for 16±2.0 hours. The grown culture was subjected to centrifugation at 10,000 rpm for 10 minutes at 4 ° C. Following centrifugation, supernatant (media components) was discarded and the cell pellet was taken. Pellet was reconstituted by the addition of 500 microliter (µL) sterile ultrapure water. Glycerol stocks were prepared by mixing equal volume, i.e. 500 µL, each of reconstituted pellet and sterile 40 % glycerol. The culture was always activated before further use by sub-culturing twice in nutrient broth and nutrient agar. Another set of cultures was stored at 4 °C and sub-cultured once a week.

2.2.2. Screening of *B. megaterium* for expression of marker enzymes

Preparation of test culture

The *B. megaterium* was streaked on a nutrient agar medium. A single colony of strain was transferred to 5.0 mL of Tryptone Glucose Yeast extract (TGY) broth and incubated for 24.0±2.0 h at 37˚C. The broth culture of the *B. megaterium* was centrifuged at 10,000 rotations per minute (rpm) for 10 min at 4 °C. Followed by washings of the pellet twice using 10 mili-molar (mM) potassium phosphate buffer (pH=6.8), to remove the broth components as supernatant. The final suspension was prepared in 10 mM potassium phosphate buffer (pH=6.8) and optical density was set at 595 nm to approximately 0.320±0.02 using a multimode plate reader. Final cell suspensions were further used for the screening of enzymes.

Screening protocol

The *B. megaterium* strain was screened for the expression of ten marker enzymes like Esterase, ß-glucosidase, α-glucosidase, α-galactosidase, α –amylase, Tryptophanase, Acid phosphatase, Protease, Alkaline phosphatase, Peorxidaseusing their respective chromogenic substrates like Indoxyl acetate, p-nitrophenyl- ß-D-glucopyranoside, p-nitrophenyl- α –D-glucopyranoside, p-nitrophenyl-α-D-galactopyranoside, Starch, 4-(Dimethylamino) cinnamaldehyde, p-Nitrophenyl Phosphatase, Azocasein, 5-Bromo,4-Chloro-3-indolyl-phosphatase, 3,3',5,5'-Tetramethylbenzidine. The protocol for screening of different marker enzymes were performed as per the protocol discussed as follows.

Esterase enzyme

For screening, 100 µL cell suspension of *B. megaterium* culture and 100 µL of the indoxyl acetate (chromogenic substrate) were taken in a microcentrifuge tube (MCT). The tube was incubated at 37 ˚C and the enzyme activity was measured in terms of color development up to 4.0 h. The control tube was added with 100 µL chromogenic substrate and 10 mM potassium phosphate buffer (pH=6.8) and the absorbance was taken at 605 nm.

α-galactosidase, α-glucosidase, β-glucosidase, acid phosphatase, and alkaline phosphatase

The same protocol was used as that of esterase except for a p-nitrophenyl (PNP) chromogenic substrate (Table 1) at 10 mM concentration (3 mg/mL in 10mM PPB). The color change was observed and recorded at 10 minutes intervals initially for the first two hours followed by after 30 minutes intervals for up to 24 hours of final incubation time. Yellow color development in the tubes was indicative of the presence and expression of the marker enzyme, absorbance was measured at 405 nm.

Table 1. Targeted marker enzymes and their respective chromogenic substrates

S. No.	Marker enzymes	Chromogenic Substrate	Concentration	Solvent used
1	Esterase	Indoxyl acetate	10 mM	PPB pH 6.8
2	ß-glucosidase	p-nitrophenyl- ß-D-glucopyranoside	10 mM	PPB pH 6.8
3	α-glucosidase	p-nitrophenyl- α –D-glucopyranoside	10 mM	PPB pH 6.8
4	α-galactosidase	p-nitrophenyl-α-D-galactopyranoside	10 mM	PPB pH 6.8
5	α –amylase	Starch	1% (w/v)	Distilled water
6	Tryptophanase	4-(Dimethylamino)cinnamaldehyde	10 mM	Chloroform,Ethanol (1:1)
7	Acid phosphatase	p-Nitrophenyl Phosphatase	10 mM	Citrate pH 4.8
8	Protease	Azocasein	10 mM	Tri base buffer pH 8.0
9	Alkaline phosphatase	5-Bromo,4-Chloro-3-indolyl-phosphatase	10 mM	Tris HClbuffer pH 8.2
10	Peorxidase	3,3',5,5'-Tetramethylbenzidine	10 mM	Na-citrate PO_4 buffer pH 4.0

Amylase

For the detection of amylase enzyme activity in *B. megaterium* culture, the method of Xiao et al.(Xiao et al., 2006) was used with some modifications. The assay was initiated by adding 40 µL of starch (Sigma S-2630) solution (10 mg/mL) and 30 µL bacterial cells of in 0.1 M phosphate buffer at pH 7.0 to MCT tubes. After the incubation of 30 minutes of incubation at 50 °C, 20 µL of 1 M HCl was added to halt the enzymatic reaction, followed by the addition of 100 µL of iodine reagent. Following color development, 100 µL of the iodine-treated samples were transferred to a transparent flat-bottomed 96 well microplate and the absorbance at 580 nm was measured.

Tryptophenase

A sterilized test tube containing 4 mL of tryptophan broth was taken and inoculated with an overnight grown culture of *B. megaterium* culture followed by incubation at 37 °C for 24-28 hours. Then 0.5 ml of Kovac's reagent was added to the broth culture and tubes were observed for the presence or absence of a cherry red color ring on the top of the tube.

Peroxidase

One hundred µL of substrate solution (7.63 M H_2O_2 + 0.921 mM TMB) in sodium citrate-phosphate buffer pH 4 was transferred to a microtiter plate containing bacterial culture followed by shaking of the plate for 15 minutes at room temperature under dark condition. Meanwhile, absorbance was taken at 650 nm.

Protease

The method was used as per Mel et al.(Mel et al., 2000) with some modifications. One hundred µL of azocasein (5 mg/mL) in 100 mMTris (pH 8.0) was taken in MCT followed by the addition of 100 µL of cell suspension of *B. megaterium* and the mixture was incubated at 37 °C for 1 hour. Then the enzyme reaction was stopped by the addition of 400 µL of 10% trichloroacetic acid (in Mili Q water). The final mixture was centrifuged at 10,000 rpm for 10.minutes and a supernatant of trichloroacetic acid was transferred in 700 µL of 525 mMNaOH. The optical density (O.D) of the final mixture was measured at 440 nm.

2.2.3. Screening of marker enzyme for pesticide inhibition

Targeted Pesticides

Twenty-four pesticides (Fenitrothion, Monocrotophos, Tetrachlorovinphos, Malathion, Dimethoate, Paraoxon methyl, Endosulfan, Aldrin, Amisulbrom, Ametoctradin, Edinfenphos Carbendazim, Maneb, Zineb, Captan, Thiram, Ziram, Glyphosate, 2-Phenylphenol, Thiobencarb, Alachlor, Atrazine, Asulam, and Aclonifen) were analyzed for their inhibition activity against targeted enzymes. The procedure for the screening of marker enzyme for its inhibition study used is as follows.

Step 1. Exposure: A 50 µL of activated bacterial cells (OD_{600} 0.320±0.02) were transferred to wells of a microtiter plate containing different concentrations of pesticide/solvent residues. Following proper mixing, the plate was allowed to incubate at 37°C for 1 hour.

Step 2. Enzyme-substrate reaction: A 50 µL of chromogenic substrate was added to each well of the

microtiter plate. After the addition of the substrate, the tubes were allowed to incubate at 37 °C for an enzyme-substrate reaction to take place. The optical density was taken at specific intervals of time using a multimode plate reader. Final observations for inhibition in enzyme activity were taken in terms of color development after the optimum incubation period depending upon the type of enzyme to be screened for inhibition.

2.2.4. Optimization of enzyme inhibition assay with a selected enzyme

Quantity of bacterial cells

10, 20, 30, 40, and 50 µL of activated bacterial cells (Optical density 0.320±0.02) of *B. megaterium* strain reconstitution with phosphate buffer was used. The enzyme reaction was observed followed by adding 50 µL (10 mM) of the respective chromogenic substrate.

2.2.5. Incubation time

Time of exposure

The activity of the selected enzyme was allowed to inhibit by exposure to a minimum concentration of pesticide for a different period ranging from 10-50 minutes.

Enzyme-substrate reaction time

After exposure with pesticide for optimized time, 50 µL(10 mM) of the chromogenic substrate was added in the wells of the microtiter plate followed by incubation for the detection of residual enzyme activity from 5 to 35 minutes.

3. RESULTS

3.1. Enzyme (s) expression

The *Bacillus megaterium* (*B. megaterium*) strain was screened for α-amylase activity as depicted in Figure 1A. A significant level of amylase, α-galactosidase, ß -glucosidase, alkaline phosphatase, α-glucosidase, α – amylase, Protease, Peroxidase, and Esterase was found at 70, 35, 30, 40, 65, 60, 15, and 15 min, respectively (Figure 1A and 1B). Whereas in case of acid phosphatase and tryptophenase enzyme activity, *B. megaterium* has not shown any enzyme activity even at or above 120 min incubation at 37 ^{o}C. In the existing literature, almost all species of the *Bacillus* genus are known to synthesize α-amylase, since this genus has the potential to dominate the enzyme industry (Vishnu et al. 2014). In another study, Gurudeeban et al. (Gurudeeban et al. 2011) have isolated a *B. megaterium* from the leaves of *Avicennia marina* able to express amylase. Stark et al. (Stark et al. 1982) who also reported the production of the α-amylase enzyme in *a B. megaterium* strain S218. However, this enzyme was found intracellular in *B. megaterium* strain M as shown by Weibull et al. (Weibull et al. 1959). Thus acid phosphatase may not be released extracellularly to show its activity. Our results are well supported by Patil et al. (A. G. G. Patil et al. 2010) who also reported the expression of α- galactosidase in *B. megaterium* VHM1. This α-glucosidase enzyme is well reported for its widespread occurrence in several species of the Bacillus (Castro et al. 1995). Our results in this regard were well supported by Kelly and Fogarty (Kelly and Fogarty 1983) who also reported the expression of α-glucosidase in *B. megaterium*. In another study by Stark et al. (Stark et al. 1982) also reported the expression of the α-glucosidase enzyme in *B. megaterium* S218.The production of β-D-glucosidases by several Bacillus species such as *B. subtilis, B. licheniformis*, etc. is well reported in the literature (Bagudo et al. 2014; Naz et al. 2010; Rehena et al. 1989). Castro et al. (Castro et al. 1995)who found that the presence of this enzyme extracellularly as well as membrane-bound. Higerd and Spizizen(1973) who also found expression of the same enzyme in vegetative cells of many other Bacillus species. Jung et al. (2003) also reported the presence of the same enzyme in cell-bound in the *B. megaterium* strain. Similarly, Priest (1977) also observed the expression of the same enzyme in the form of extracellular enzymes in the genus Bacillus including *B. megaterium* and *B. licheniformis*. Zheng et al. (2017) also reported the activity of the same enzyme *B. megaterium* who studied stereoselectivity and catalytic activity of this enzyme.Tariq et al. (2016) who also reported negative tryptophanase activity in *B. megaterium*. Our finding in this regard was well supported by Priya et al. (2014) and Wood and Tristram(1970) they also reported the production of alkaline phosphatase by several Bacillus species such as *B. megaterium*, *B. subtilis*, etc. Our study in this regard was well supported

by findings of other researchers they also reported production of protease by several Bacillus species such as *B. megaterium, B. subtilis*, etc. The production of peroxidase by several Bacillus species such as *B. megaterium, B. subtilis*, etc. (Patil 2014; Rao andKavya 2014).Further, all expressed enzymes in the *B. megaterium* were subsequently screened for inhibition by all targeted pesticides of different groups.

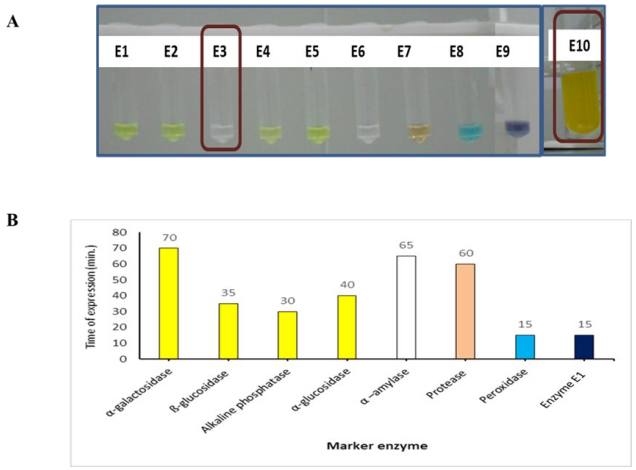

Figure 1. Enzyme expression in Bacillus megaterium. A. Enzyme expression in *Bacillus megaterium* using specific chromogenic substrate. B. Time of expression (or change in color of the chromogenic substrate) of enzymes of *Bacillus megaterium* strain

3.2. Screening enzymes for their inhibition by pesticides

Results of inhibition of α-glucosidase, ß-glucosidase, α-galactosidase, and esterase enzyme activity by different pesticide screened by microtiter assay are shown in Table 2-5 and summarized limit of detection of enzymes are given in Table 6. Based on visual observation, inhibition of α-glucosidase enzyme activity was found up to 100 ppb with maneb followed by thiram and ziram up to 1 ppm in terms of no yellow color i.e., p-nitrophenol development due to the inability of the α-glucosidaseenzyme to hydrolyze PNP substrate. Other twenty-two pesticides showed inhibition up to a maximum of 1 ppm only. In case of ß-glucosidase enzyme, inhibition of activity was found up to 200 ppm with asulam and followed by glyphosate up to 300 ppm with ß-glucosidase indicated by no p-nitrophenol production or yellow color development. Other pesticides were not inhibitory to ß-glucosidaseactivity up to the highest concentration of 500 ppm. Further, α-galactosidase was sensitive to most pesticides and more interestingly, pesticides that showed the highest inhibition were fungicides. Based on visual observation, the highest inhibition was observed up to 10 ppb with maneb followed by edinfenphos, thiram, ziram up to 1 ppm. Some pesticides were not inhibitory to enzyme activity up to 100 ppm. The alkaline phosphatase activity of was not inhibited by any of the pesticides in terms of yellow color i.e., p-nitrophenol development due to the enzymatic hydrolysis of PNP substrate. The activity of α-amylase was also not inhibited by any of the pesticides in terms of no blue color development due to the enzymatic hydrolysis of starch. The inhibition of protease enzyme activity was observed only with zineb at 100 ppm in terms of lesser red color development due to the partial ability of the enzyme to utilize azo-casein. The inhibition of enzyme activity was observed only with ziram at 100 ppm in terms of lesser blue color development due to the partial ability inhibition of the enzyme activity. Later, esterase enzyme activity studies wherein no change in color of indoxyl acetate from pink to purple in the presence of pesticides may be due to inhibition by 10 ppb for fenitrothion, monocrotophos, malathion, paraoxon methyl amisulbrom, ametoctradin, carbendazim, maneb, captan, asulum was observed. Our findings in this regard were very well supported by Ahmed et al. (Ahmed et al. 2002) who also reported the significant role of protease for insecticide resistance in a strain of *Muscadomestica* L. Our results in this regard are very well supported by Niemi et al. (2009). Similarly, another study was done by Bhardwaj and Shekhar (Bhardwaj and Shekhar 2005) who also reported inhibition of α-glucosidaseand α-galactosidaseactivity in the midgut of the last instar naiad of *Trithemis aurora* by quinalphos, chlorpyrifos, cypermethrin, and deltamethrin pesticides. Inhibition in the ß-glucosidase enzymatic activity in tea garden soil containing residues of organophosphate and organochlorine pesticides were shown by Bishnu et al. (2008). A significant role of protease for insecticide resistance in a strain of *Muscadomestica* L was reported by Ahmed et al. (2002). Moccelini et al. (2010)developed a

biosensor based on the inhibition of peroxidase for the detection of thiodicarb and carbamate pesticides. Using microtiter assay, among eight enzymes screened for their inhibition by twenty-four pesticides, only seven enzymes namely esterase, β-glucosidase, α-glucosidase, α-galactosidase, α-amylase, protease, alkaline phosphatase, and peroxidase showed sensitivity towards pesticides. However, a higher level of sensitivity in terms of lower LOD, i.e., 10 ppb for pesticides was obtained with α-galactosidase and enzyme esterase but enzyme esterase has shown a lesser time i.e. 15 minutes for enzyme reaction as compared to other enzymes. Therefore, esterase enzyme activity in *Bacillus* species was selected for further optimization study.

Table 2. Visual perception based inhibition of α-glucosidase activity

S. No.	Pesticide	LOD (ppm)	S. No.	Pesticide	LOD (ppm)
1	Fenitrothion	>100	**13**	**Maneb**	0.1
2	Monocrotophos	>100	**14**	Zineb	100
3	Tetrachlorovinphos	>100	**15**	**Captan**	10
4	Malathion	>100	**16**	**Thiram**	1
5	Dimethoate	>100	**17**	**Ziram**	1
6	Paraoxon methyl	>100	**18**	Glyphosate	>100
7	Endosulfan	100	**19**	2-Phenylphenol	>100
8	Aldrin	>100	**20**	Thiobencarb	>100
9	Amisulbrom	>100	**21**	Alachlor	>100
10	Ametoctradin	>100	**22**	Atrazine	>100
11	Edinfenphos	>100	**23**	Asulam	100
12	Carbendazim	>100	**24**	Aclonifen	>100

Table 3. Visual perception based inhibition of ß-glucosidase activity

S. No.	Pesticide	LOD (ppm)	S. No.	Pesticide	LOD (ppm)
1	Fenitrothion	>500	**13**	Maneb	>500
2	Monocrotophos	>500	**14**	Zineb	>500
3	Tetrachlorovinphos	>500	**15**	Captan	>500
4	Malathion	>500	**16**	Thiram	>500
5	Dimethoate	>500	**17**	Ziram	>500
6	Paraoxon methyl	>500	**18**	**Glyphosate**	300
7	Endosulfan	>500	**19**	2-Phenylphenol	>500
8	Aldrin	>500	**20**	Thiobencarb	>500
9	Amisulbrom	>500	**21**	Alachlor	>500
10	Ametoctradin	>500	**22**	Atrazine	>500
11	Edinfenphos	>500	**23**	**Asulam**	200
12	Carbendazim	>500	**24**	Aclonifen	>500

Table 4. Visual perception based inhibition of α-galactosidase activity

S. No.	Pesticide	LOD (ppm)	S. No.	Pesticide	LOD (ppm)
1	Fenitrothion	100	13	Maneb	0.01
2	Monocrotophos	100	14	Zineb	10
3	Tetrachlorovinphos	100	15	Captan	10
4	Malathion	>100	16	**Thiram**	1
5	Dimethoate	>100	17	**Ziram**	1
6	Paraoxon methyl	>100	18	Glyphosate	>100
7	Endosulfan	100	19	2-Phenylphenol	>100
8	Aldrin	>100	20	Thiobencarb	100
9	Amisulbrom	>100	21	Alachlor	100
10	Ametoctradin	>100	22	Atrazine	>100
11	**Edinfenphos**	1	23	Asulam	100
12	Carbendazim	>100	24	Aclonifen	>100

Table 5. Visual perception based inhibition for esterase activity

S. No.	Pesticide	LOD (ppm)	S. No.	Pesticide	LOD (ppm)
1	**Fenitrothion**	0.01	13	**Maneb**	0.01
2	**Monocrotophos**	0.01	14	Zineb	0.1
3	**Tetrachlorovinphos**	0.01	15	**Captan**	0.01
4	Malathion	0.1	16	Thiram	0.1
5	Dimethoate	0.1	17	Ziram	0.1
6	**Paraoxon methyl**	0.01	18	Glyphosate	10
7	Endosulfan	10	19	2-Phenylphenol	0.1
8	Aldrin	1	20	Thiobencarb	50
9	**Amisulbrom**	0.01	21	Alachlor	50
10	**Ametoctradin**	0.01	22	Atrazine	10
11	**Edinfenphos**	0.1	23	**Asulam**	0.01
12	**Carbendazim**	0.01	24	Aclonifen	10

Table 6. Summary of observations on maker enzymes screening using microtiter assay

S. No.	Marker enzymes	Sensitivity	Pesticide	Reaction time
1	α-glucosidase	100 ppb	Thiram, Ziram, Maneb	2.50 hour
2	ß-glucosidase	200 ppm	Asulam	35 minutes
3	Acid phosphatase	-	-	-
4	α-galactosidase	10 ppb	Maneb	5.50 hour
5	Alkaline phosphatase	>100 ppm	-	30 minutes
6	α –amylase	>100 ppm	-	2 hours
7	Protease	100 ppm	Zineb	1 hour
8	Peorxidase	100 ppm	Ziram	15 minutes
9	Esterase	10 ppb	Captan, Maneb, Asulam etc.	15 minutes
10	Tryptophanase	-	-	-

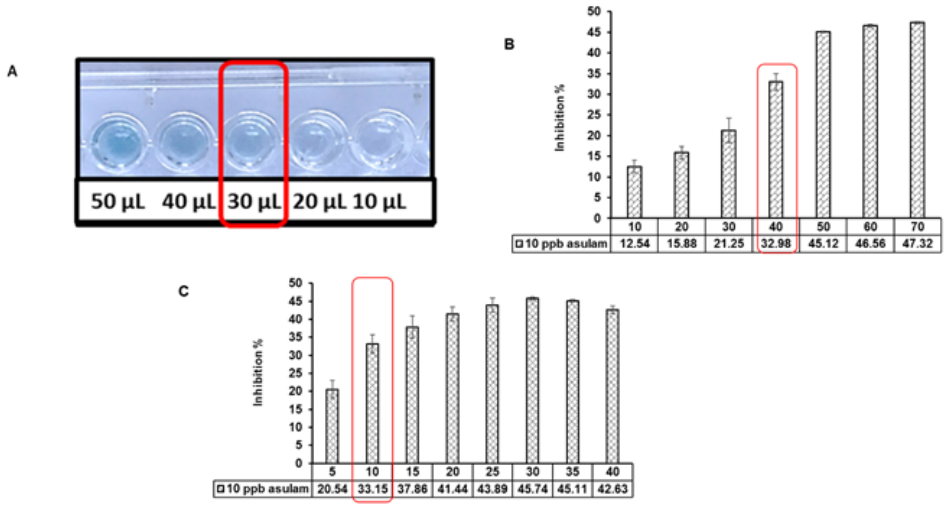

Figure 2. Optimization of chromogenic microtiter assay for detection of pesticide residues in water. A. Volume of spores B. Time of exposure (minutes) C. Time of reaction (minutes)

Table 7. LOD of different groups of pesticides achieved using microtiter assay

S. No.	Pesticide Name	LOD	S. No.	Pesticide Name	LOD
1.	**Fenitrothion**	1 ppb	**13.**	**Maneb**	1 ppb
2.	**Monocrotophos**	1 ppb	**14.**	**Zineb**	1 ppb
3.	**Tetrachlorvinphos**	1 ppb	**15.**	**Captan**	0.1 ppb
4.	**Malathion**	10 ppb	**16.**	**Thiram**	10 ppb
5.	**Dimethoate**	10 ppb	**17.**	**Ziram**	10 ppb
6.	**Paraoxon-methyl**	1 ppb	**18.**	**Glyphosate**	5 ppm
7.	**Endosulfan**	5 ppm	**19.**	**O-phenylphenol**	10 ppb
8.	**Aldrin**	750 ppb	**20.**	**Thiobencarb**	10 ppm
9.	**Amisulbrom**	1 ppb	**21.**	**Alachlor**	10 ppm
10.	**Ametoctradin**	1 ppb	**22.**	**Atrazine**	10 ppm
11.	**Edifenphos**	10 ppb	**23.**	**Asulam**	1 ppb
12.	**Carbendazim**	1 ppb	**24.**	**Aclonifen**	10 ppm

3.3. Optimization of colorimetric microtiter assay with an esterase enzyme

As shown in Figure 2A, the activity of esterase at 30 µL of spores was observed with optimum color development within a short period of incubation, i.e., 10 minutes. Exposure is the pre-incubation of the enzyme with pesticide residues before the addition of substrate. In the current work, the activity of esterase was allowed to inhibit by exposure to 10 ppb of asulam for a different period ranging from 10-50 minutes. As shown in Figure 2B, an increasing trend in the degree of the inhibitory signal from 12.54 to 32.98 % at 10 ppb as the time of exposure was increased from 5 min. to 40 min. An increasing trend in inhibition of esterase activity was found to increase from 20.50 to 45.11 % at 10 ppb with an increase in time of incubation from 5 minutes to 35 minutes (Figure 2C). After that, no significant increase in inhibition of esterase activity was observed at 30 minutes of incubation. However, at 40 minutes of incubation, the activity of esterase was increased and masked inhibition. Thus by these findings, an incubation time of 10 minutes was selected for enzyme-substrate reaction to take place. Our findings are similar to the outcomes of Bucur et al. (Bucur et al. 2006) who observed an increase in inhibition of AChE by carbaryl with the simultaneous increase in exposure time.

3.4. Determination of the limit of detection (LOD)

The LOD of each pesticide by developed assay was taken as the pesticide concentration that produced blue color for microtiter plate as well as an inhibition at ≥ 30 % in esterase activity with an O.D. at 605 nm was measured in microtiter plate assay to avoid any false-positive results. LOD obtained for 24 pesticides of insecticide, herbicide, and fungicide group with microtiter assay are summarized in Table 7. With the optimized protocol, the esterase enzyme showed the most significant inhibition (i.e. limit of detection) at a with a further optimized protocol, the esterase enzyme showed the inhibition at 1 ppb concentration of fenitrothion, monocrotophos, tetrachlorovinphos, paraoxon methyl, amisulbrom, ametoctradin, carbendazim, maneb, zineb, and asulam in a reaction time of 10 minutes. Most significantly a LOD of 0.1 ppb for captan pesticide was achieved in a reaction time of 15 minutes. The percent inhibition in esterase activity was found to increase with a simultaneous increase in the concentration of pesticide which is in line with the findings of other studies (Ayat et al. 2021; Yunhe et al. 2010). Comparing the analytical data obtained for pesticides of three groups indicated the lower LOD and thus higher sensitivity for members of fungicides followed by insecticides.

For insecticide detection, several biosensors are developed and achieved the different limits of detection. The higher sensitivity of some of the organophosphate pesticides can be explained by the fact that these pesticides cause inhibition by forming stable covalent intermediates (Kumaran and Tran-Minh 1992; Sharma et al. 2021; Wu et al. 2021). Montes et al. (Montes et al. 2018) developed an electrochemical biosensor based on optimized biocomposite for organophosphorus and carbamates achieved LOD of 0.25 ± 0.03 to 1.03 ± 0.05 ppb for malathion. Gan et al. (Gan et al., 2010) developed a disposable organophosphorus pesticides (OPs) enzyme biosensor based on magnetic composite nanoparticle-modified screen-printed carbon electrodes (SPCE) and detected dimethoate up to the lowest limit of 56 ppb. Gabaldónet al. (Gabaldón et al. 1999) reported a commercial kit available for fenitrothion detection with a LOD of 25 ppb. Wu et al. (Wu et al. 2011) developed amperometricacetylcholinesterase (AChE) biosensor which was fabricated based on mesocellular silica foam achieved LOD 0.05 ppb for monocrotophos. For fungicides, Marty et al. (Marty et al., 1993) developed a sensor based on the enzyme aldehyde dehydrogenase for detection of dithiocarbamate fungicide, i.e., maneb, and reported LOD of 0.05 ppm. An enzyme aldehyde dehydrogenase-based biosensor for the detection of zineb was developed with a LOD of 8 ppb (Noguer et al., 1999). Pita *et* al. (Pita et al. 1997) developed a biosensor based on enzyme tyrosinase for the detection of ziram and reported a LOD of 22.63 ppb with the developed sensor. The least sensitivity was achieved for the herbicides group at 10 ppm for some of its members. This shows that these pesticides are low inhibitory to esterase. For herbicide detection, several biosensors are developed and achieved different LOD, but most of the sensors are based on other than esterase. For example, Koblizek et al.(Koblizek et al. 1998) developed a biosensor based on photosystem-II (PSII) particles from *Synechococcus elongates* for detection of atrazine and got LOD up to 0.43 ppb. Oliveira et al. (Oliveira et al. 2012) developed a biosensor based on heme-containing enzymes and achieved a LOD of 30 ppb for glyphosate. An immunoassay (ELISA) is commercially available by Abraxis LLC for glyphosate, atrazine, and alachlor with LOD of 0.05 ppb, 3 ppb, and 0.08 ppb respectively (Abraxis LLC).The pesticide paraoxon is the strong known organophosphate inhibitor for the activity of various enzymes and was used as a model pesticide in the development of several enzymatic inhibitions based biosensors in existing prior-art (Arduini et al. 2007; Heo and Crooks 2005; Ivanov et al. 2012; Mulchandani et al. 2006; Pohanka et al. 2010). Our findings show that paraoxon does not represent the inhibition pattern of other pesticides against enzymes. That infers that paraoxon methyl is not a model pesticide for the development of enzymatic inhibitions-based biosensors for another pesticide.

4. CONCLUSION

All expressed enzymes were screened for their activity inhibition by twenty-four pesticides of different groups using the enzyme inhibition assay. The limit of detection of β-D-glucosidase, α-D-glucosidase, α-D-galactosidase, protease, peroxidase, and esterase was observed at pesticide concentrations of 200 ppm, 100 ppb, 10 ppb, 100 ppm, 100 ppm, and 10 ppb respectively for different pesticides. With the optimized esterase inhibition assay, a LOD of 0.1 ppb for captan pesticide was achieved in a reaction time of 10 minutes. The findings of the current study are consistent with the existing studies indicating the potential of esterase to target the detection of a broad range of pesticides. The enzyme inhibition-based assay using esterase enzyme would be a promising approach for the rapid and cost-effective assay for pesticide detection.

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