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PII: S0141-8130(19)37825-0

DOI: https://doi.org/10.1016/j.ijbiomac.2019.12.192

Reference: BIOMAC 14220

To appear in: *International Journal of Biological Macromolecules*

Received date: 28 September 2019

Revised date: 4 December 2019

Accepted date: 20 December 2019

Please cite this article as: S. Sirajuddin, M.A. Khan, S.A.U. Qader, et al., A comparative study on degradation of complex malathion organophosphate using of Escherichia coli IES-02 and a novel carboxylesterase, *International Journal of Biological Macromolecules*(2019), https://doi.org/10.1016/j.ijbiomac.2019.12.192

A Comparative study on degradation of complex Malathion organophosphate using of *Escherichia coli* IES-02 and a novel Carboxylesterase

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ABSTRACT

Malathion organophosphates considered as the major constituent of herbicides, pesticides and insecticides. Extensively used in agricultural, horticultures and for numerous household applications contributes to precedence organic pollutants leading antagonistic effects on human health and environment. Therefore detoxification of malathion from contaminated site is of general interest. Simultaneously it is very emerging to isolated novel indigenous microbial strains from contaminated site with a record of pesticide application. In this study *Escherichia coli* IES-02 isolated from malathion contaminant effluent and the strain showed maximum efficiency in malathion degradation that utilized it as the sole source of carbon. Carboxylesterase (33.0, 30.0, 28.0 kDa) were purified (1685.71 U/mg) from *Escherichia coli* IES-02 showed significant results in malathion degradation approximately 81% within 20 minutes as compared with *Escherichia coli* IES-02 cells within 4 hour (99.0 to 95.0%) into monocarboxylic acid and diacid derivatives. The generation time of *Escherichia coli* was also observed at 60 minutes with 0.1ppm, 68 minutes with 0.5ppm, 74.5 minutes with 2.0ppm and 91.37 minutes with 50ppm of malathion. The degradation rate and transformation metabolites were estimated by Gas Chromatography-Mass Spectrometry respectively. Malathion metabolites pathway proposed in this study which revealed the potential application against lethal environmental pollution.

Keywords: Malathion; Carboxylesterase; *Escherichia coli* IES-02

1. INTRODUCTION

Malathion organophosphorus pesticides (MOPs) have been broadly used throughout the world to control weeds and pest mainly spread in agricultural and horticulture. In late 19th century, organochlorine pesticides were the only choice to regulate these insect. Previously it was considered that malathion are comparatively less persistent in the environment and also

associated with less or negligible health risks to non target aquatic organisms as compared to organochlorine pesticides. However, recently several government agencies including the USEPA, have started to reevaluate the extensive use of organophosphorus pesticides due to disquiet about their major effects on the central nervous systems of humans, children in particular **[1],[2].** Currently, detoxification of malathion organophosphate depends on chemical treatment, incineration and landfills **[3]**.However, these methods are feasible but produces large volume of acids and alkali or leaching of pesticides into surrounding soil and ground water supplies which is another alarming environmental concern and through incineration of organophosphate destructive chemical compounds which can further develop toxic emissions in the whole ecosystem **[3]**. Microbial biodegradation of malathion organophosphate is one of the promising, cost effective, efficient and reliable approach of green biotechnology to degrade toxic chemicals and generate by products that are comparatively far more less toxic to previously reported methods **[4], [5], [6]**. Carboxylesterase are a major class of detoxification enzymes used to degrade complex structure of pesticides. Several bacterial species including *Bacillus Thuringiensis MOS-5,* **[7]** *Sphingobium yanoikuyae* strain P4 **[8]** carboxylesterase have been used. Rare literature has been reported for production and screening of carboxylesterase from microbial sources and relatively little research has been carried out on comprehensive product identification. This study represents the malathion biodegradation potential of carboxylesterase purified from newly isolated *Escherichia coli* IES-02.

2. EXPERIMENTAL SETUP

2.1. Reagents and chemicals

All the chemicals and reagents were used in this study are of analytical grade. Malathion - 98% (AccuStandard, USA) were obtained from FQSRI, Pakistan Agricultural Research Council, and

University of Karachi. Analytical grade n-hexane was purchased from Sigma-Aldrich, USA.

2.2. Sample collection

Wastewater samples were collected from Lyari River Karachi at 24°56'44.13" N 67°5'14.53" E, Pakistan. Samples were collected aseptically and transferred in autoclaved flacons and stored at 4°C for further analysis.

2.3. Isolation and screening of indigenous bacteria capable of malathion degradation

For isolation and screening of malathion degrading strain, wastewater samples were serially diluted up to 10^{-6} from final dilution 100µl sample was spread on minimal agar plate containing 50ppm malathion. Incubation of malathion containing samples plates were kept at 37°C for 24hr. The bacteria capable of growing in the presence of malathion appeared with clear halo zones on minimal agar palate. The isolates showing halo zone were selected and purified by repeated streaking. The purified strains were maintained on nutrient agar slants and stored at 4°C.

2.4. Identification of malathion degrading bacterial isolate

The selected strains were identified and characterized on the basis of morphological, biochemical and molecular analysis. Berge's manual of determinative bacteriology was considered for further confirmation up to species level **[9]**. Scanning electron microscope (SEM) was used for identification of bacterial cells morphology. Biochemical characterization was achieved by catalase, oxidase, starch hydrolysis, citrate utilization, nitrate reduction, , MRVP, growth on EMB agar, growth on SDA, growth on nutrient agar with 7.5% NaCl and sugar fermentation.

2.5. Growth kinetic of *Escherichia coli* IES-02 in absence of malathion

For growth kinetics of *Escherichia coli* IES-02, 10% inoculum was transferred in 225ml of minimal salt medium flasks containing different concentrations of malathion (0.1, 0.5, 2.0, 50

ppm) against blank which contain glucose as a sole carbon source and incubated at 37˚C 120rpm for 24hours. Samples were collected after every interval of one hour. The growth of the pesticide degrading bacterial isolate was assessed by using UV – spectrophotometer (Kanza Max Biochemis Try, Biolebo, France) at 600nm. Generation time (g) and specific growth was calculated by analyzing growth curve.

2.6. Production of malathion degrading enzyme by *Escherichia coli* IES-02

For enzyme production, fresh minimal salt media supplemented with 50ppm of malathion was inoculated with *Escherichia coli* IES-02. The culture was incubated at 37°C for 24 hours 500 mL-Erlenmeyer flasks containing 250mL of medium on a rotary shaker (Thermo electron corporation model no. 2873, USA) at 150 rpm, harvested by centrifugation (5810 R, Eppendorf AG, Hamburg, Germany) at 40006x g for 30 min at 4°C. Cell free filtrate was analyzed for enzymatic activity and total protein content was also estimated by Lowry's method using BSA (Bovine serum albumin) as a standard **[10]**.

2.7. Purification of malathion degrading enzyme

Ammonium sulfate precipitation approach was selected for purification of carboxlesterase. To accomplish this, gradient precipitation approach was followed by using 20%–60% concentration of ammonium sulfate. Gradually ammonium sulphat is added in in cell free filtrate with constant stirring at 4 °C and left it for overnight at cooling condition (4 ºC) to achieve complete equilibration. Precipitates were collected by centrifugation at 4000 x g for 30 min and dissolved in the smallest possible volume of 50mM PBS (pH 7.5). Enzyme activity and total protein was determined after ammonium sulfate precipitation.

Partially purified malathion degrading protein sample was desalted with PD-10 desalting column (GE Healthcare UK Ltd. Little Chalfont, Buckinghamshire, UK) that contain sephadex G-25 of 5

KDa. The column was first washed and equilibrated thrice by 50 mM PBS (pH 7.5) at spin down at 4,000 x g for 2 min. The flow through was discarded. After equilibration of the column, the sample was loaded slowly and allowed to pass through the column under 4,000 x g for 2 min. Enzyme activity and total protein was determined after de-salting the samples.

Partially purified desalted sample was further concentrated by Centricon® centrifugal filter device Ultracel YM-10 (Millipore corporation USA) of 10.0 kDa membrane. Sample was poured in sample reservoir vial of centricon® tubes which was attached with filtrate vial. Sample was centrifuged at 4,000 x g until desired concentration was achieved. Enzyme activity and total protein was determined after filtration of the samples.

2.8. Enzyme Assay

Malathion catalytic activity was assayed by adding 100µl enzyme solution to 5 ml of 50 mM Phosphate Buffer Saline (pH 7.5) containing 5 ppm of malathion and incubated for 1 hour at 37°C. After that, samples were extracted with 5 ml n-hexane and the residues of malathion were determined by Gas chromatography **[11]**. A control experiment without insecticide in minimal salt media was used for comparison. One unit of enzyme activity (U) was defined as the amount of enzyme required to catalyze formation degrade 1mmol of malathion under standard assay conditions.

2.9. Estimation of Molecular weight of malathion degrading enzyme

Native polyacrylamide gel electrophoresis (PAGE) was performed with 12.5% resolving gel and 4.5% stacking gel for the assessment of molecular weight and enzyme purity by comparing its migration rates with known molecular weight standard markers. The technique was performed according to the method described by Ornstein (1964) with some modification **[12]**. Low molecular weight markers were used (SDS7, Sigma-Aldrich, USA).Proteins were stained by the

silver staining procedure of Morrissey **[13]**.

2.10. Biodegradation of Malathion by *Escherichia coli* IES-02

Malathion biodegradation by an enzyme produced by *Escherichia coli* IES-02 were analyzed using a GC system (Agilent 6890N) equipped with an electron capture detector (GC-ECD). The degrading ability of the isolated culture was ascertained by adding 25ml of inoculum in five flasks of 500mL, each containing 225mL of minimal salt medium and different concentration of Malathion (0.1, 0.5, 2.0, 50 ppm). While control flask was taken without adding bacterial isolates to assess the degradation capability of isolated bacteria. All flasks were placed in shaking water bath at 37°C for 24 hours at 120rpm. 25mL of sample was taken after 0, 8 and 24 hours of inoculation and residues of Malathion were extracted twice with n-hexane (2 x 25mL) by the addition of equal amount of n-hexane to each aliquot. After vigorous shaking for 5 min, the organic layer was separated and dehydrated by passing through anhydrous sodium sulphate. The solvent layer was then allowed to evaporate completely by using rotary evaporator (BUCHI Rotavapor B-740). The dried residue was then dissolved in 10mL analytical grade n-hexane and subjected to Gas Chromatography analysis in Food Quality and Safety Research Institute, Pakistan agricultural research council (PARC), Karachi, Pakistan. Each sample was injected three times and the mean, standard deviation and standard error was calculated.

2.11. Identification of metabolites produced by malathion biodegradation

The non-degraded residues of malathion were monitored through Gas Chromatography - Mass Spectrometry (GC/MS) analysis. In this assay, conical flask containing 225mL minimal salt medium supplemented with malathion (50ppm) as a sole carbon source, was inoculated with 25mL of 24 hour grown culture and incubated at 37°C for 24 hours. 25mL of sample was taken after 24 hours of inoculation and the residual malathion was extracted twice with n-hexane (2 x

25mL) by the addition of equal amount n-hexane to each aliquot. After vigorous shaking for 5 min, the organic layer was separated and dehydrated by passing through anhydrous sodium sulphate. The solvent layer was then allowed to evaporate completely by using rotary evaporator (BUCHI Rotavapor R-200, BUCHI heating bath B-490, BUCHI-recirculating chiller B-740). The dried residue was then dissolved in 10mL analytical grade n-hexane and subjected to gas chromatography (GC/MS) analysis in HEJ, Research Institute of Chemistry, University of Karachi. The transformation products of malathion by the isolated bacteria were identified on gas chromatography-mass spectrometry (GC-MS) system Agilent 7890A (G3440A) equipped with mass spectroscopy detector (MS Agilent 7000 GC/MS triple quadrupole) with auto-sampler, the injector mode was splitless and injector volume was 2.0 µL at an inlet temperature of 250°C. The analyses were performed in electron ionization (EI) mode (70eV). AnAgilent USB 393752HHP-5MS column (30m × 250 µm × 0.25 µm) was used with a temperature program of 70°C for 1 min; increased to 280°C at 5°C/min and held for 20 min. Helium was used as a carrier gas at a flow rate of 3mL/min. The products identified by mass spectrometry analysis and were matched with authentic standard compounds from the National Institute of Standards and Technology (NIST, USA) library database available at HEJ, Research Institute of Chemistry, University of Karachi.

3. RESULTS AND DISCUSSION

3.1. Isolation, screening and identification of malathion degrading bacterial strain

Ten different bacterial isolates were isolated form pesticide polluted wastewater of Lyari river samples. All isolates were tested for malathion degrading abilities. The isolates that showed maximum tolerance to malathion were selected. Among various isolates maximum malathion degrading organism was identified as *Escherichia coli* IES-02 [GenBank: KU593482]. Pure

culture analysis and scanning electron micrographs of *Escherichia coli* are shown in **Figure 1**. It is clear from (**Figure 1a)** that the *Escherichia coli* IES-02 cells represented green metallic sheen with pin pointed and circular shaped medium size colonies on eoisen methylene blue (EMB) agar plate. However, when the cells were exposed with 50ppm malathion (**Figure 1c)** the cells were swollen as compared to cells without malathion (**Figure 1b)**. It might be due to the culture was adapted and consumes pesticide as carbon source. During the process of adaptation, the bacteria entered into stressed phase and slowed further growth. In this phase rod shaped morphology of *Escherichia coli* IES-02 changed into coccus because of increased in malathion concentration. However, this change was interim and the cells changed into original rod shaped form after adapting time period.

3.2. Growth kinetics of *Escherichia coli* IES-02 in minimal salt medium

The growth kinetics for potential malathion degrading *Escherichia coli* IES-02 was evaluated under varying concentrations of malathion in minimal salt media. (**Figure 2a)** demonstrated that the phase of adaptation of *Escherichia coli* IES-02 continued up to 1h, however a significant increase in the cell number observed during 6 hours of incubation, indicating that the culture after remaining in lag phase of 1h entered into phase of positive acceleration and achieved stationary phase after 6 hours with generation time of 40.90 min and specific growth rate of 0.024.

3.3. Growth kinetics and degradation studies of *Escherichia coli* IES-02 at different concentrations of Malathion

Growth and degradation potential of *Escherichia coli* IES-02 observed at different concentrations (0.1, 0.5, 2.0, 50 ppm) of malathion. It was observed that the generation time (g) of *Escherichia coli* IES-02 was increased with the increase in the concentration of malathion.

However, it was affected on the specific growth rate which decreased to 0.010 min^{-1} at 50ppm as compared to the control 0.024 min^{-1} **(Table 1)**. The study revealed that the *Escherichia coli* IES-02 could be used to minimize the time required for degrading environmental samples containing high concentrations of pesticide. An inverse relationship between pesticide concentration and the bacterial growth was observed in **Figure 2**. At low concentrations of malathion (0.1, 0.5 ppm) **(Figure 2b) (Figure 2c),** the adaptation time period of bacteria was about 1 hour as the concentration increased (2.0, 50 ppm) **(Figure 2d) (Figure 2e)** the bacterial adaption time period was also increased about 2 hours. When compared with the control (growth without pesticide), the growth pattern was different as in control sample bacteria does not required extra adaptation time for growth and remain in exponential phase. After 7 hours of incubation the generation time was calculated 60 min with 0.1ppm malathion dose, 68 min with 0.5ppm of malathion dose, 74.5 min with 2.0ppm of malathion dose and 91.37 min with 50ppm of malathion dose after 6 hours of inoculation. It is suggested that malathion concentration in the range of 0.1, 0.5 and 2.0ppm stimulated the growth of *Escherichia coli* IES-02 however, a marked reduction in bacterial count noted when 50ppm malathion dose were used with generation time of 91.37 min. From others findings it has been assumed that the bacterial enzymes suppressed at high concentration with decreased in growth rate **[14]** It was further noted that *Escherichia coli* IES-02 grow faster at low concentration of malathion. However at higher concentration with decreased in growth rate, the growth of *Escherichia coli* IES-02 significantly decreased or very slightly increased during 24 hours of incubation, when compared with control. This suggested that, at high concentration the appropriate catabolic enzymes may be repressed and bacteria may need an acclimation period to induce the necessary degradation enzymes because of this reason the prolonged lag phase observed at high concentration of pesticide. Therefore, it can be

concluded that the *Escherichia coli* IES-02 play a significant role in pesticide detoxification. Biodegradation was quantified by comparison of malathion concentration between inoculated (**Figure 2b, 2c, 2d, 2e)** and un-inoculated samples (**Figure 2a)** Substrate utilization was increased in microbial biomass and subsequent biodegradation of the contaminant. Degradation of pesticide (50ppm) by *Escherichia coli* IES-02 was found to be high and complete degradation was achieved within 4 hours of incubation.

3.4. Effect of Malathion concentration and enzyme secretion by *E. coli* IES-02:

Different concentration of malathion (0.1ppm, 0.5ppm, 2.0ppm and 50.0ppm and 100.0ppm) production medium were prepared in minimal salt medium (**Table 2**). Medium containing 0.1ppm to 2.0ppm malathion showed relatively low production of carboxylesterase whereas, maximum carboxylesterase activity (74.18 U/ml) was attained with 50.0ppm malathion. This huge difference in overexpression of carboxylesterase in 50.0ppm concentration of malathion is specifically due to the presence of sufficient amount of substrate organophosphate malathion. Carboxylesterase displayed as an inductive enzyme which augmented in the presence of malathion. However, at 100.0ppm of malathion displayed decreased in the carboxylesterase activity (50.0 U/ml). This might be due to the accumulation of organophosphate in the production medium which ultimately decreased enzymatic production. These results are also correlated with other findings in which carboxylesterase activity is augmented in the presence of malathion when *Pseudomonas putida* IS168 used 50.0ppm of malathion as a sole carbon source in the production medium **[15].**

3.4. Purification of malathion degrading protein

Ammonium sulfate precipitation method was selected to purify and concentrate carboxylesterase by changing its solubility within cell free filtrate. It was observed that 60% ammonium sulfate

concentration gave maximum precipitation of carboxylesterase with reference to specific activity. Fold purification of enzyme was investigated by using 60% ammonium sulfate concentration. It was observed that fold precipitation increased as activity increased and enzyme precipitates showed 155.80 U mg^{-1} specific activity with 5.14 fold purification whereas, after desalting and centricon filtration the specific activity and fold purification showed 236.0 U mg^{-1} specific activity with 38.39 fold purification. It could be concluded that purification of carboxylesterase from *E.coli IES-02* yields 38.39 fold purified enzyme with 1685.72 Umg^{-1} of specific activity (**Table 3**).

3.5. Monitoring of residual malathion by GC analysis

A highly sensitive method Gas chromatography (GC-ECD) was used for the determination of malathion residues degraded by the enzyme produced by *Escherichia coli* IES-02. Malathion peak was observed after the retention time of 7.81 min **(Figure 3a)**. The result of percentage degradation is shown in **(Table 4) (Figure 3c)**. The non-degraded residual malathion was monitored in minimal salt medium containing malathion (0.1, 0.5, 2.0, 50 ppm) through GC analysis. It was found that the considerable removal of malathion with elapsed time in inoculated media and more than 50% of malathion (0.1, 0.5 ppm) and 90% of malathion (2.0, 50 ppm) was degraded respectively during the first four hours later, malathion was further degraded to more than 90% (0.1, 0.5 ppm) and 99% of malathion (2.0, 50 ppm). For calculating the final concentration of pesticides peak corrected area (peak area-control area) were used. The results also confirmed the reduction in pesticide concentration due to the reduction in peak height and area compared with control. **(Figure 3b)** at each step of purification malathion degradation was observed **(Table 5)**. These results justified that purified enzyme strongly degraded malathion (50ppm) within 1 hour and has a potential applicability for detoxification of malathion residues.

Previously, carboxylesterase from other microbial sources have been reported **[16]** however, the role of carboxylesterase in *Escherichia coli* IES-02 had not been characterized yet. This is the first study representing that *Escherichia coli* IES-02 carboxylesterase provides easier and safer microbial source to use for the degradation of organophosphorus pesticides.

3.6. Estimation of molecular weight of malathion degrading enzyme

Native PAGE was performed for the identification of molecular weight of malathion degrading enzyme. The results showed three sharp bands of 33.0, 30.0, 28.0 kDa **(Figure 4a)**. Approximate molecular weight of carboxylesterase was determined by corresponding it with the relative mobility of standard molecular weight marker. These results are also justify by similar findings with the molecular weight of carboxylesterase band I (33kDa), II (30kDa) and III (28kDa) represented from extract of wheat kernel **[17]**. Carboxylesterase are low molecular weight protein and different researcher reported molecular weight in the range of 25-60 kDa. Previous studies reported that there are slow moving and fast moving esterase with hydrolyzing activity of malathion **[18]**. *Bacillus licheniformis* S-86 strain is able to produce type I and II bands of 38.4 kDa esterases **[19]**. Pesticide degrading enzymes mentioned different molecular masses of carboxylesterase, such as type-B carboxylesterase (53 kDa) from *Bacillus sp*. BP-7 **[20]**, carboxylesterase B1 (65 kDa) from *Culexpipiens* **[21]** and carboxylesterase (31 kDa) from *Sphingobium sp*. strain JZ-1 **[22]**

3.7. Identification of degradation metabolites

The degradation products of malathion by *Escherichia coli* IES-02 were extracted and identified by GC-MS. The metabolite peaks were identified using documented data from National Institute of Standards and Technology (NIST) library database. The GC analysis peaks revealed that there were seven metabolites peaks occurring around 8–28 min corresponded with isomers of

malathion and different metabolites including, malathion monocarboxylic acid (MMA), malathion dicarboxylic acid (MDA), succinic acid, mercapto, diethyle ester, S-ester with O, S-dimethyl phosphorodithioate, Oxalic acid isobutyl nonyl, ethyl hydrogen fumarate and diethyl maleate which appeared after 8hours of incubation **(Figure 5)**. The mass spectrum of malathion metabolites are shown in **(Figure 6 a, b, c, d, e, f)**. Different reports suggested that malathion monocarboxylic acid is considered as universal primer hydrolytic metabolite of malathion and predominantly produced by the electron theory**.** Malathion dicarboylic acid were produced as a minor metabolite**.** The result have been reported previously by **Yoshii et al. 2008** that the isozymes of the band I (33kDa), II (30kDa) carboxylesterase tended to produced comparatively large amount of malathion monocarboxylic acid and III (28kDa) lowest molecular weight carboxylesterase have a tendency to produce a relatively small amount of malathion dicarboxylic acid **[17].** The proposed pathway of malathion metabolites were identified and confirmed by GC-MS based on the resemblance of their fragment and molecular ions with those of corresponding authentic compounds. Novel detoxification pathway for malathion was proposed on the basis of transformation products **(Figure 7)**. Mono and diacid metabolites formed through carboxylesterase activity **[23]**. The obtained data indicated that malathion was detected as a parent compound at 26.53 minutes, M.W 285. Malathion esterase transformed malathion into most common degradation product malathion monocarboxylicacid (MMA) at 26.25 minutes, M.W 302 **(Figure 6a)** and malathion monocarboxylestease converted MMA into malathion dicarboxylic acid (MDA), at 23.48 minutes **(Figure 6b),** M.W 274 with parallel formation of diethyl malate 8.19 minutes, M.W 172 **(Figure 6f)**. Small amount of other metabolite were also detected including ethyl hydrogen fumarate 8.58 minutes, M.W 143 **(Figure 6e) [24].** Malathion dicarboxyloxidoreductase transformed MDA into succinic acid, mercapto, diethyle ester, S-ester

with O, S-dimethyl phosphorodithioate 23.24 minutes, M.W 283 **(Figure 6c)[25]** and oxalic acid 13.89 minutes, M.W 113 **(Figure 6d)**. A similar degradation pathway was suggested by **Paris et al. 1951 [26]**. These results suggested the formation of these metabolites by the activity of carboxylesterase **[27], [28], [29].** Results were in the same trend with those obtained by **Kamal et al. 2008** declared that strain of *Bacillus thuringiensis* was able to utilize malathion and degrade it into malathion monocarboxylic acid and malathion dicarboxylic acid **[4]**. Carboxylesterase activity, which degrades malathion to its monoacid and diacid, is the predominant degradative pathway **[30]**. Identification of metabolites has been detected in previous studies and thus mapping the entire decomposition processes may contribute to a better comprehension of malathion biodegradation processes. In this study novel metabolites were also formed by degradation of malathion.

Malathion Monocarboxylic acid (MMA) and malathion dicarboxylic acid (MDA) are the metabolites obtained from esterase activity. Some other peaks of succinic acid , mercapto, diethyle ester, S-ester with O, S-dimethyl phosphorodithioate, Oxalic acid isobutyl nonyl, ethyl hydrogen fumarate and diethyl maleate have also been observed in the chromatogram **(Figure 5)** but the percent abundance of those traces are not considerable. And there is no significant impact in the study. These findings are parallel with other findings in which malathion was degraded during biodegradation process and could be converted into these metabolites **[15, 31].**

This finding can beneficially be applied in the prospective practical application in biodegradation of organophosphate that has potential significance in reduction of environmental pollution from ecosystem.

4. Conclusions

The current study justified the comparation between biodegradation rate of malathion organophosphate by *Escherichia coli* IES-02 and its carboxylesterase. Maximum malathion degradation 99% by *Escherichia coli* IES-02 was observed within 4 hour with 50ppm malathion. Carboxylesterase was successfully purified and exhibited 81% degradation rate of malathion (50ppm) within 20 minutes. By comparison of degradation rate of malathion, carboxylesterase showed maximum efficiency towards malathion degradation in short time. The degradation pathway of malathion by carboxylesterase of *Escherichia coli* IES-02 is first proposed which further confirmed degradative end product by GC/MS analysis. *Escherichia coli* IES-02 detoxifying enzyme carboxylesterase revealed remarkable application in control of malathion residues in pesticides wastewater. It can be suggested that *Escherichia coli* IES-02 consumed malathion as a carbon source which reflected highly potential candidate in the biodegradation of organophosphorus in contaminated site.

Conflict of interest

The authors declare that there is no conflict of interest relevant to this manuscript.

Acknowledgments

The authors are grateful to the Institute of Environmental Studies University of Karachi Pakistan and Dr. AQ Khan's Institute of Biotechnology & Genetic Engineering (KIBGE), University of Karachi Pakistan for providing research facilities to conduct this study. Further the authors are thankful to Pesticide laboratory of Pakistan Agricultural Research Council (PARC), Karachi Pakistan for helping in all pesticides related issues.

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Table Caption

Table 1 Generation time and specific growth rate of *Escherichia coli* IES-02

Malathion Concentration	Generation time (minute)	Growth rate constant of *E. coli* population ($minute^{-1}$)
Control	40.90	0.024
0.1ppm	60.40	0.016
0.5ppm	68.05	0.014
2ppm	74.5	0.013
50ppm	91.37	0.010

Table 2 Effect of Malathion concentration and enzyme secretion

Malathion Concentration (ppm)	Total activity (U*)	Total Protein (mg)	Specific activity (U/mg)
0.1	8.0	3.9	2.05
0.5	15.0	3.1	4.83
2.0	29.0	2.7	10.7
50	74.15	1.8	40.12
100	50.50	1.8	27.7

Table 3 Purification steps of Malathion degrading protein from *Escherichia coli* IES-02

Purification steps	Malathion % Degradation	Total Volume (ml)	Total Protein (mg)	Total activity (U*)	Specific activity (U/mg)	Purification Fold
Crude	**88.11**	**100**	**1.848**	**74.159**	**43.9**	**1**
20%*	**41.29**	**10**	**1.54**	**98.0**	**63.63**	**1.449**
40%*	**85.97**	**10**	**0.99**	**125.79**	**127.06**	**2.89**
60%*	**99.1**	**10**	**0.69**	**155.80**	**225.79**	**5.14**
Desalting	**97**	**5**	**0.345**	**290**	**840.5**	**19.13**
Filtration	**98.27**	**5**	**0.14**	**236**	**1685.71**	**38.39**

* ***Ammonium sulfate precipitation***

Table 4 Residual Percentage of malathion in minimal salt media analyzed by peak area

Initial Malathion concentration (µg/ml)	Retention Time (min)	Peak corrected area	Initial Malathion concentration (µg/250ml)	Malathion concentration After 24hrs (µg/250ml)	Malathion remaining after 24 hours of incubation (%)
Control	7.81	79689	-	-	-
Standard	7.81	64052897	12.5µg/50ml	-	-
0.1	7.81	4589537	25µg/250ml	0.917	0.37
0.5	7.81	2811753	125µg/250ml	5.623	4.50
2	7.81	901246	500 µg/250ml	1.802	0.37
50	7.81	2568214	12500µg/250ml	51.364	0.42

Table 5 Malathion degradation by carboxylesterase

Samples	Corrected Area	Malathion % remaining After 20 min	After 40 min	After 60 min
Crude	9521527	54.67	29.54	11.89
20%*	1888539	79.44	64.38	58.71
40%*	2217356	49.10	20	14.03
60%*	1773200	29.27	5.44	0.9
Desalting	1200000	21.28	3.18	2.96
Filtration	700000	18.76	1.98	1.728

Figure Captions

Figure 1. (a) Morphological Analysis of *Escherichia coli* IES-02 on Eoisen methylene blue (EBM) agar plates. (b) Scanning electron micrographs of *Escherichia coli* IES-02 at magnification of 11,000 ×. (c) Scanning electron micrograph of *Escherichia coli* IES-02 with malathion concentration (50ppm) at magnification of 9,000×.

Figure 2. Evaluation of Growth kinetics and degradation rate of malathion (a) Control: Growth kinetics of *Escherichia coli* IES-02 in minimal salt medium; (b) Growth kinetics and degradation of malathion at 0.1ppm; (c) Growth kinetics and degradation of malathion at 0.5ppm, (d) Growth kinetics and degradation of malathion at 0.1ppm 2.0ppm, (e) Growth kinetics and degradation of malathion at 50ppm.

Figure 3: GC analysis of malathion degradation (a) GC chromatogram of malathion standard peak at 12.50ppm; (b) Analysis of GC chromatogram of malathion degradation at 50ppm (1) peak at 4hr, (2) peak at 8hr, (3) peak at 24hr; (c) Percentage of residual amount of malathion in minimal salt media inoculated with *Escherichia coli* IES-02.

Figure.4. (a) Crude and purified fractions of carboxylesterase were analyzed by SDS-PAGE:(Lane M) contains protein standards with their mass indicated in kDa on the left, (Lane 1) contain crude protein, (Lane 2) contains from partially purified carboxylesterase obtained from purification step, (Lane 3) contain the purified carboxylesterase from filtrate; (b) GC analysis of different fractions of crude and purified carboxylesterase at 5ppm malathion; (c) Percentage of

residual amount of malathion in minimal salt media inoculated with purified carboxylesterase.

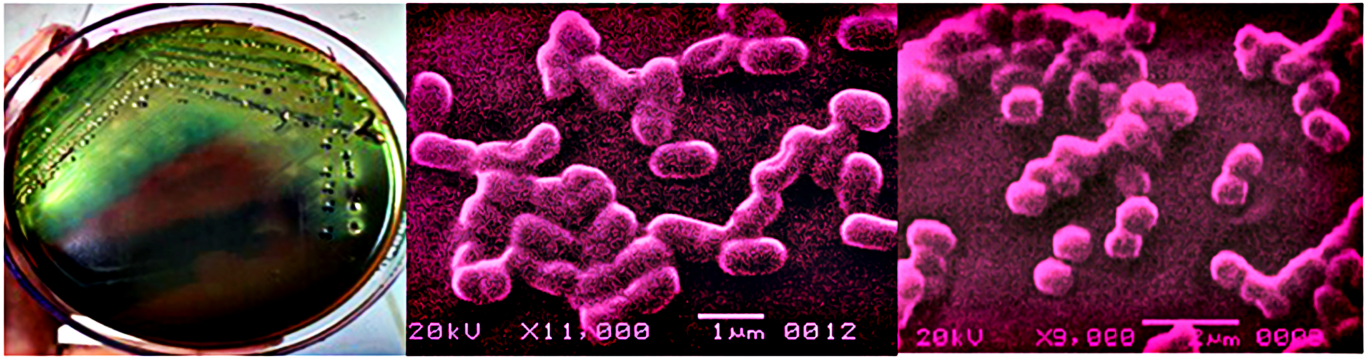
Figure 5: Degradation products of malathion after 8hours of incubation.

Figure 6: Mass-spectrum studies of proposed malathion metabolites; (a) malathion monocarboxylic acid with m/z 302at 26.253 min; (b) malathion dicarboxylic acid with m/z 274 at 23.48 min; (c) succinic acid , Mercapto, diethyle ester, S-ester with O, S-dimethyl phosphorodithioate with m/z 283 at 23.24 min; (d) Oxalic acid isobutyl nonyl ester with m/z 113 at 13.89 min; (e) Ethyl Hydrogen Fumarate with m/z 143 at .8.58 min; (f) Diethyl maleate with m/z 1172 at 8.19 min.

Figure 7: Proposed metabolic pathway of malathion degradation.

Highlights

- *Escherichia coli* IES-02 isolated from malathion contaminant effluent and the strain served as potential malathion degrader.
- A novel carboxylesterases were purified from *Escherichia coli* IES-02 and served as Malathion degrading protein.
- Purified carboxylesterase showed 81% Malathion degradation within 20 minutes as compared to *Escherichia coli* IES-02 cells metabolizes 99% Malathion within 4 hours.
- In this study Malathion transformation products pathway was proposed.



a **b** **c**

Figure 1

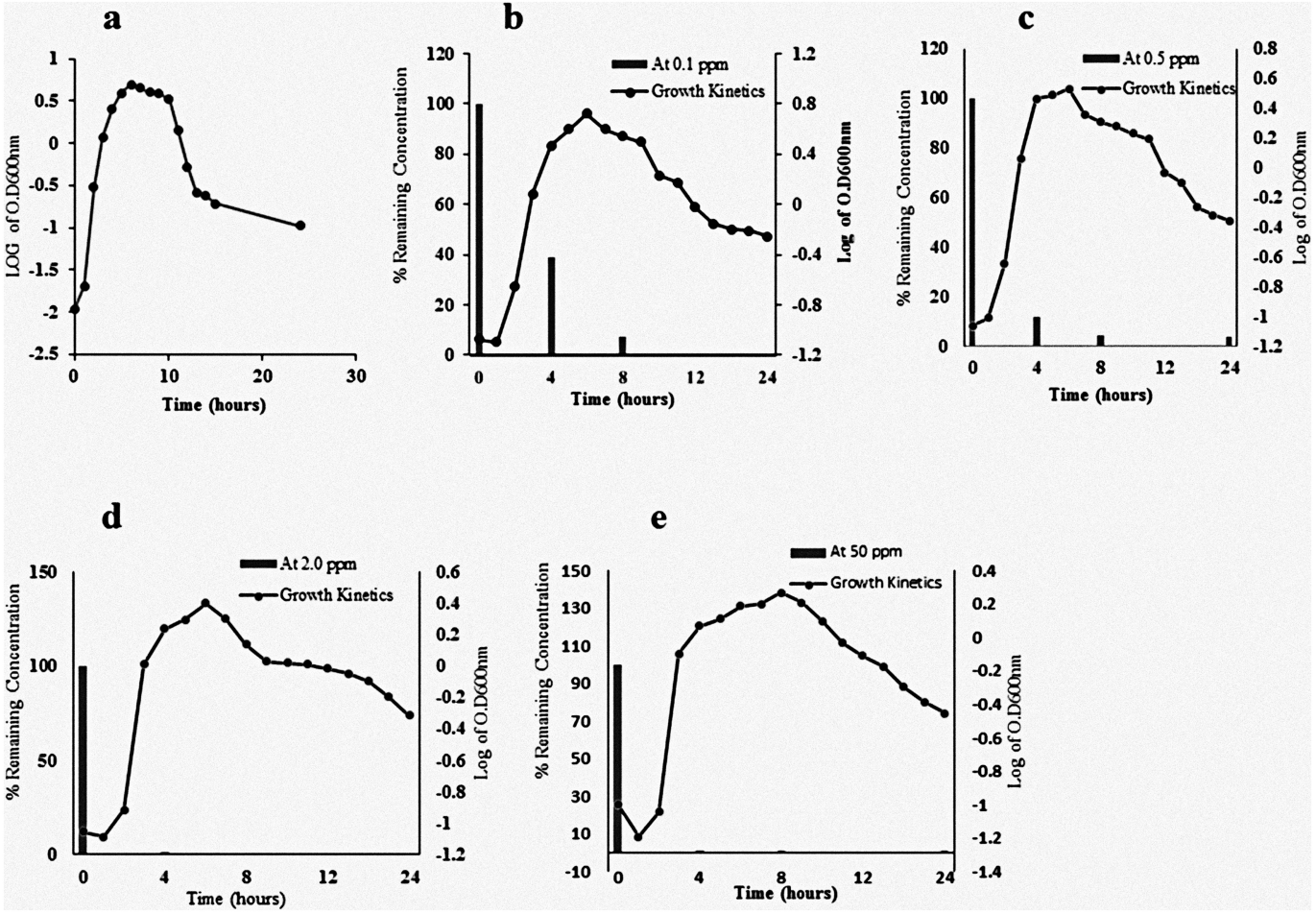
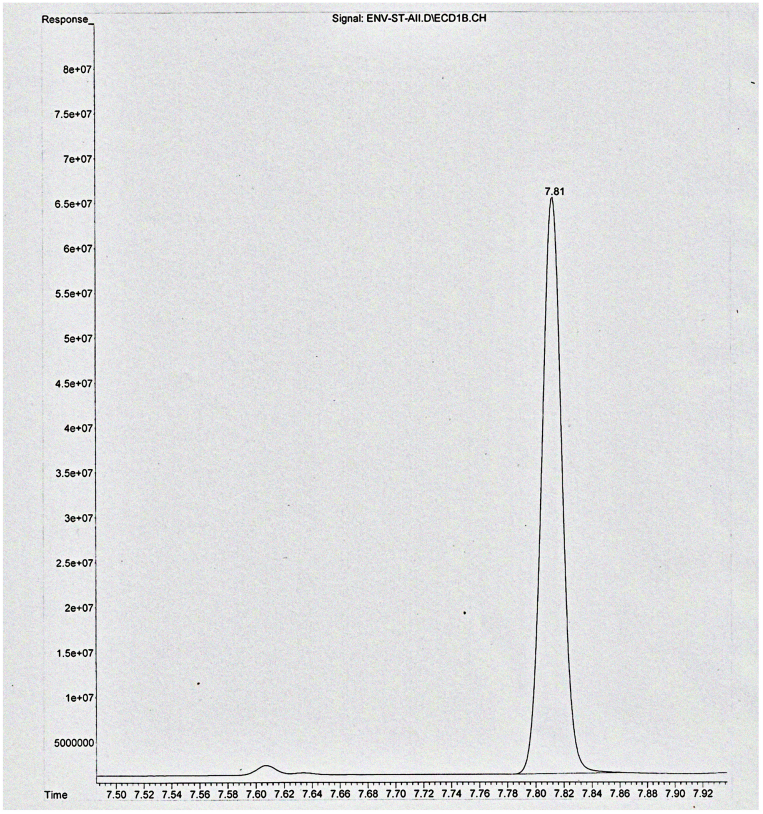
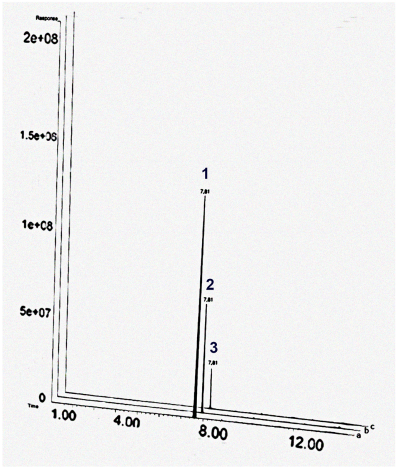

a
LOG of O.D600nm
Time (hours)
b
At 0.1 ppm
Growth Kinetics
% Remaining Concentration
Log of O.D600nm
Time (hours)
c
At 0.5 ppm
Growth Kinetics
% Remaining Concentration
Log of O.D600nm
Time (hours)
d
At 2.0 ppm
Growth Kinetics
% Remaining Concentration
Log of O.D600nm
Time (hours)
e
At 50 ppm
Growth Kinetics
% Remaining Concentration
Log of O.D600nm
Time (hours)

Figure 2


a
Response_
Signal: ENV-ST-All.D\ECD1B.CH
8e+07
7.5e+07
7e+07
6.5e+07
6e+07
5.5e+07
5e+07
4.5e+07
4e+07
3.5e+07
3e+07
2.5e+07
2e+07
1.5e+07
1e+07
5000000
7.81
Time
7.50 7.52 7.54 7.56 7.58 7.60 7.62 7.64 7.66 7.68 7.70 7.72 7.74 7.76 7.78 7.80 7.82 7.84 7.86 7.88 7.90 7.92


b
2e+08
1.5e+08
1e+08
5e+07
0
1
2
3
1.00
4.00
8.00
12.00

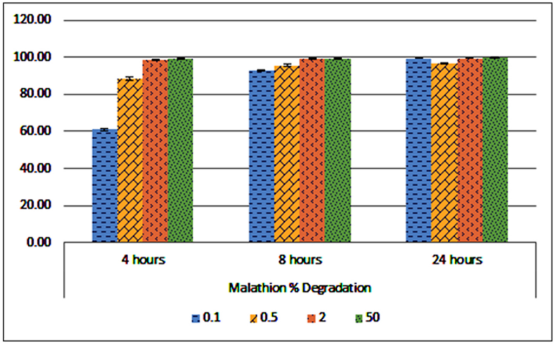
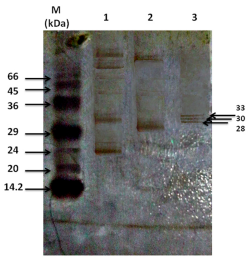
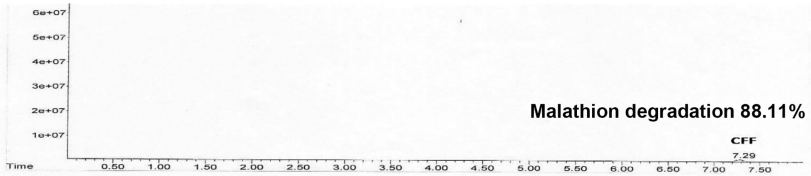

c
120.00
100.00
80.00
60.00
40.00
20.00
0.00
4 hours
8 hours
24 hours
Malathion % Degradation
0.1
0.5
2
50

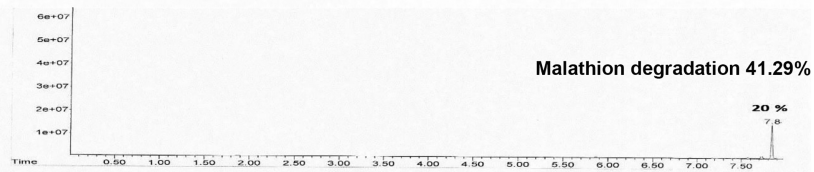
Figure 3

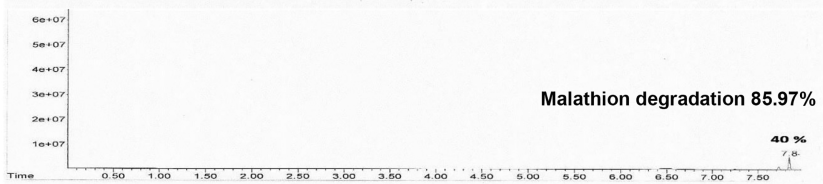
a

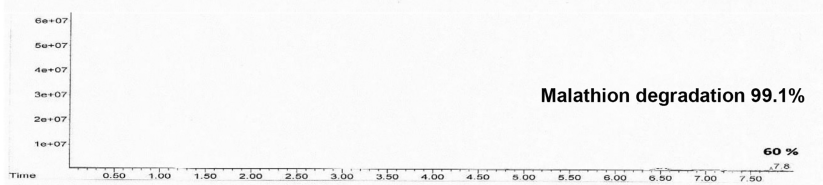


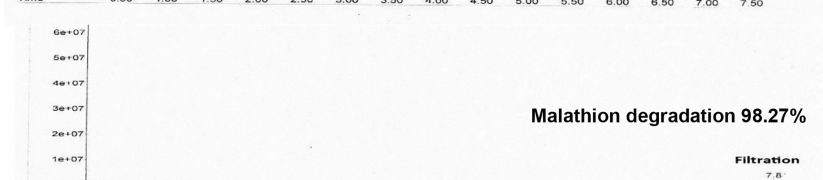
b



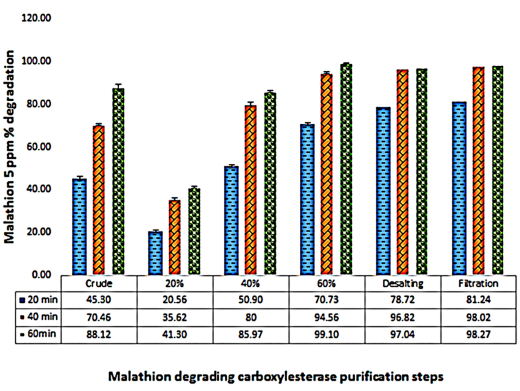






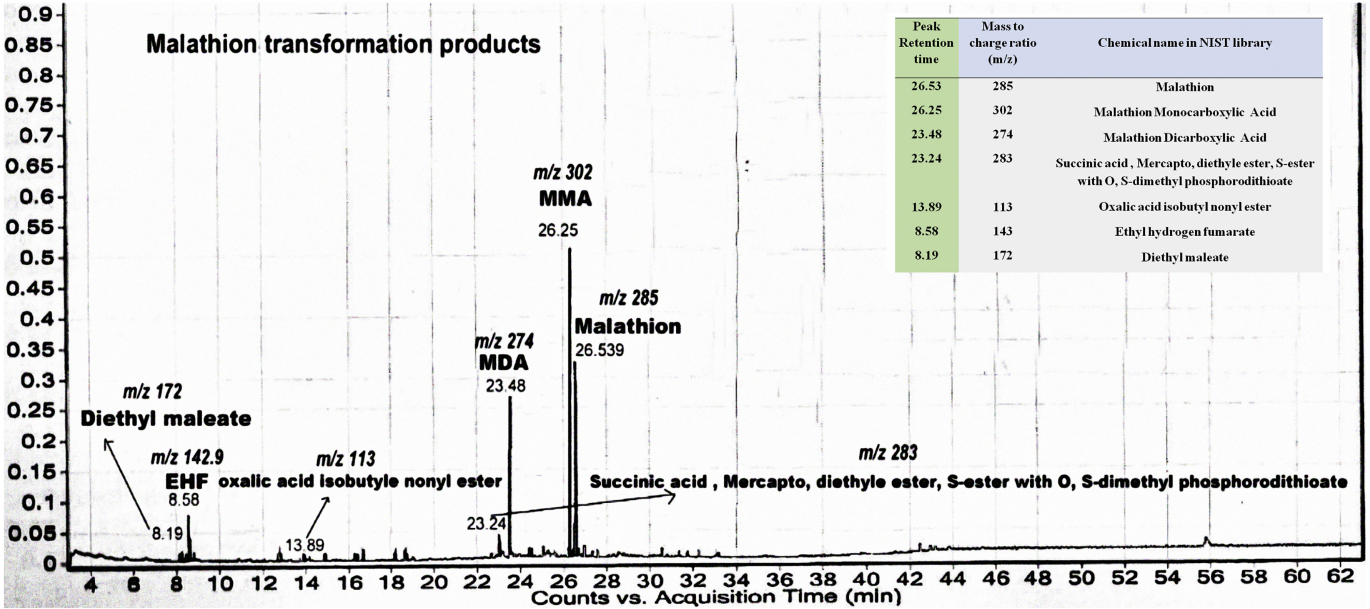


c



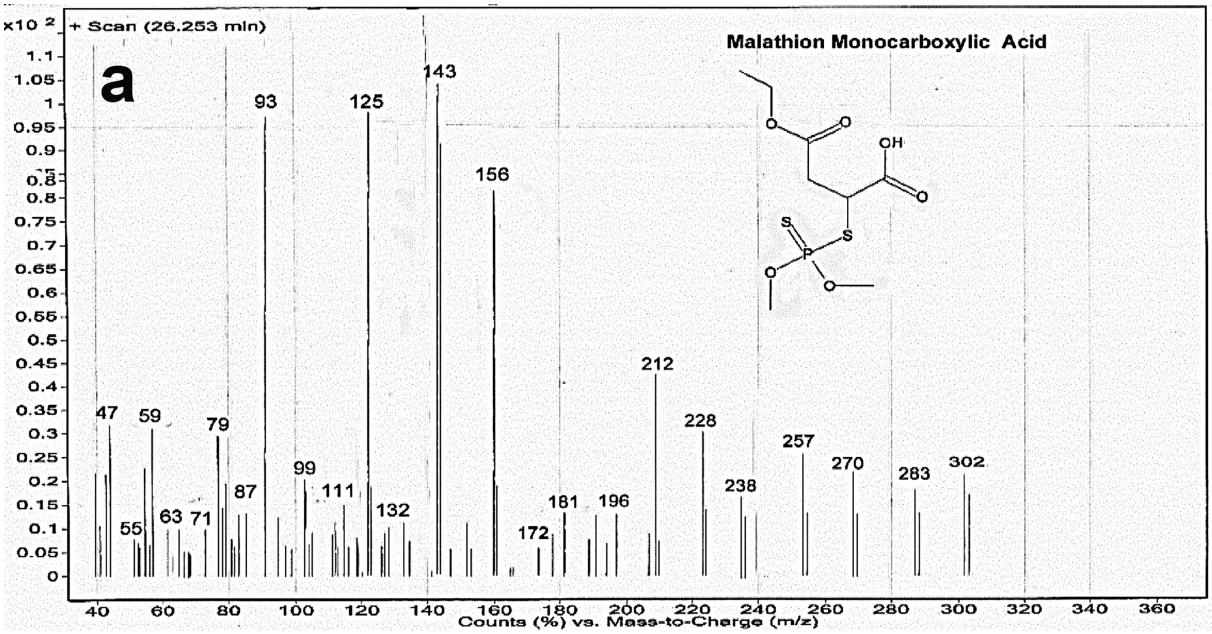
	Crude	20%	40%	60%	Desalting	Filtration
20 min	45.30	20.56	50.90	70.73	78.72	81.24
40 min	70.46	35.62	80	94.56	96.82	98.02
60min	88.12	41.30	85.97	99.10	97.04	98.27

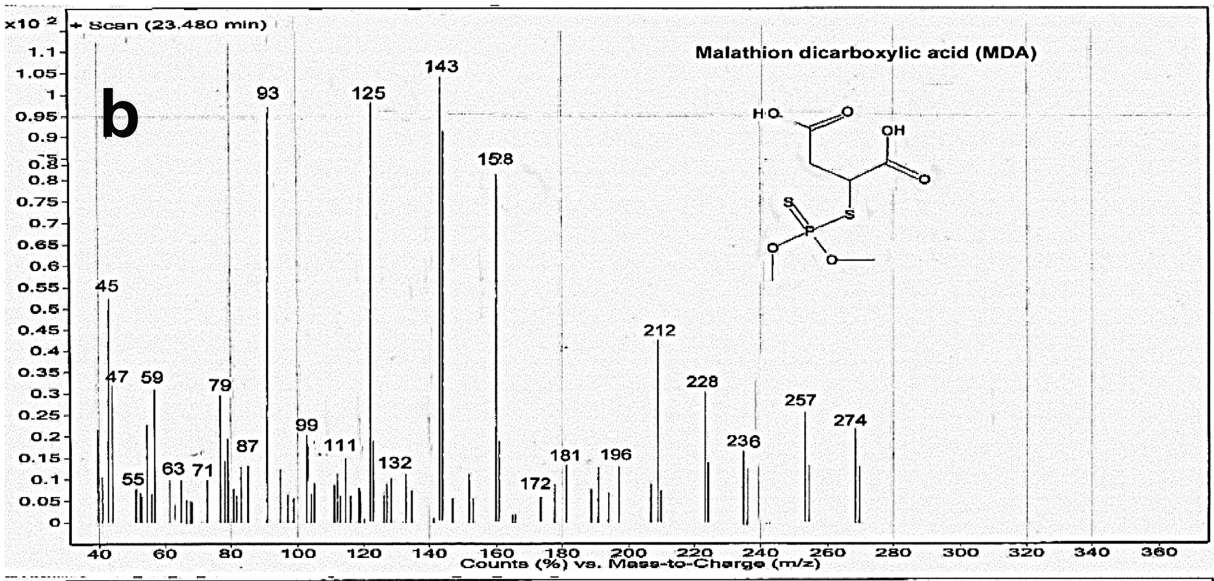
Figure 4



Peak Retention time	Mass to charge ratio (m/z)	Chemical name in NIST library
26.53	285	Malathion
26.25	302	Malathion Monocarboxylic Acid
23.48	274	Malathion Dicarboxylic Acid
23.24	283	Succinic acid , Mercapto, diethyle ester, S-ester with O, S-dimethyl phosphorodithioate
13.89	113	Oxalic acid isobutyl nonyl ester
8.58	143	Ethyl hydrogen fumarate
8.19	172	Diethyl maleate

Figure 5


a
+ Scan (26.253 min)
Malathion Monocarboxylic Acid
Counts (%) vs. Mass-to-Charge (m/z)


b
+ Scan (23.480 min)
Malathion dicarboxylic acid (MDA)
Counts (%) vs. Mass-to-Charge (m/z)

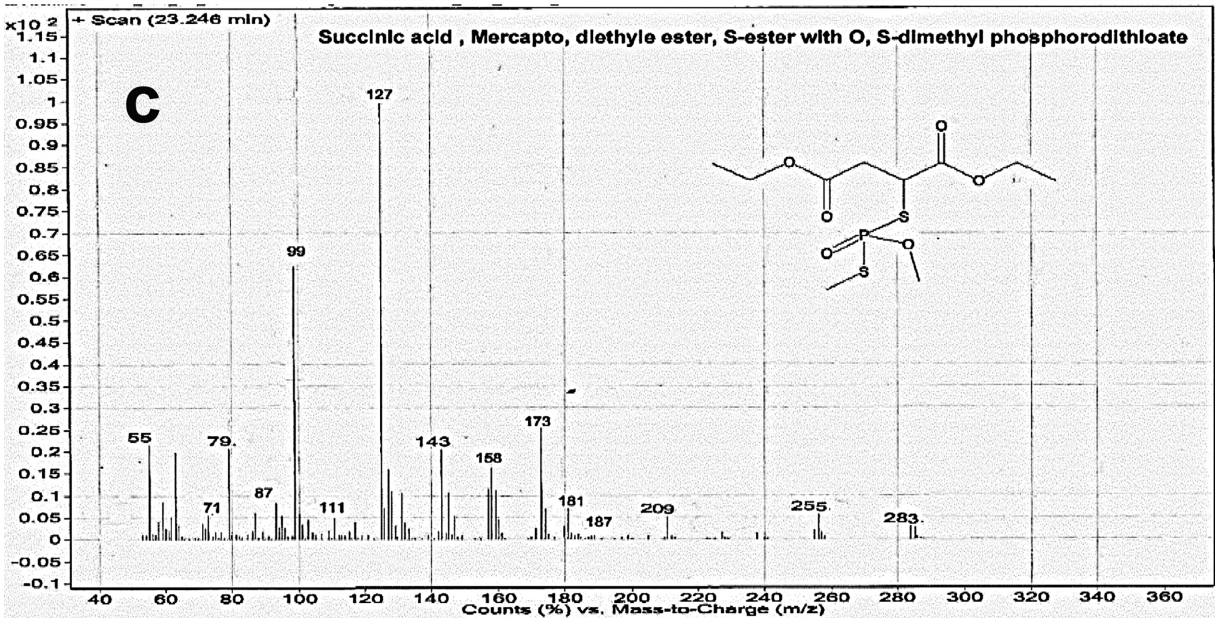
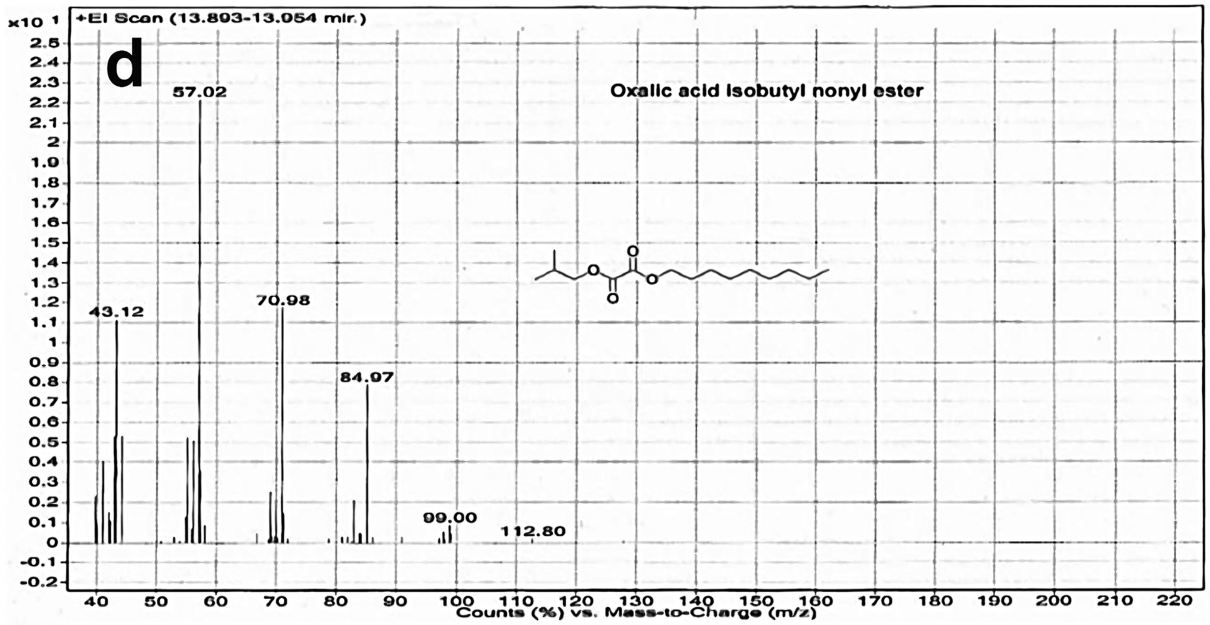
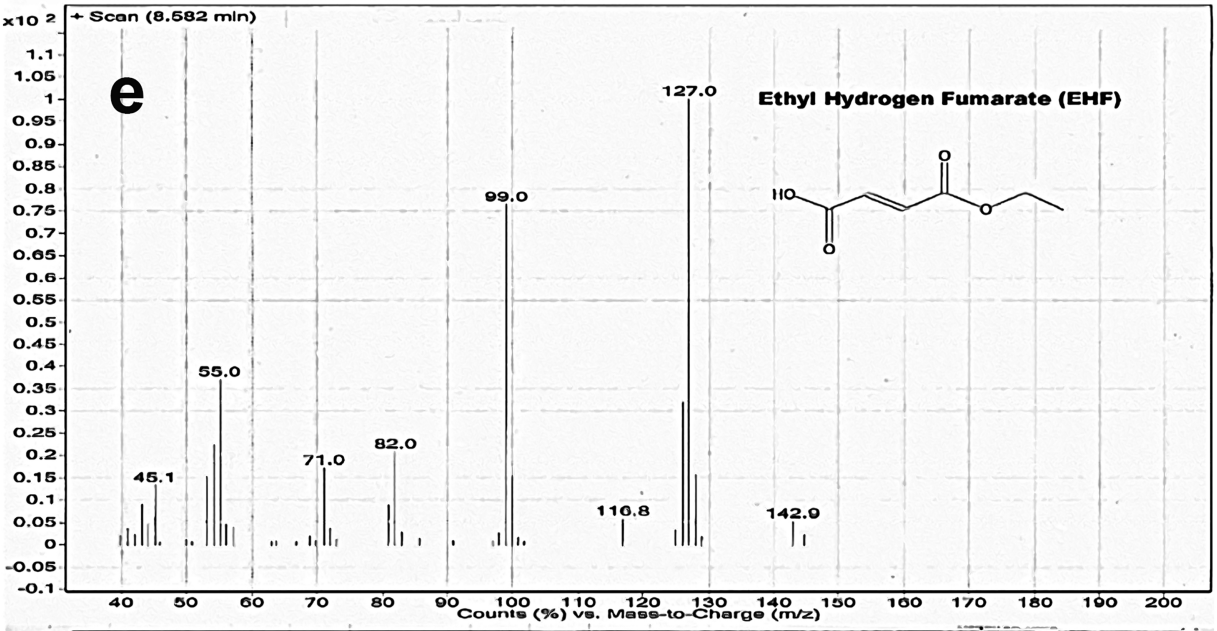

c
+ Scan (23.246 min)
Succinic acid , Mercapto, diethyle ester, S-ester with O, S-dimethyl phosphorodithioate
Counts (%) vs. Mass-to-Charge (m/z)

Figure 6ac


d
+EI Scan (13.803-13.054 min.)
Oxalic acid isobutyl nonyl ester
57.02
43.12
70.98
84.97
99.00
112.80
Counts (%) vs. Mass-to-Charge (m/z)


e
+ Scan (8.582 min)
Ethyl Hydrogen Fumarate (EHF)
127.0
99.0
55.0
82.0
71.0
45.1
116.8
142.9
Counts (%) vs. Mass-to-Charge (m/z)

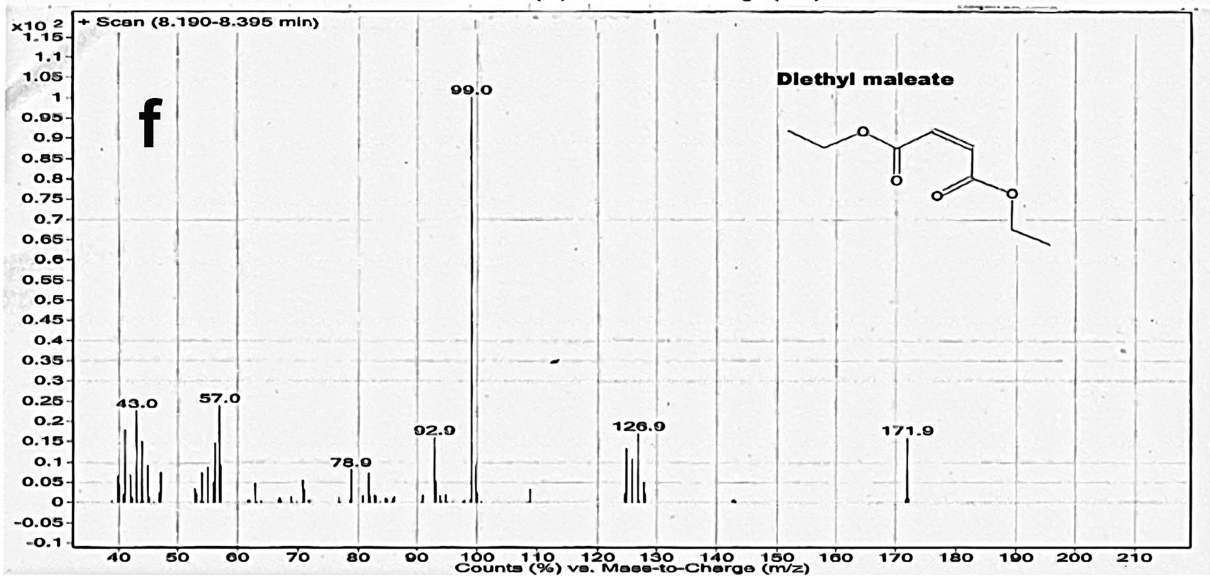

f
+ Scan (8.190-8.395 min)
Diethyl maleate
99.0
43.0
57.0
92.0
126.9
78.0
171.9
Counts (%) vs. Mass-to-Charge (m/z)

Figure 6df

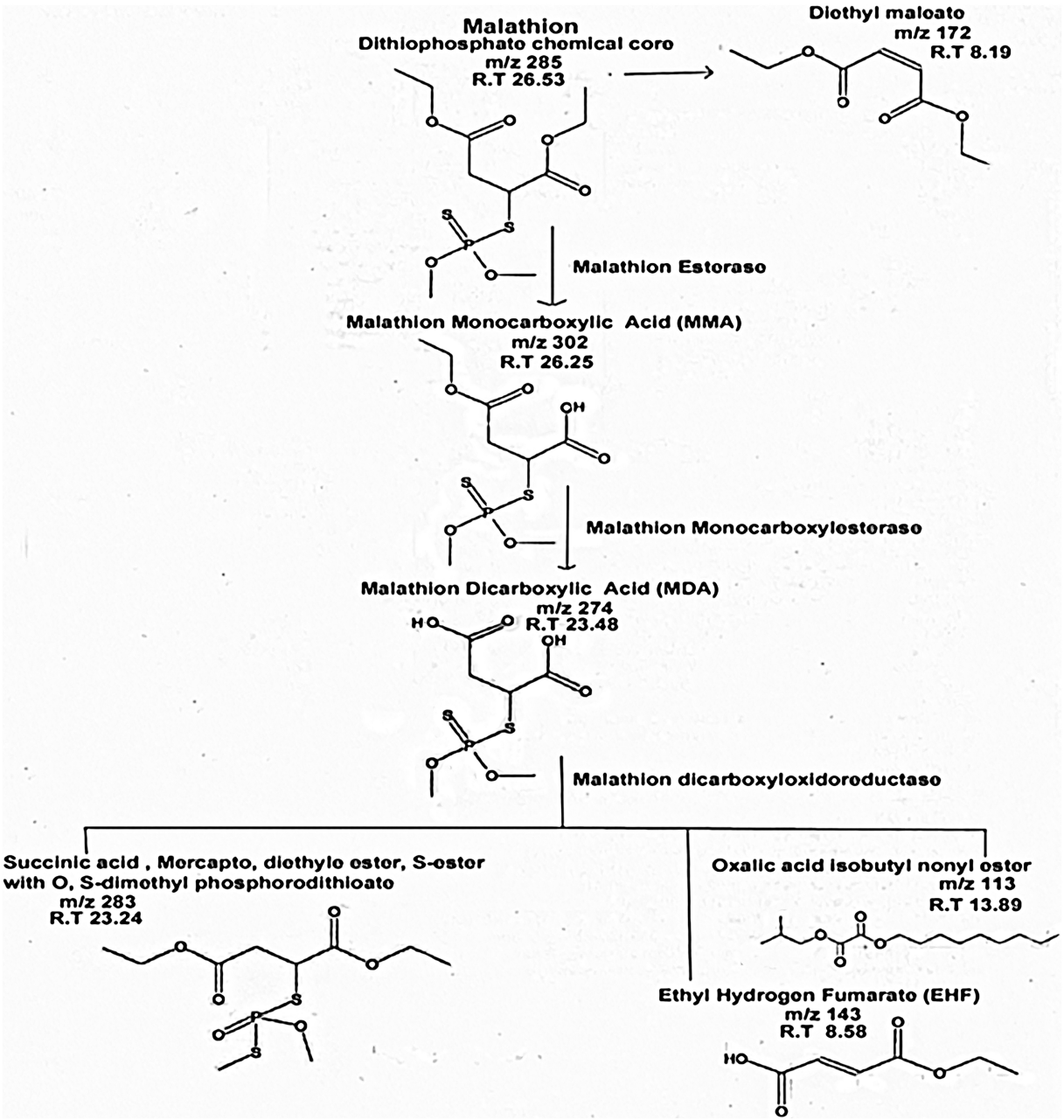

Malathion
Dithiophosphato chomical coro
m/z 285
R.T 26.53
Diethyl maloato
m/z 172
R.T 8.19
Malathion Esterase
Malathion Monocarboxylic Acid (MMA)
m/z 302
R.T 26.25
Malathion Monocarboxylesterase
Malathion Dicarboxylic Acid (MDA)
m/z 274
R.T 23.48
Malathion dicarboxyloxidoreductase
Succinic acid , Mercapto, diethyle ester, S-ester with O, S-dimethyl phosphorodithioate
m/z 283
R.T 23.24
Oxalic acid isobutyl nonyl ester
m/z 113
R.T 13.89
Ethyl Hydrogen Fumarate (EHF)
m/z 143
R.T 8.58

Figure 7