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Role of a few bacterial species on biodegradation of organophosphorous pesticide (methyl parathion): An approach to access the outcome of biodegradation by GC-MS & HPLC

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ABSTRACT

Biodegradation is a promising tool for complete destruction of organic pollutants present in the environment. Organophosphorus compound (OPs) like methyl parathion (MP) is widely used as pesticide in agricultural as well as non-agricultural fields. The pesticides of organophosphorus type is less bio accumulative and more readily degradable in the environment. The present study was focused on the biodegradation of methyl parathion pesticide using soil bacteria. Among the several bacterial isolates from soil, three species identified as Pseudomonas aeruginosa, Enterobacter sp and Klebsiella sp. The growth of these pesticide degrading bacteria was assessed in nutrient broth. The experimental study was conducted to investigate the efficiency of the chosen bacterial species on the degradation of methyl parathion and its effect on bacterial growth. The degradation of the pesticide was tested in three concentrations like 100 ppm, 200 ppm and 300 ppm. Among the three bacterial species used for degradation of methyl parathion at different concentrations, maximum degradation was observed by Pseudomonas aeruginosa at a temperature of 35^0C at pH 7 in 59 hrs at 300 ppm concentration. The degradation products of all the three bacterial samples were characterized by FTIR, HPLC and GC-MS.

Key words: Biodegradation, Methyl Parathion, *Pseudomonas aeruginosa, Enterobacter sp. and Klebsiella sp.*

INTRODUCTION

Agriculture is the back bone of Indian economy. Ensuring food security for more than one billion Indian population with decreasing cultivable land resource is very poor. This could be ensured by the use of high yielding variety of seeds, balanced use of fertilizers and sensible use of quality pesticides. To meet the needs of food requirements for increasing population in the country, currently farmers are using higher amounts of fertilizers and pesticides in the field of crop production [1]. Millions of tons of pesticides are applied annually in modern agriculture to increase the production through controlling harmful effects caused by the target organisms including insects, fungi, worms and nematodes [2]. Pesticides can be generally classified into three groups namely organochlorine, organo-phosphorous or phosphates and carbamides [3]. Organophosphorous pesticides are an essential group of pesticide used widely all over the world, the degradation of this pesticide is very faster than compared to other groups of pesticides [4,5]. Organophosphorous pesticides such as parathion, methyl parathion, carbamate and chlorpyrifos are a group of highly toxic agricultural chemicals commonly used in plant protection and to kill the insects [6,7]. Though, the majority of the applied pesticides, even if sprayed on greenery of crops, vegetable plants and weeds, the excessive

use of pesticides leads to an accumulation of a huge amount of pesticide residues in the food chain and eventually reach the air, soil, surface water and ground water environment that further leads to a substantial health hazard for the current and future generations due to uptake and accumulation of these toxic compounds, which may affect the growth and activity of soil microbial communities [8].

Methyl parathion (O, O-Dimethyl-O-(4-nitrophenyl) phosphorothioate) is an important broad spectrum organophosphorus insecticide. It is used to control insects such as aphids, mealybugs and mites on a wide variety of crops, including cereals, fruits, vegetables, ornamentals, sugarcane and cotton [9]. It is also used to increase agricultural yields, but after usage they remain in soil and water. MP inhibits acetylcholinesterace (AChE) and also suppresses the activity of acetylcholinesterase as it has neurotoxicological properties [10]. It causes irreversible phosphorylation of esterase in the central nervous system of insects, mammals and also in nontarget organisms, including humans, resulting in headache, nausea, skin damage, cancer, birth defects and even death. These healthiness and environmental worries have led to considerable interest in emerging ecofriendly, safe and economical alternatives for the detoxification of MP in soil, ground water and surface water matrices. At present year one of the major environmental problems facing the world, contamination of soil, water and air by toxic chemicals such as dyes, chemical industrial waste and pesticides. The various cleanup technologies available for degradation of pesticides are photocatalytic degradation, electrochemical degradation and biodegradation process [11,12]. Although MP is considered a relatively persistent insecticide in the environment, it can be degraded in natural soil and water by microorganisms in a short period of time [13,14].

Bioremediation is a promising alternative to physicochemical and biological methods of degradation of pesticides because it is less expensive, cost-effective and environmental friendly technique. Several researchers have focused on the degradation of herbicides [15,16] algicides [17] maticides [18], and some other researcher has reported the fungal degradation of different pesticides [19-25]. Reports are available on the biodegradation of various pesticides under different conditions [26,27] by using contaminated water [28,29], fresh water and marine water [30], while few of them have reported the degradation of various pesticides by using isolated bacteria in various agricultural fields such as rice fields [31-35] tomato crop field [36] and coffee farm soil [37]. Many bacterial strains from soil such as *Brevibacterium aureum* and *Pigmentiphaga sp*. have been reported completely or partially degradation of pesticide in environment [38,39]. The present investigation is focused on biodegradation of MP by the soil bacteria under different ranges of temperature, pH and varying concentrations of MP. Experiments were therefore designed and executed to study the role of soil bacteria in biodegradation of MP and also to optimize the conditions to maximize the degradation process.

MATERIALS AND METHODS

Chemicals

Analytical grade Organophosphorous (Methyl Parathion), (99.9%) and all other solvents were purchased from Sigma Aldrich Mumbai, India. All other reagents used in the study were of Analytical Grade. All the chemicals were stored in dark bottles at 4^0 C prior to use.

Collection of soil samples

The soil samples were collected from the different cultivated fields mainly (crop, sugarcane, vegetables and fruits fields) from the top layer 0-10 cm which had been exposed to MP pesticide in Tiruchirapalli District, Tamil Nadu, India. In the laboratory, the soil samples were stored in sterile polythene bags at 4^0 C.

Isolation and identification of the soil bacterium

The collected soil samples were subjected to serial dilution and pour plate technique for the isolation of pesticide degrading bacteria with the help of Nutrient agar and King's B agar plates. Well grown bacterial colonies were picked and further purified by streaking. The isolated strains were maintained on Nutrient agar and King's B agar slants and stored at 4^0 C. Identification process for the three different bacterial isolates were carried out by following the routine bacteriological methods.

Studies on bacterial growth and degradation of methyl parathion using soil bacteria

After the isolation of bacteria from soil, they were identified and characterized by biochemical tests. The pure bacterial cultures obtained were selected for degradation of methyl parathion. The effect of temperature, pH and pesticide concentration on pesticide degradation were studied in this experiment, where the temperature ranged from

15^0C to 45^0C and pH from 3 to 11, with methyl parathion pesticide concentration from 100 ppm to 300 ppm respectively. The growth curve obtained for the three chosen bacteria *Pseudomonas aeruginosa, Klebsiella sp* and *Enterobacter sp* was checked using UV- spectrophotometer at various time intervals.

Effect of Temperature and pH on the growth of pesticide degrading bacteria

Temperature is one of the most important factors on microbial survival and growth. To study the ability of the bacteria for the degradation of the pesticide, an experiment was conducted in an Erlenmeyer flask containing 100 ml minimal salt broth where 100, 200 and 300 ppm pesticide was incorporated. After sterilization, the flasks were cooled and inoculated with the bacterial culture and maintained at different temperatures (15^0C, 25^0 C, 35^0C and 45^0C). 5ml of culture was drawn and centrifuged at 5000 rpm for 10 min and the pellet was discarded and the supernatant was collected to evaluate the growth of pesticide degrading bacteria. The optical density was taken at 560nm using UV- spectrophotometer at various time intervals. Similar input was carried out at different pH (3.0, 5.0, 7.0, 9.0 and 11.0) ranges and the optical density was taken at 560 nm. Identification of pesticide degradation products of residual concentration of MP and its metabolites were characterized by using FTIR, HPLC and GC-MS.

Analytical characterization of pesticide degrading bacteria

Fourier Transform Infrared (FTIR) Spectroscopic analysis of methyl parathion degradation

The FTIR spectra of all the samples were recorded using PerkinElmer IR spectrometer. All the samples were recorded without using any solvent. FTIR spectroscopy was used in this study to obtain qualitative analysis & functional group information for methyl parathion samples. The non-distractive nature of FTIR additionally to analyze the samples by alternative technique was not exposed in this particular study. This method was developed with consideration of possible future adaptation to include both quantification & coupled analytical techniques.

HPLC analysis of pesticide degradation

High Performance Liquid Chromatography (HPLC) was utilized to determine the pesticide residue in the medium containing bacterium. The experiments of pesticides degradation were conducted in Erlenmeyer flasks containing 100 ml nutrient broth in different concentration of pesticide (methyl parathion 100 ppm, 200 ppm, 300 ppm) in separate flasks. Bacterial strains were incubated at pH 7 at 300C for 72 hours. After incubation, the well grown pesticide degrading sample was analyzed by HPLC analytical method.

Extraction of biodegradation products (GC-MS)

The procedure employed for the extraction of sample after four day degradation were separated to analyses by GC/MS. The 5 ml of homogenized liquid samples were collected at regular interval of time after degradation and centrifuged at 8000 rpm for 10 min at 25 ^{0}C. After centrifugation the solid portion (pellet) at the bottom was extracted with 5 ml acetonitrile by sonication. The extract was then filtrated. The supernatant clear solution was centrifuged and the supernatant sample was collected.

GC/MS analysis

A GC/MS was used for the separation and detection of intermediate products of pesticide degradation. An PerkinElmer clarus 500 GC system was used for separation and detection of pesticide degraded products. Turbo mass ver 5.2.0 software was used for GC/MS analysis. The GC separation of Elite-5 capillary column (30m × 250 µm id), helium was used as a carrier gas at a flow rate of 1 ml/ min, the injection temperature was 250 ^{0}C, the column temperature was 280 ^{0}C for 10 min. the temperatures corresponding to transfer line and the ion trap were 200 ^{0}C and 160 ^{0}C respectively. Mass spectra were obtained by the electron-Ionization (EI) mode at 70 ^{0} ev. Scan mode was chosen and the range of amu was form 40-450. The injection volume was 2.0 µl with split range of (1:10) was used to detect and separate the degradation products.

RESULTS AND DISCUSSION

Isolation and characterization of methyl parathion degrading bacterium

Soil samples collected from three different fields (paddy field, sugarcane field and vegetable field) were processed for isolation of the bacterial species. Based on microscopic and biochemical tests, the bacteria identified were, *Pseudomonas aeruginosa, Enterobacter sp,* and *Klebsiella sp.*

Table. 1 The morphological and biochemical characteristics of bacterial strains isolated from agriculture soil

Characteristics	Principle	Results		
		Pseudomonas aeruginosa	***Enterobacter sp***	***Klebsiella sp***
Gram staining	Selective staining of cell wall	Gram -ve	Gram -ve	Gram-ve
Cell Shape	Detects the physical nature	Rod	Rod	Rod
Indole test	Detects deamination of tryptophan	Gram-ve	Gram -ve	Gram-ve
Methyl red	Detects acid production	-	-	-
Voges - Proskauer	Detects acetoin production	-	+	+
Citrate utilization test	Detects capability of organism to utilize citrate as a sole carbon source	+	+	+
Carbohydrate test	Detects gas production	+	+	+
Litmus milk	Detect the coagulation	+	+	+
Oxidase test	Detects cytochrome oxidase production	+	-	-
Catalase test	Detects catalase production	+	+	+
Triple sugar test	Detects alkaline or acid but	Alkaline slant / acid but	Alkaline slant/ acid but	Acid slant/ Alkaline but

In this study the growth of a few bacterial isolates like *Pseudomonas aeruginosa*, *Enterobacter sp*, and *Klebsiella sp* were investigated in mineral salt broth culture of different pH (3.0, 5.0, 7.0 and 9.0). Maximum growth of all the three bacterial isolates were observed at pH 7.0 and the growth rate decreased when the pH increased, but it was strongly inhibited at acidic pH. The biodegradation of methyl parathion by *Pseudomonas aeruginosa, Enterobacter sp,* and *Klebsiella sp* bacteria was investigated further at different temperature. The optimal temperature for the growth of all the three tested isolates was found to be 37^0C, and above and below which, a decrease in the bacterial growth was observed.

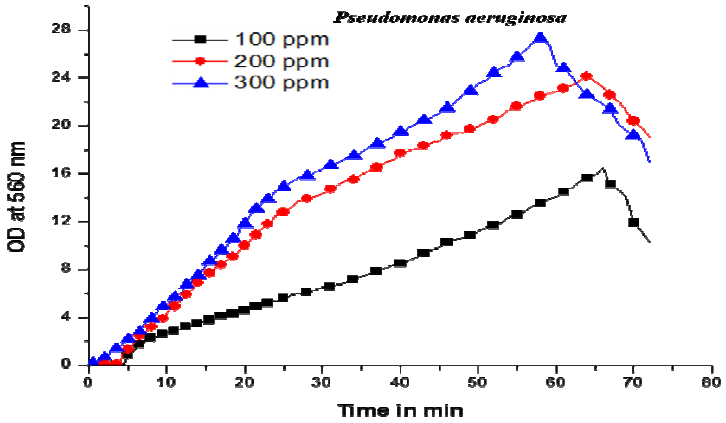


Fig. 1: Degradation of methyl parathion by *Pseudomonas aeruginosa* in nutrient agar medium containing different concentrations of methyl parathion pesticide as the sole source of carbon and energy

FTIR analysis of MP pesticide degradation by isolated soil bacteria

The FT-IR spectral data provides more insight into the structural changes of MP and confirms the degradation of pesticide by the bacteria taken for present investigation. The FT-IT spectra of MP before and after treatment with bacteria were represented in Fig 5,6,7. It is evident that the shifting of characteristic absorption peaks of MP corresponding to the N=O, C-H, P=S, C=C and P-O-CH3 bond groups from 1402.2 cm-1 and 3410.0 cm-1, 694.7 cm-1, 1635.5 cm-1, and 1123.7 after the treatment with bacteria indicating degradation of MP. The shifted peak positions of MP after the treatment with bacteria *Pseudomonas aeruginosa, Enterobacter sp and Klebsiella sp* are represented in table 2 reveal clearly the structural changes occurred due to the degradation process caused by the bacteria.

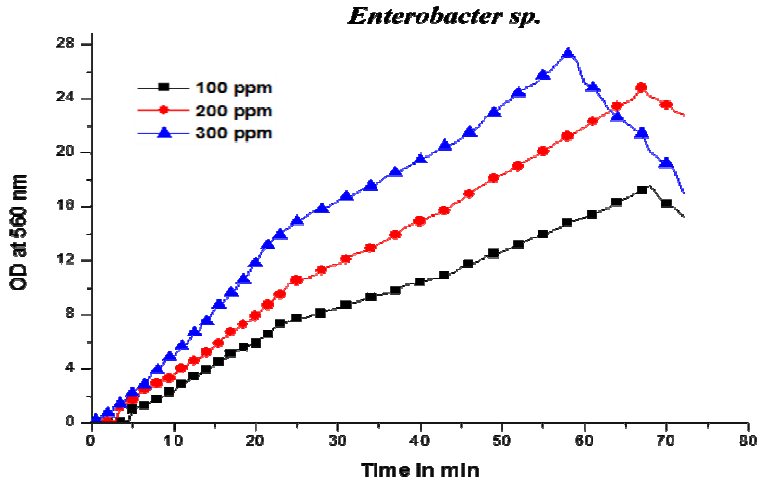


Fig. 2: Degradation of methyl parathion by *Enterobacter sp* in nutrient agar medium containing different concentrations of methyl parathion pesticide as the sole source of carbon and energy

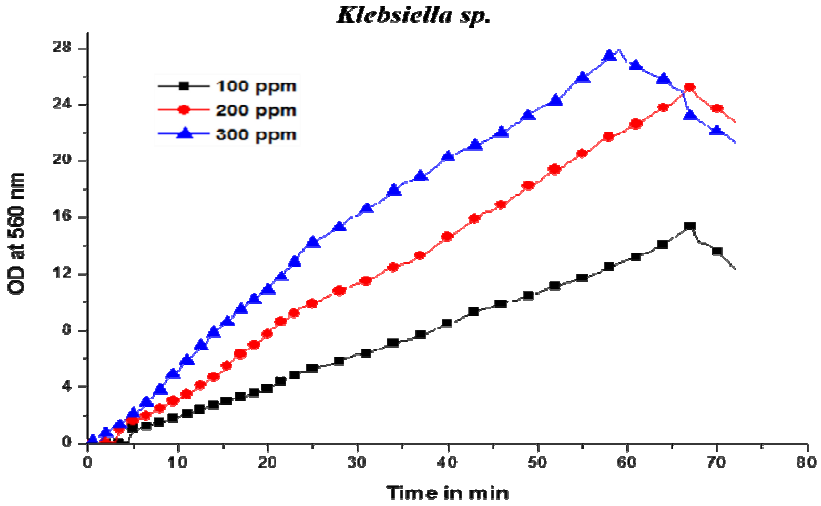


Fig. 3: Degradation of methyl parathion by *Klebsiella sp* in nutrient agar medium containing different concentrations of methyl parathion pesticide as the sole source of carbon and energy

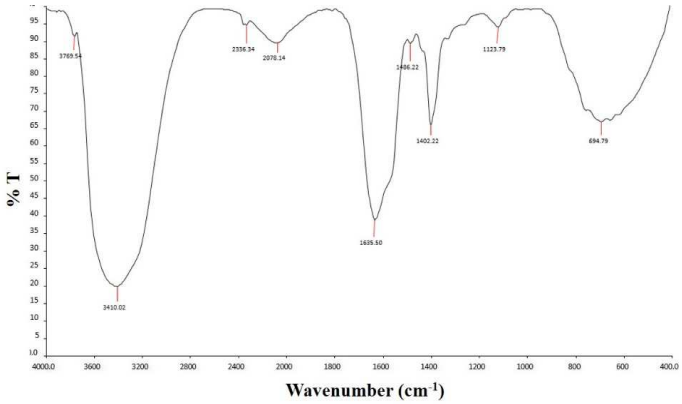


Fig. 4: FT-IR spectrum of methyl parathion pesticide

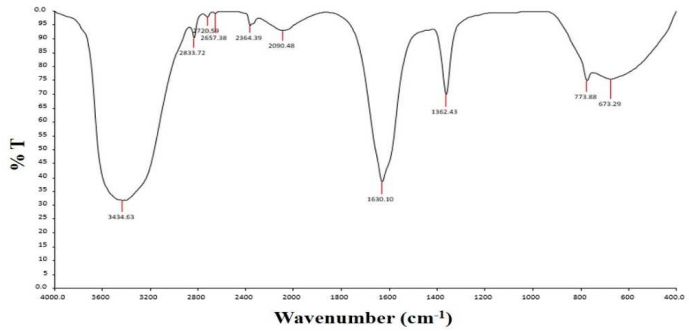


Fig. 5: FTIR spectrum of biodegradation of methyl parathion pesticide in 72 hours by *Pseudomonas aeruginosa*

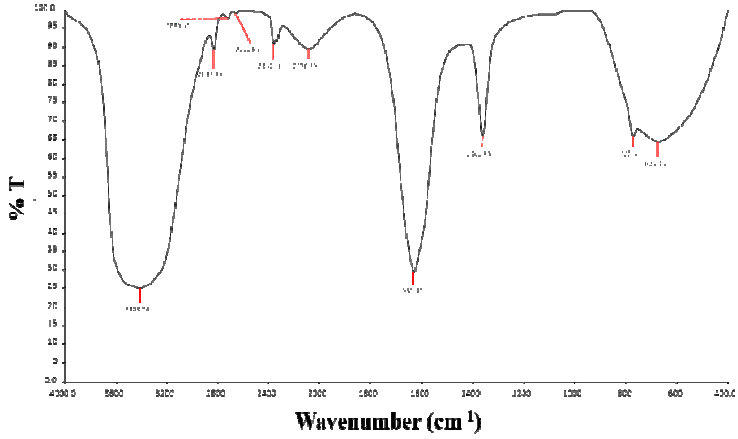


Fig. 6: FTIR spectrum of biodegradation of methyl parathion pesticide in 72 hours by *Enterobacter sp*

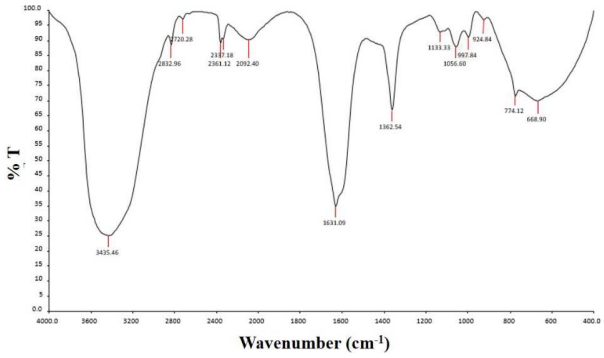


Fig. 7: FTIR spectrum of biodegradation of methyl parathion pesticide in 72 hours by *Klebsiella sp*

Table. 2 FT-IR analysis of pesticide degrading bacteria

MP.F,GP	Frequensis	Treatment after bacteria (1) *pseudomonas aeruginosa*	Treatment after bacteria (2) *enterobacter sp*	Treatment after bacteria (3) *klebsiella sp*
N=O	1402.2	1362.4	1361.8	1362.5
C=H	3410.0	3434.6	3408.9	3435.4
P=S	694.79	673.2	674.4	668.9
C=C	1635.5	1630.1	1631.4	1631.0
P-O-Ar	1123.7	-	-	-

HPLC analysis of pesticide degradation

High Performance Liquid Chromatography technique (HPLC) was employed to determine the pesticide residue in the medium containing bacterium. The experiments of pesticides degradation were conducted in Erlenmeyer flasks containing 100 ml nutrient broth in different concentration of pesticide (methyl parathion 100 ppm, 200 ppm, 300 ppm) in separate flasks. Bacterial strains were incubated at pH 7 at 300C for 72 hours. After incubation, the well grown pesticide degrading sample was analyzed by HPLC analytical method.

The bacterial degradation of the pesticide sample was monitored by HPLC (shimadzu HPLC Vp series (Japan)) with two CBM-20 A 230V pumps and LC8A, LC8B pump , wavelength of UV detector at 250 nm with reverse phase, C18 (2) 60mm for the length of the reverse phase column was used. The mobile phase components that contain acetonitrile & water (1:9) ratio and a buffer solution containing NH_2PO_4 (0.5%) & KH_2PO_4 (0.5%) were used. The column temperature was maintained at 30^0C (room temperature). The chromatographic analysis was carried out for the given sample by injecting 20 µl by using Rheodyne Syringe. Finally the pesticide degrading bacterial culture was identified by their retention time & levels of the peaks were calculated by reference standards.

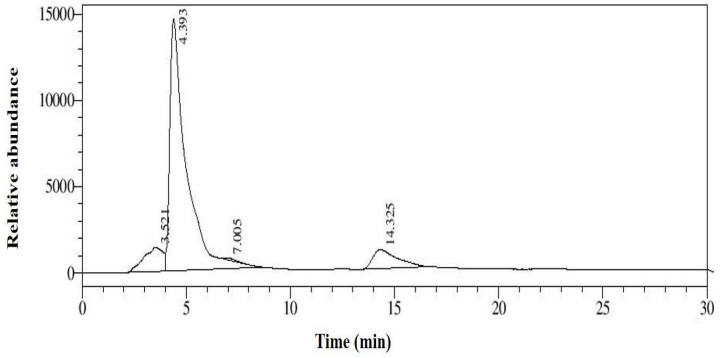


Fig. 8: HPLC Analysis of methyl parathion

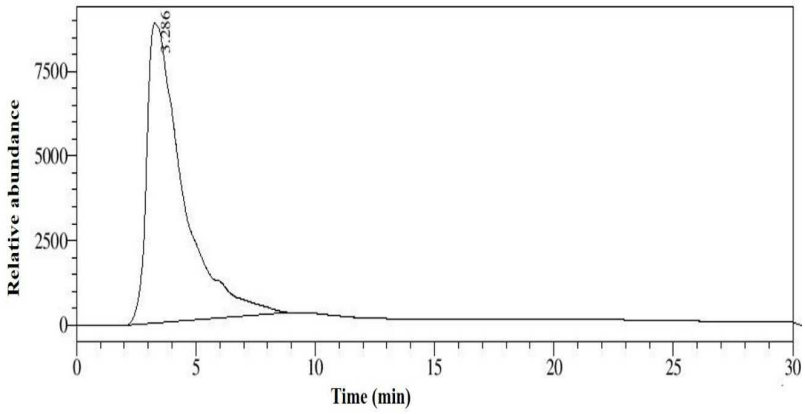


Fig. 9: HPLC Analysis of degradation of MP by *Pseudomonas aeruginosa*

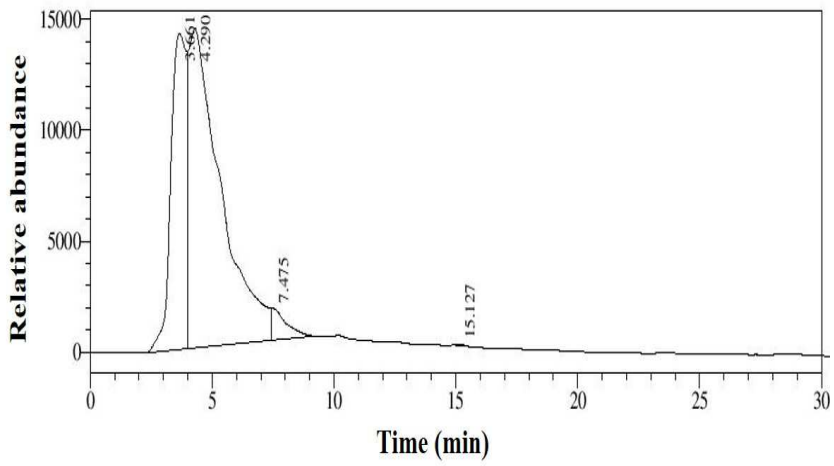


Fig. 10: HPLC Analysis of degradation of MP by *Enterobacter sp*

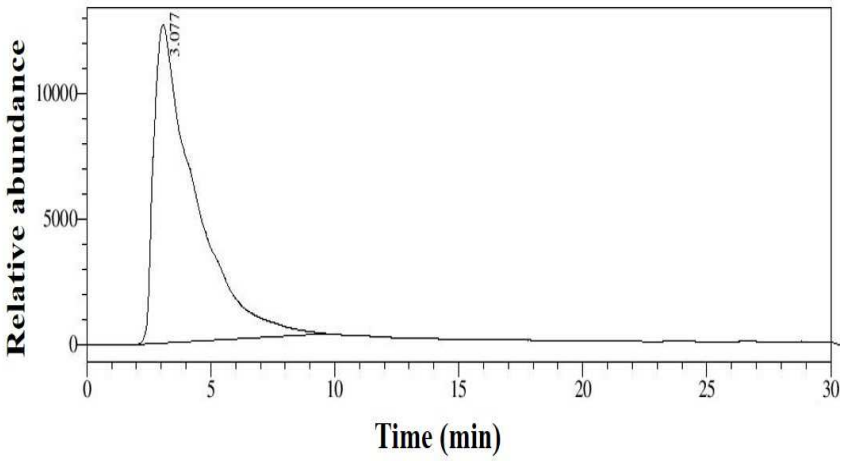


Fig. 11: HPLC Analysis of degradation of MP by *klebsiella sp*

Analysis of reduction of MP concentration in the bacterial cultures by HPLC studies

The HPLC analysis was performed to confirm the rate of degradation of the pesticide methyl parathion and the HPLC chromatogram shown in Fig 9-11 clearly indicate the reduction of MP concentration with respect standard MP concentration shown in Fig 8. The peak analysis of HPLC was carried out for four samples which includes (The standard -Methyl Parathion, Biodegraded samples 1-*Pseudomonoas aeruginosa,* 2 -*Enterobacter sp* and 3-*Klebsiella sp*). The standard HPLC profile of Methyl Parathion enumerated a peak with a retention time of 4.393. The above value was taken as the reference to compare the biodegradation of MP by other three test samples. Among the three bacterial, the rate of degradation was high in the case of *Klebsiella sp.* which bestowed the best result with a retention time value of 3.077. Subsequently the biodegradation consummated by *Pseudomonoas aeruginosa* elucidated a retention time of 3.286 which also facilitated the degradation of the pesticide methyl parathion. The retention value (4.290) of the biodegraded sample by *Enterobacter sp* also showed better result with the retention time of 3.661. From the experimental result it is proved that all the three bacteria were found to be more effective for the degradation of MP.

GC/MS analysis of degradation process

In order to substantiate the biodegradation of methyl parathion by HPLC analysis and ensure the biodegradation products, the samples were examined using GCMS analysis. The results obtained from GCMS were shown in Fig.

12 and the ion chromatogram clearly indicates the fragmented ions corresponding to the various degraded products of methyl parathion.

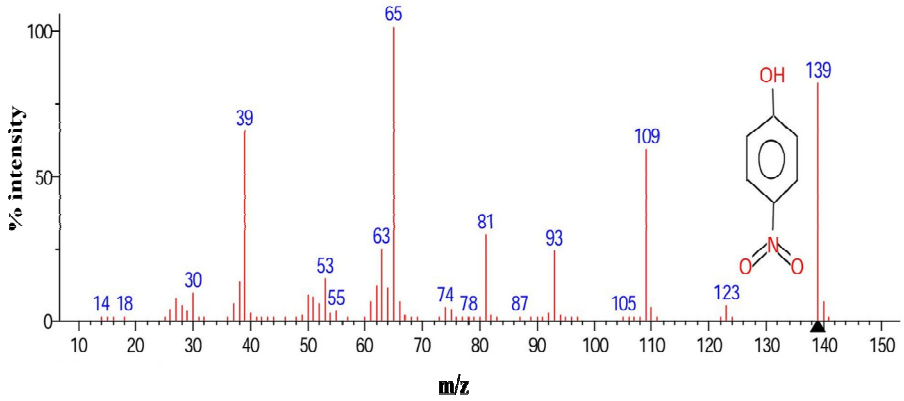
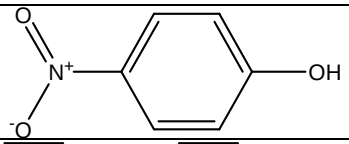
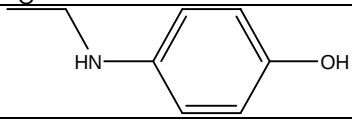


Fig. 12: GC MS Identified compound 1 obtained by MP degradation by *Pseudomonas aeruginosa*

Table. 3 Structure of some biodegradation products determined by GCMS in the EI mode

Compounds	Peak name and molecular weight	Structure	RT (min)	% Peak area
1	4-nitrophenol Molecular weight: 139		22.41	95.55
2	Phenyl,4-ethylamino Molecular weight:137		17.29	0.219

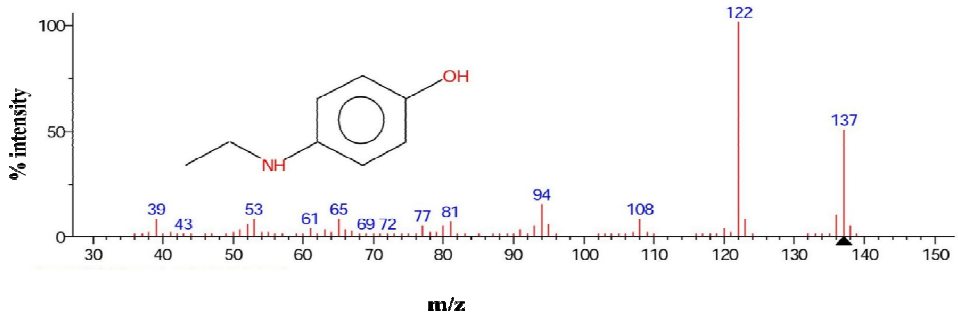


Fig. 13: GC MS Identified compound I1 obtained by MP degradation by *Pseudomonas aeruginosa*

Compounds obtained by the degradation were detected at their respective retention times. The major peak 95.5% represents the degraded product 4-nitrophenol (compound I) of molecular weight corresponds to 139. Hence it is evident that MP is degraded completely in the presence of bacteria. It is also observed that without the presence of bacteria the degradation of MP was very slow**.** GCMS ion chromatogram also revealed that the MP peak is disappeared rapidly after the treatment with the bacteria. In addition to the major product nitrophenol other bi products were also found to be present and it is less than five percentage. The peak correspond to the fragment of molecular weight 137 can be accounted for the compound II. The table 3 represents the details of the degraded production along with their retention time.

CONCLUSION

Biodegradation is an important and challenging research area which in turn brings a suitable remedy for pesticide contamination. Biological methods are more attractive compared to conventional methods as they support sustainability of earth leading to protection of natural resources. The present work was aimed to identify the

application and ability of the microorganisms, such as *Pseudomonas aeruginosa*, *Enterobacter sp* and *Klebsiella sp* against MP which is used widely in the field of agriculture. From the spectral and analytical results of the experimental study it is inferred that *Pseudomonas aeruginosa* is more effective in biodegradation at pH 7 and temperature 35 ^{0}C and also it is evident that the degradation process is complete and it is further confirmed by HPLC and GCMS analysis. This research work can be extended further to focus on the kinetics, physiological and ecological process that occurs at low concentration. Biodegradation strategies for pesticide concentration in various species of natural resources can be focused and the present work will provide useful insights for environmental production.

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