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Research Article
Microbial Degradation of the Organophosphorus Insecticide, Methyl Parathion Using the Natural Bacterial Isolate, *Pseudomonas aeruginosa*

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Abstract
Background and Objective: Pesticides are hazardous to human beings, flora, fauna and ecosystems. Though they are applied to increase the productivity in crop fields for controlling pests, they cause pollution in air, water and soil. Bioremediation is employed to clean polluted sites using organisms. Microbes are ubiquitous and some of them can degrade pesticides. Hence the present study has been designed to isolate a bacterial strain capable of degrading methyl parathion and to test its efficiency of degradation. **Materials and Methods:** Soil samples collected from contaminated crop fields were subjected to serial dilution, plating and incubation. From the grown colonies one colony was chosen and it was identified using biochemical tests. It was tested for its efficiency after exposing to 50, 100, 150 and 200 ppm for 30 h by monitoring changes in pH, orthophosphate, turbidity and the influence of sugars and immobilization. UV-visible spectrophotometry, HPLC analysis and statistical analysis were carried out to confirm the degradation efficiency of the natural isolate. **Results:** The natural isolate was identified as *Pseudomonas aeruginosa* based on the results of biochemical tests. Maximum orthophosphate was released in 200 ppm methyl parathion. The pH declined during degradation while turbidity exhibited an increase which indirectly indicated the degradation by the natural isolate. Orthophosphate level increased steadily when immobilized cells were tested. All the tested carbohydrates enhanced the release of orthophosphate. Both UV-visible spectrophotometry and HPLC analysis confirmed the degradation of the pesticide by the natural isolate. **Conclusion:** The natural isolate can be used to degrade pesticides like methyl parathion and its capacity can be enhanced by immobilization or supplementation with carbohydrates.

Key words: Methyl parathion, *Pseudomonas aeruginosa*, biodegradation, orthophosphate, bioremediation

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

One of the primary human concerns the world over is health. Increasingly, people in developed and developing countries have realized that the extensive use of chemical pesticides, which are used to keep crops healthy, has led to the contamination of the soil, crops and the resulting food products and drinks. It is a well-known fact that the water used for agriculture gets contaminated with chemical fertilizers and pesticides[1-3]. Pesticide residues accumulate in agricultural land and can remain active for upto 30-50 years, affecting crops grown on that land. In this way, pesticides find their way into the food chain and since they are toxic to humans, pose serious health hazards. As a result hundreds of thousands of people are being slowly poisoned[4-5].

Bioremediation is advantageous as it brings about the degradation of many organic contaminants quickly and effectively. In consequence, over the last 20 years, bioremediation has grown from a virtually unknown technology to a technology that is considered for the remediation of a wide range of contaminating compounds. Microbes as agents of bioremediation exhibit characteristics like small size, ubiquitous distribution, high specificity, surface area, growth rate and enzymatic and nutritional versatility[6,7] Biodegradation is a metabolic process that involves the complete breakdown of an organic compound. When this compound is broken down into its inorganic compounds, the process is referred as mineralization. Biodegradability represents the susceptibility of substances to be altered by microbial processes. The alteration may occur by intra/extra cellular enzymatic attack that is essential for the growth of the microorganisms. The attacked substances are used as a source of carbon, energy, nitrogen or other nutrients. Biodegradation may occur in either aerobic or anaerobic conditions. The rate of biodegradation is influenced by the numbers and types of microbes present in the site and the structure of the target molecule[8-12].

Methyl parathion is an organophosphorus insecticide that was first synthesized in 1940 and is used to control aphids, boll weevils and mites on cotton, soybeans, wheat, rice, alfalfa, onion, sugar beets and lettuce. It is used increasingly in agriculture and public health as an effective replacement of its ethyl analogue, parathion, which has been banned in many countries because of its higher mammalian toxicity. The detoxification procedure of methyl parathion is the use of strong alkali (1N NaOH) which is slow, a large amount of salt is required and the final pH of the treated solution is above 13. Therefore, the microbial application for methyl parathion-detoxification would represent a significant improvement in waste disposal technology[13,14].

Microbes can utilize methyl parathion as a source of carbon and the concentration upto 5 ppm could increase multiplication. Bacteria and actinomycetes showed a positive effect on methyl parathion while fungi and yeasts were less able to utilize this compound[15]. Hence in the present study an attempt has been made to isolate a bacterial strain capable of degrading methyl parathion and to test its efficiency of degradation. Experiments were also designed to study the effects of immobilization, supplementation of sugars on degradation process and the degradation products by UV-visible spectrophotometry and HPLC methods.

MATERIALS AND METHODS

The present study was conducted from June, 2017 to April, 2018 in the laboratory of the PG Department of Microbiology, The American College, Madurai, Tamil Nadu, India.

Pesticide used: The pesticide used in the present study belongs to the class of organophosphates which is commercially available as methyl parathion. It is selected on the basis of its wide application and present market trends.

Collection of soil sample: Pesticide, especially methyl parathion applied soil samples were collected from agricultural fields near Thirupuvanam, 25 km away from Madurai, Tamil Nadu, India in sterile containers and immediately brought to the laboratory for analysis.

Isolation and maintenance of bacteria: The bacteria capable of degrading methyl parathion were isolated from the collected soil with varying concentrations of methyl parathion in the medium. The bacterial strain exhibiting highest tolerance to methyl parathion was isolated, identified and preserved for further studies. Isolated culture was maintained on nutrient agar slants and stored at 4°C. The maximum concentration of methyl parathion for bacterial growth was determined by the inoculation of the selected bacterial strain on minimal medium containing 50, 100, 150 and 200 ppm concentrations of methyl parathion. The plates were incubated at 37°C for 24 h.

Identification and characterization of bacteria: The identification and characterization of the selected bacterial strain were carried out using morphological, cultural and biochemical tests according to Bergey's manual of determinative bacteriology[16].

Sample preparation: The organism was inoculated into minimal broth containing different concentrations of the pesticide (50, 100, 150 and 200 ppm). The flasks were incubated at room temperature and the samples were then subjected for the estimation of orthophosphate.

Estimation of orthophosphate: One milliliter of sample was taken in a flask and 1 mL of ammonium molybdate and 3 drops of stannous chloride solution were added and kept for 10 min for the development of blue colour and the absorbance was recorded in a colorimeter at 650 nm. Distilled water blank was subjected in a similar manner. Similarly the standard phosphorus solution of different strengths was processed and standard curve was plotted between absorbance and the concentrations of standard phosphorus solution. The orthophosphate content of the sample was deduced by comparing its absorbance with the standard curve.

Measurement of pH: pH was analyzed every 6 h up to 30 h of treatment for the sample containing minimal medium, culture and different concentrations of methyl parathion using pH meter and readings were recorded.

Measurement of turbidity: Growth was measured as turbidity at 600 nm with 6 h interval for 30 h.

Supplementation of sugars: The efficiency of pesticide degrading ability of the bacterium was tested by providing different carbon sources like fructose, glycerol, lactose, maltose and sucrose of 1% concentration in minimal medium containing 200 ppm concentration of methyl parathion. The flasks were incubated at 37°C and orthophosphate released was estimated every 6 h up to 30 h.

Immobilization of cells: The pure culture of the isolate was grown in nutrient broth and the cells were harvested by centrifugation at 10,000 rpm for 10 min and the cells were washed and suspended in 0.1% NaCl. Then, 3.5% of sodium alginate was added to the cell suspension and mixed thoroughly without forming any air bubble in the slurry. The slurry containing the cells was extended as drops through a tube (2 mm diameter) into 4% $CaCl_2$ solution. The drops formed into spherical beads of 2 mm size. The gel beads were kept in 4% $CaCl_2$ solution at 5°C for about an hour for complete gelation. Then the beads were washed with sterile distilled water and used for methyl parathion degradation study[17].

UV-visible spectrophotometry: The sample containing minimal broth, 200 ppm concentration of methyl parathion and the inoculum was centrifuged at 6 h interval for 30 h and the clear supernatant was used for spectral analysis. The clear supernatant was scanned from 200-600 nm in a spectrophotometer (Elico SL: 159) and analysed for specific absorption in the spectrum.

High pressure liquid chromatography (HPLC) analysis: The sample containing minimal medium, inoculum and 200 ppm concentration of methyl parathion was taken on subsequent treatment periods (6, 12, 18, 24 and 30 h) and they were subjected to HPLC analysis by UV detection.

Statistical analysis: Two way analysis of variance (ANOVA) was performed on the factors like orthophosphate released, turbidity, pH and influence of sugars for the 2 variables namely treatment period and methyl parathion concentration using MS-Excel latest version 2019.

RESULTS

Isolation and biochemical characterization: Table 1 illustrates the responses of the isolated bacterial strain to the various biochemical tests. Based on these results, it is identified as *Pseudomonas aeruginosa*. It is a Gram negative rod and exhibits positive response for sorbitol, lysine decarboxylase test and nitrate reductase test while negative response for urease, indole methyl red, Voges Proskauer and oxidase tests.

Table 1: Biochemical characterization of *Pseudomonas aeruginosa* isolated from methyl parathion pretreated soil

Biochemical test	Results
Gram staining	G-ve rod
Sorbitol	+
Indole	-
Methyl red	-
Voges-Proskauer	-
Citrate utilization test	+
TSI	K/K
Urease	-
Lysine decarboxylase test	+
Nitrate reductase test	+
Mannitol	-
Motility	+
Blood agar	Alpha HC
Lactose	-
Sucrose	-
Catalase	+
Oxidase	+

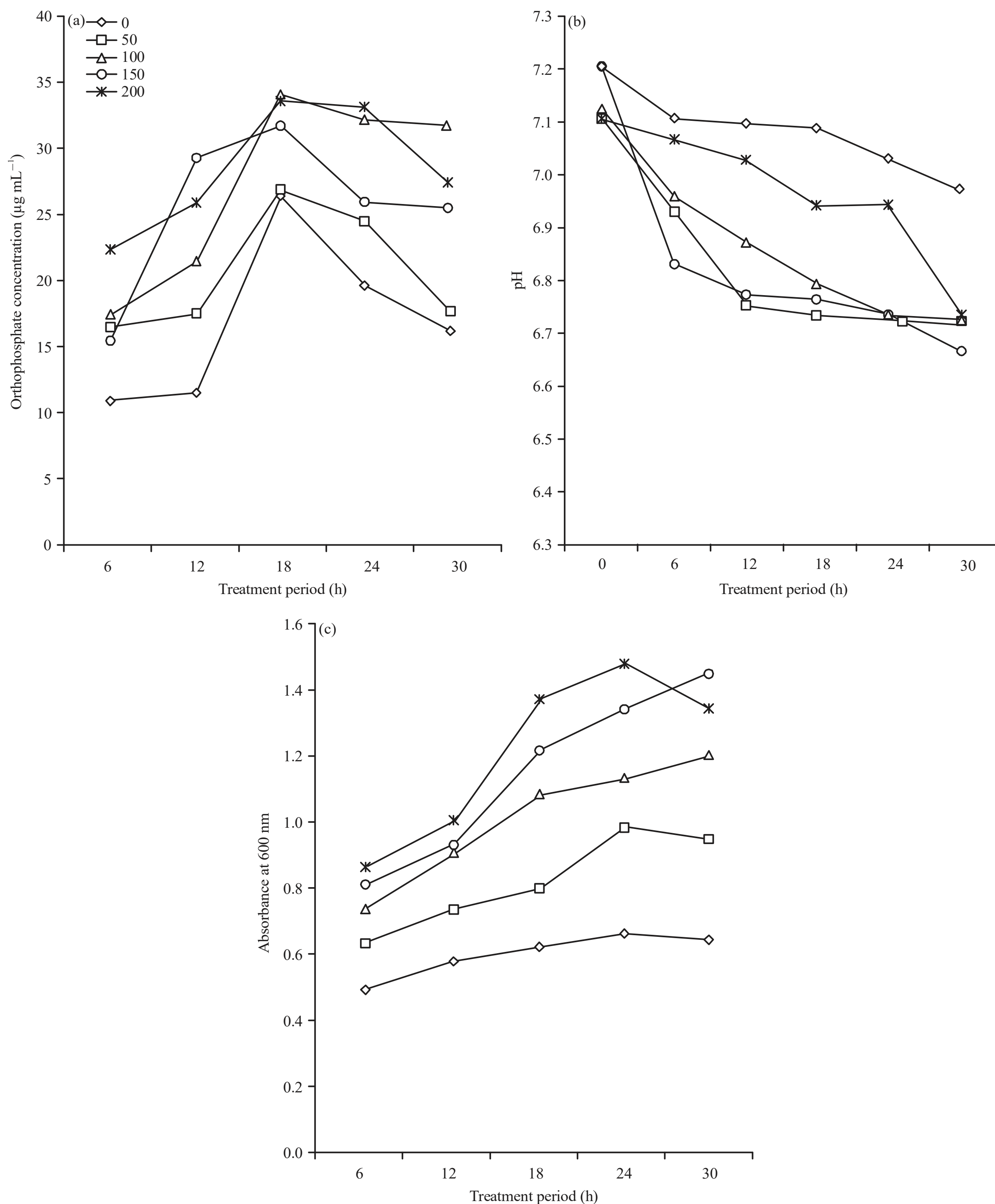

Fig. 1(a-c): (a) Orthophosphate released, (b) Changes in pH and (c) Turbidity during the degradation of methyl parathion by *Pseudomonas aeruginosa*

Biodegradation: Orthophosphate released during the degradation of methyl parathion at different concentrations of methyl parathion (50, 100, 150 and 200 ppm) by the isolate *P. aeruginosa* seems to be fluctuating (Fig. 1a). The isolate released maximum orthophosphate at 200 ppm concentration of methyl parathion which was selected for further analysis.

Figure 1b shows the effective degradation of methyl parathion by *P. aeruginosa* taking place at the pH range

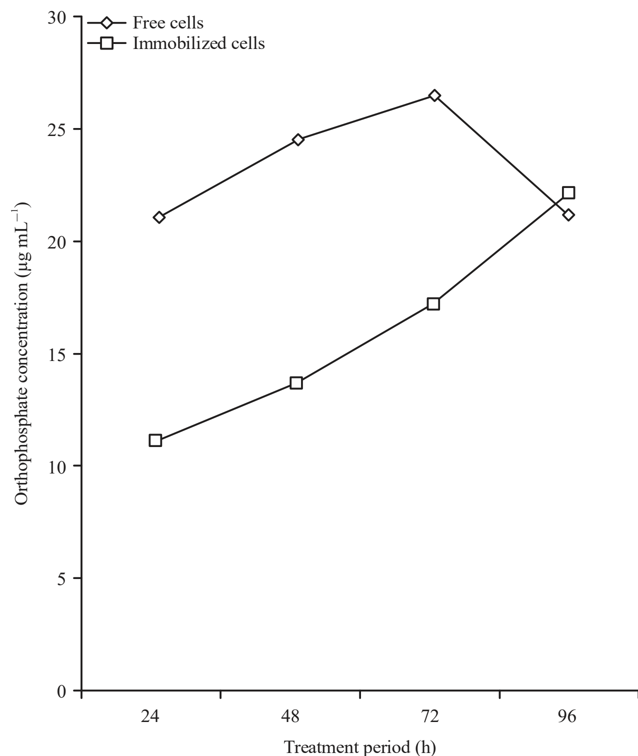

Fig. 2: Orthophosphate released during the degradation of 200 ppm methyl parathion by free and immobilized cells of *Pseudomonas aeruginosa*

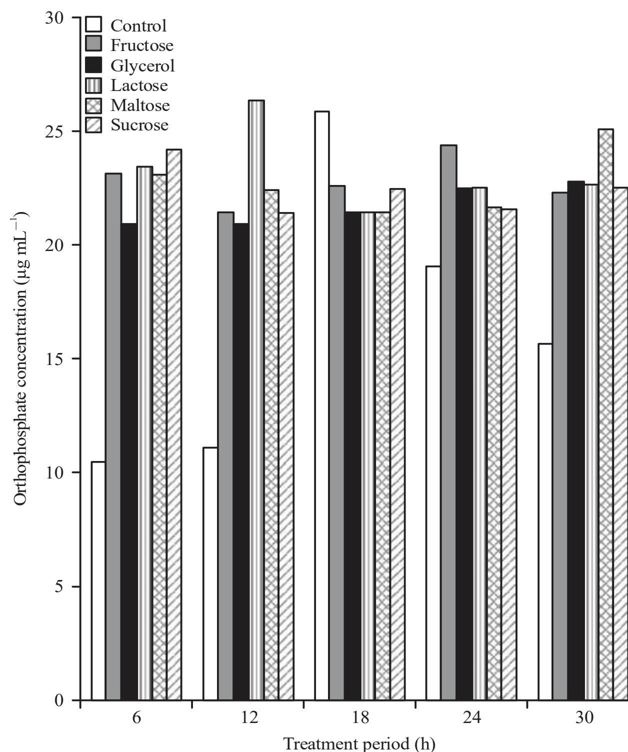

Fig. 3: Orthophosphate released during the degradation of 200 ppm methyl parathion by *Pseudomonas aeruginosa* when supplemented with various sugars of 1% concentration

of 6-8. With the increase in treatment period, the pH was shown to decrease at 12 h treatment period and then decreased. Turbidity measurements indicated that there was a significant increase in the growth during the treatment period until 24 h which shows that the organism effectively utilized the pesticide as the sole source of carbon and phosphorus (Fig. 1c).

Immobilized cells and influence of sugars: Immobilized cells of *P. aeruginosa* released orthophosphate with a constant, stable and gradual increase but in the case of free cells it seems to decrease after 72 h (Fig. 2). When carbon sources are supplemented in the minimal medium (fructose, glycerol, lactose, maltose and sucrose), they enhanced the degradation process, where *P. aeruginosa* utilized sucrose, maltose and lactose effectively for methyl parathion degradation (Fig. 3).

HPLC and UV-visible spectroscopic analysis: The residual concentration of methyl parathion and its metabolites (p-nitrophenol and p-aminophenol) at various time intervals (0, 6, 12, 15 and 24 h) were analyzed by HPLC (Fig. 4). Change in absorbance with reference to increase in incubation period

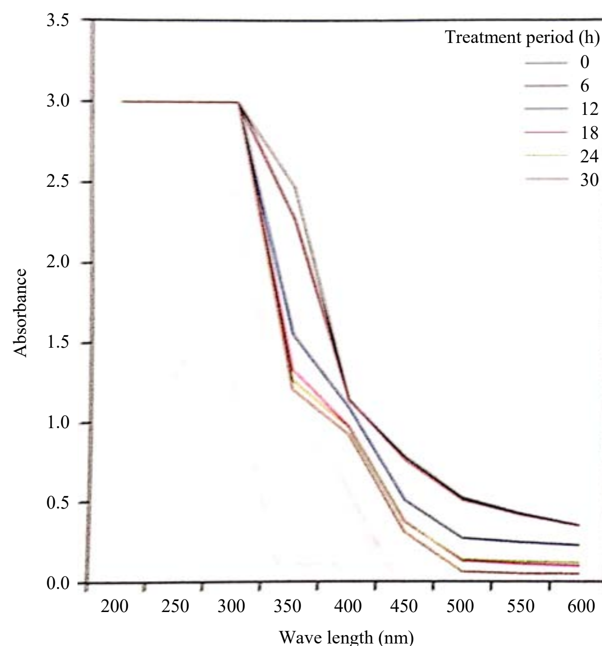

Fig. 4: UV-visible absorption spectrum taken during the degradation of 200 ppm methyl parathion by *Pseudomonas aeruginosa*

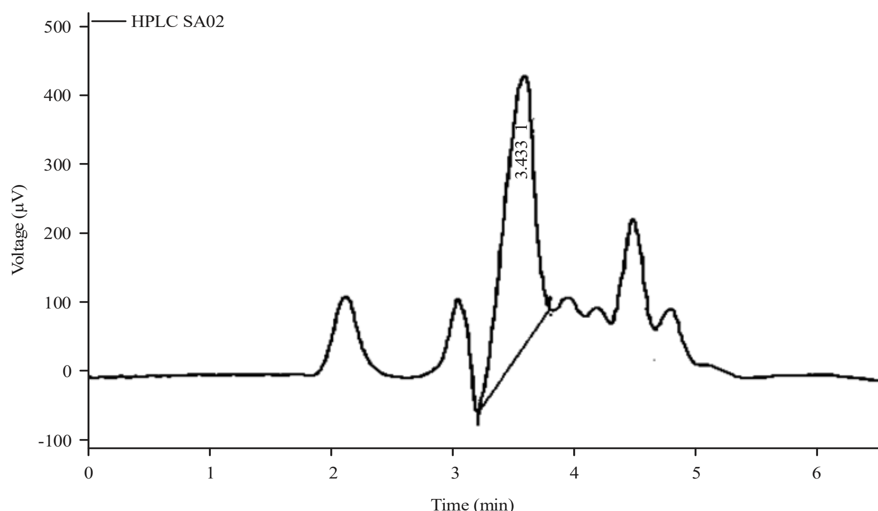

Fig. 5: HPLC analysis report for 200 ppm methyl parathion

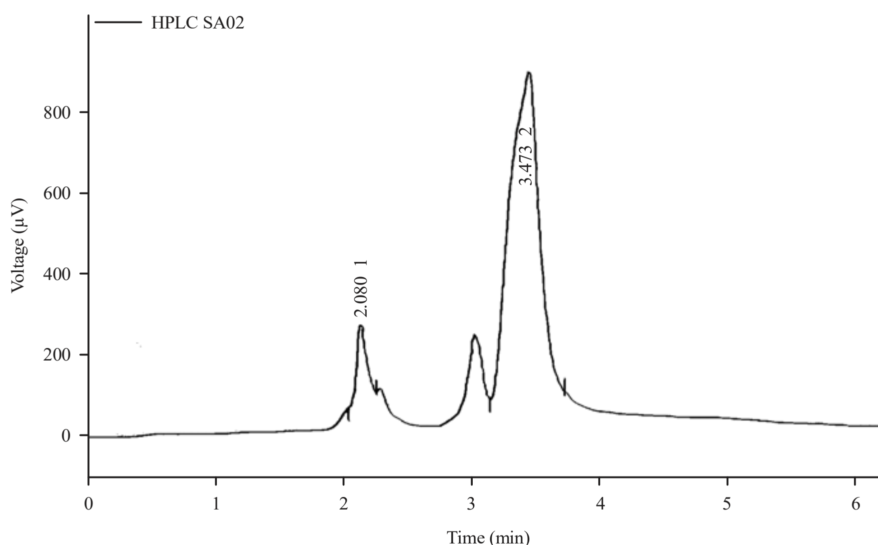

Fig. 6: HPLC analysis report for 200 ppm methyl parathion degradation by *Pseudomonas aeruginosa* after 30 h

was noticed in spectroscopy studies. This is an evidence for the degradation of methyl parathion. The retention time for standard methyl parathion was 3.433 min with the peak area of 7.345 mV.s and peak height of 0.444 mV (Fig. 5). The retention time for 200 ppm of methyl parathion after degradation by *P. aeruginosa* was 3.413 and 2.080 min with the peak area of 1.058 and 13.585 mV.s and with the peak height of 0.201 and 0.838 mV which indicated the formation of intermediate compounds during methyl parathion degradation (Fig. 6) at 30 h of treatment period.

Statistical analysis: Table 2 exhibits the two way ANOVA for the factors such as orthophosphate released during degradation by *P. aeruginosa*, pH and turbidity with the variables, treatment period and methyl parathion concentration. Variables of treatment period and methyl parathion concentration caused significant variations which were statistically significant at 5% level.

DISCUSSION

In the present study, the bacterial strain was isolated from the collected soil sample and was identified as *Pseudomonas aeruginosa*. The biodegradation ability of the isolate against the organophosphorus insecticide, methyl parathion was tested and analyzed in detail with the help of

Table 2: Two way analysis of variance for the factors with the variables, treatment period and methyl parathion concentration

Factors	Source of variation	SS	df	MS	Calculated F-value	Table value at 5% level	Level of significance
Orthophosphate released	Treatment period	499.3	4	124.83	13.577	3.007	Significant (p<0.05)
	Methyl parathion concentration	595.6	4	148.9	16.196	3.007	Significant (p<0.05)
pH	Treatment period	0.346	4	0.086	38.412	3.007	Significant (p<0.05)
	Methyl parathion concentration	0.137	4	0.034	15.226	3.007	Significant (p<0.05)
Turbidity	Treatment period	1.319	4	0.330	32.436	3.007	Significant (p<0.05)
	Methyl parathion concentration	0.682	4	0.171	16.795	3.007	Significant (p<0.05)

UV-visible spectroscopy, HPLC and statistical analysis. Organophosphorus compounds are widely used as pesticides, insecticides and chemical warfare agents and their widespread contamination of soil, sediments and ground water continues to be a concern today[18]. Due to their extreme toxicity there is an urgent need for safe, economical and reliable methods for detoxification or remediation of these compounds. With the developments in biotechnology, new efforts have been emphasized on the use of microorganisms for the degradation of pollutants rather than disposal[19].

In the present study, methyl parathion degrading bacterium was isolated from pesticide polluted agricultural soil. Begum and Arundhati[20] isolated bacterial species from agricultural soils in and around Visakhapatnam, India where pesticides were used severely. Nature of soil is mainly determined by the occurrence of numerous xenobiotic organic components because of modern agricultural practices. The natural microbial populations present in that soil are experiencing the pesticide stress and as a result of that, they start to develop the degrading ability against the exposed pesticides[21]. Many investigations proved that the microorganisms are capable of degrading the pesticides and detoxify the pesticide polluted sites. It is well known that methyl parathion has a significant influence over the microbial activity and biodegradation of methyl parathion is most realistic using the newly isolated bacterial strains[22].

Several bacterial species were isolated from the organophosphorus pesticide, methyl parathion pre-treated agricultural fields using aqueous medium and solid medium[23]. Growth and maintenance of isolate on nutrient agar, exhibited a confluent growth in accordance with studies showing biodegradation of organophosphorus pesticides in the presence of additional energy sources in, nutrient broth. When different concentrations of methyl parathion were mixed with minimal medium, the isolate was able to resist up to 200 ppm of methyl parathion. The isolate was capable of degrading the commercially available methyl parathion used in this study, implying the compound used in preparation of this formulation might promote the growth of these bacteria in combination with components of minimal medium[23].

The isolate obtained from the methyl parathion polluted soil sample was identified as *Pseudomonas aeruginosa* based on morphological, cultural and biochemical characterization. It is Gram-negative, rod shaped, highly oxidative and metabolically versatile in nature. Balamuruguan *et al.*[24] also isolated methyl parathion degrading *P. aeruginosa* from pesticide polluted soil. The levels of orthophosphate released were tested at varying concentrations of methyl parathion (50, 100, 150 and 200) and was found that *P. aeruginosa* could degrade 200 ppm of methyl parathion. In the control, level of orthophosphate released was very meager amount which indicated that the *Pseudomonas* sp. utilized methyl parathion as a sole source of phosphorus and carbon[25]. pH of the sample was found to be around 6-8 and this indicates that the degradation had taken place effectively. The optimum pH range for enzymatic hydrolysis by *Pseudomonas* sp. was reported from 6-9. From the decrease in pH, it can be understood that the acidity of the medium increases i.e., during the process of degradation, an acid was formed as a product and thus decrease in the pH was noted in the medium[26]. Increase in the turbidity in medium indicates the growth of the organism. Turbidity was shown to increase during the treatment intervals (6, 12, 18, 24 and 30 h). From the increase in the turbidity of the medium, it can be inferred that the organism uses methyl parathion as a source of energy as it grows on minimal medium with methyl parathion and causing the degradation of methyl parathion. Similar kinds of observations were noticed in the earlier investigations related with biodegradation of pesticides using different bacterial strains[27-29].

Immobilized cells are more effective in pesticide degradation which provide more advantages than the traditional biological methods that use free cells. The advantages are high cell concentrations, reuse of cells, the elimination of "cell wash" problems at a high dilution rate, high productivity yields and the capacity to retain catalytic activity for more time[30,31]. The degradation activity of immobilized cells and free cells of *P. aeruginosa* isolate were compared in the present study. The results indicated a significant increase in methyl parathion degradation

with immobilized cells compared to free cells. Fernández-López *et al.*[32] enhanced methyl parathion degradation by the immobilization of *Burkholderia* sp. isolated from agricultural soils and also noticed the residual concentration of methyl parathion and its metabolites (p-nitrophenol and p-aminophenol) at various time intervals using HPLC. The present investigation helped to find out the potential of *P. aeruginosa* isolate in biodegradation of methyl parathion and its metabolite, p-nitrophenol from agricultural soil. In HPLC analysis, p-aminophenol was not found in any media, but the residual methyl parathion and p-nitrophenol were detected in the media. UV-visible spectroscopic studies indicated the degradation of methyl parathion after 30 h of treatment using the natural isolate, *P. aeruginosa*. The peak value shows the presence of an intermediate compound and the difference in the retention time indicates the occurrence of intermediate compound. p-Aminophenol is a metabolite of methyl parathion from reductive reaction and p-nitrophenol is a metabolite of methyl parathion from hydrolytic reaction[32]. The results indicated that methyl parathion in the minimal medium was hydrolyzed to its intermediates by *P. aeruginosa*.

The present study was conducted under laboratory conditions. Therefore, in future studies, *P. aeruginosa* should be tested with industrial waste and in natural environments, because microorganisms which are able to degrade organic pollutants in culture may sometimes fail to function when they are inoculated into a sludge system of industry and natural environments. They may lose their degrading ability because they may be susceptible to toxins or predators in the environment, they may use other organic compounds in preference to methyl parathion, or they may be unable to move through soil to sites containing methyl parathion. Genetic engineering of *P. aeruginosa* may be conducted in order to improve biodegradation. Furthermore *P. aeruginosa* should be tested for its pathogenicity to aquatic organisms, such as fish, prawn and crab before being applied for the detoxification of pesticides in the environment[33-35].

CONCLUSION

The natural isolate, *Pseudomonas aeruginosa* was identified based on the biochemical tests. It was able to degrade methyl parathion upto 200 ppm concentration in 30 h. Immobilized cells degraded better than that of free cells. All the sugars tested enhanced the release of orthophosphate during degradation.

SIGNIFICANCE STATEMENT

This study discovered the efficiency of *Pseudomonas aeruginosa* on the biodegradation of methyl parathion that can be beneficial for bioremediation programmes for restoring soil quality. This study will help the researchers to uncover the critical areas of using the natural isolate, *P. aeruginosa* for biodegradation of pesticides that many researchers were not able to explore. Thus a new theory on using *P. aeruginosa* for treating pesticide polluted soil may be arrived at.

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