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Research paper

Biodegradation of malathion and evaluation of kinetic parameters using three bacterial species

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Abstract

Efficacy of three different bacterial species for biodegradation of malathion and its secondary products have been investigated. The concentration range of malathion under investigation was 25–200 mg·L^{-1}. It has been observed that *Pseudomonas putida* was found to be most efficient for degradation of malathion. The removal of malathion was 72% at its concentration of 125 mg·L^{-1}. The optimum parameters were studied for all three bacterial species in batch mode. The average values of K_s and μ_{max} were obtained for all these species for degradation of malathion. Results indicate that *P. putida* has high degradation potential than *Rhodoccocus rhodochrous* and *Sphingomonas sp*. The degradation of *P. putida* was maximum at concentration of 125 mg·L^{-1}, pH and temperature at 7 ± 0.2, 80 °C respectively. Metabolites were obtained using GCMS analysis.

Keywords: Biodegradation; Kinetic constant; FT-IR; SEM; Monod model; Metabolites

1. Introduction

Productivity in agriculture mainly depends on land, water, fertilizers, seed and pesticides. Pesticides are an integral part of agriculture. In current scenario approximately, 35–45% crop production is lost due to insects, weeds and several diseases, while 35% crops are lost during the storage [1]. According to a report on the outlook of pesticide consumption in India, the domestic demand for organophosphate is growing at the rate of 8–9% and export demand at 15–16%. In the USA alone at the end of last century; 27,000 tons were used in agriculture and about 7650 tons were used in mono-agricultural applications [2]. Organophosphates are preferred in agriculture due to their low persistence in the environment, but its indiscriminate use affects much toxic to animals and human beings [3]. Organophosphorus compounds such as malathion, chlorpyrifos, parathion, etc. are commonly used as insecticides for over 50 years. Organophosphate pesticides are commonly used pesticides since long for agricultural practices accounting an estimate of 34% global insecticide sales [4,5].

The literature reveals that the first recorded use of organophosphate insecticide, malathion [S-(1,2-dicarbethoxyethyl)-O,Odimethyl-dithiophosphate], by the United States Department of Agriculture (USDA) was in USA in 1956. It is currently regulated by the United States Environmental Protection Agency [6]. Malathion is a broad spectrum insecticide widely used worldwide because of its efficiency for controlling insects and pests. Physico-chemical properties of malathion are shown in Table 1 [7]. It is estimated that over 13,500 tons of malathion are used annually in the USA according to U.S. EPA [7]. Malathion has been best suited for the control of sucking insects on field crops, fruits, vegetable, livestock, etc. [8–10]. Malathion is a highly toxic compound and is listed by the United State Environmental Protection Agency (USEPA) as toxicity class (Group 2A) [11,12]. It is classified as carcinogenic to humans and animals. Its high-level exposure will affect nerve fibers and is neurotoxic in animals and immunity of higher vertebrates [12–16]. Malathion irreversibly inhibits the acetylcholinesterase enzyme that hydrolyzes acetylcholine [17]. Several international agencies such as Food and Drug Administration (FDA) and Environmental Protection Agency (US EPA) have allowed a maximum malathion concentration of a residue on specific crops used as foods [12]. The National Institute of Occupational Safety and Health (NIOSH) also recommends that malathion

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Table 1
Physico-chemical properties of malathion.

Sl. no.	Properties	Malathion
1	CAS Reg. No.	121-75-5
2	Synonyms	Dimethyl dithiophosphate of diethyl mercaptosuccinate
3	Molecular weight	330.35 g · mol^{-1}
4	Physical state	Liquid
5	Color	Colorless to light yellow
6	Melting point	285 °C
7	Boiling point	156 °C
8	Density/Specific gravity	1.2076
9	Odor	Skunk/garlic like
10	Solubility water (mg·L^{-1})	145 mg·L^{-1}
11	Vapor pressure (mmHg)	1.78×10^{-4} mmHg at 25 °C
12	Henry's law constant	$2.0\ (\pm 1.2) \times 10^{-7}$

ambient air concentration of 250 mg·m^{-3} be considered as highly hazardous to human health and life.

Malathion reacts with other chemicals in the presence of sunlight in the atmosphere to produce 40 times more toxic compound malaoxon but breaks down very quickly [12,13,18,19]. The solubility of malathion is shown in Table 1. The liquid phase malathion is degraded microbially.

The conventional removal techniques of malathion include electrochemical oxidation, Fenton oxidation, solvent extraction, chemical oxidation and adsorption [20–24]. These techniques have their own demerits like sludge generation, the formation of toxic by-products, cost expensive, energy expensive and also these methods can't be used for a wide spectrum of pesticides. Therefore, the degradation of malathion by biological means is more promising due to its merit of low investment, low operational costs, and eco-friendliness.

Table 2 shows the recent literature available on the degradation of malathion. The literature survey reveals that the mala-

Table 2
Literature survey of malathion biodegradation by different microbial species.

Sl. no.	Compound	Micro-organism	Experimental condition	% Removal of malathion	Reference
1	Malathion	*Fusarium oxysporum f.sp.*	Malathion concentration = 100–500 mg·L^{-1} Temperature = 37 °C pH = 8 rpm = 250	65%	[25]
2	Malathion	*Acinetobacter johnsonii MA19*	Malathion concentration = 100–250 mg·L^{-1} Temperature = 30 °C pH = 7–7.5 rpm = 160	–	[26]
3	Malathion	*Bacillus sp.*	Malathion concentration = 50–200 mg·L^{-1} Temperature = 37 °C pH = 4–9	49.96%	[27]
4	Malathion	*Bacillus sp.S14*	Malathion concentration = 25 mg·L^{-1} Temperature = 30 ± 1 °C pH = 7 ± 0.2 rpm = 120	64.4%	[28]
5	Malathion	*Bacillus thuringiensis*	Malathion concentration = 250 mg·L^{-1}	50%	[29]
6	Malathion	*Bacillus cereus*	Malathion concentration = 20–200 mg·L^{-1} Temperature = 30 °C pH = 7 ± 0.2	49.31	[30]
7	Malathion	*Lysinibacillus sp.*	Malathion concentration = 20–250 mg·L^{-1} Temperature = 30 °C pH = 7 ± 0.1	–	[31]
8	Malathion	*Consortium*	Malathion concentration = 5–140 mg·L^{-1} Temperature = 25 ± 1 °C pH = 7 rpm = 100	–	[32]
9	Malathion	*Acinetobacter baumannii strain AFA*	Malathion concentration = 20–100 mg·L^{-1} Temperature = 30 °C pH = 7–7.5	–	[33]
10	Malathion	*Bacillus licheniformis strain ML 1*	Malathion concentration = 25 mg·L^{-1} Temperature = 32 °C pH = 7.5 rpm = 250	78%	[34]
11	Malathion	*Pseudomonas putida*	Malathion concentration = 125 mg·L^{-1} Temperature = 30 °C pH = 7 ± 0.2 rpm=150	72%	Current study
		Rhodococcus rhodochrous	Malathion concentration = 125 mg·L^{-1}	70%	
		Spingomonas sp.	Malathion concentration = 100 mg·L^{-1}	64%	

thion degradation organisms are *Acinetobacter johnsonii*, *A. baumannii, Bacillus sp., Lysinibacillus Pseudomonas sp.,* etc. [26–34]. Kim et al. [25] have isolated the fungi *Fusarium oxysporum sp.* capable of degrading malathion effectively.

Various researches have been performed on the biodegradation of malathion using bacterial and fungal species. Earlier researchers have worked on the degradation of malathion by varying the operating conditions like pH, temperature, and concentrations. In the present study, the operating parameters such as pH and temperature were kept constant and concentrations were varied for bacterial species in order to understand the potential of most efficient bacterial species involved in degradation of malathion at high concentrations. Further, there is no report on the biodegradation kinetics of malathion in the open literature. To predict and evaluate the biodegradation rate of malathion there is a need to develop models that help to combine transport and sorption with various reaction terms, including substrate utilization, bacterial growth, and decay during the experimental process. Hence, the aim of the present study was to find most efficient bacterial species for degrading malathion and metabolites formed during the process of degradation using GC-MS. In addition to this kinetic parameter such as μ_{max}; maximum specific growth rate, K_s; half saturation constant and ratio of $\frac{\mu_{max}}{K_s}$ were also calculated.

2. Materials and methods

2.1. Chemicals and microorganisms

Malathion was purchased from Sigma-Aldrich (India) and other chemicals were purchased (AR grade) with more than 99.9% purity from Merck (Darmstadt, Germany).

Three bacterial cultures such as *Pseudomonas putida* (MTCC 1072), *Rhodococcus rhodochrous* (MTCC 1767) and *Spingomonas sp.* (MTCC 8992) were procured from MTCC, Chandigarh-160036 (India).

2.2. Cultivation of bacteria

The bacterial cultures were grown in a defined mineral salts medium (MSM), containing per liter of deionized water 1.6 g K_2HPO_4; 0.4 g KH_2PO_4; 0.2 g $MgSO_4$.$7H_2O$; 0.1 g NaCl; 0.02 g $CaCl_2$. The following sterile filtrated solutions were added to MSM medium 10 mL of a sodium citrate stock solution (100 g · L^{-1}) to a final concentration of 1 g · L^{-1}; 1 mL of a trace element solution; 1 mL of a vitamin stock solution and 1 mL of a $FeSO_4.6H_2O$ stock solution (5 g/L). The trace element solution contained 2 g/L boric acid; 1.8 g · L^{-1} $MnSO_4.H_2O$; 0.2 g · L^{-1} $ZnSO_4$; 0.1 g · L^{-1} $CuSO_4$; 0.25 g · L^{-1} Na_2MoO_4 [35]. The malathion with varying concentrations (25–200 mg·L^{-1}) was added to MSM as sole carbon sources for cell growth, using microsyringe. The pH of the medium was adjusted to 7 ± 0.2 by adding either HCl or NaOH. In a separate set of experiments, the growth of three bacterial species was studied in the presence of 25–200 mg·L^{-1} of malathion. The bacterial growth was regularly monitored by Elico UV-VIS spectrophotometer Model no SL-159 at 600 nm for 10 days. The bacterial biomass was evaluated as described elsewhere [36].

2.3. Batch experimental study

Biodegradation studies were carried out using three bacterial sp. at initial malathion concentrations of 25, 50, 75, 100, 125, 150, 175 and 200 mg·L^{-1}. For the biodegradation studies, all the morphologically different species were initially pre-cultured in nutrient broth medium to increase the bacterial density, cell suspensions with the volume of 500 µL were inoculated in 100 mL MSM medium in 120 mL of serum bottles containing 25, 50, 75, 100, 125, 150, 175 and 200 mg·L^{-1} of malathion. Serum bottles without any bacterial inocula but having the same concentration of respective chemicals were used as controls. The incubation was done at 30 °C on a shaker at 150 rpm for 10 days. All the runs were made in triplicate to minimize the error. Malathion remaining in the culture was extracted using an equal volume of chloroform and HCl in separating funnel. Residual Malathion was analyzed by GC-FID, respectively, after 24, 48, 72, 96, 120, 144, 168, 192, 216, and 240 h of incubation periods.

The rates of degradation of malathion at various substrate concentrations were calculated using the equation as under:

$$r_{ave(d^{-1})} = \frac{C_0 - C_t}{C_o(\Delta t)} \quad (1)$$

C_o and C_t are the concentrations of the substrate at t = 0 and at time t, respectively,

r_{avg} is the rate of degradation and Δt is the duration of degradation time.

The kinetic constants were calculated using the Monod model corresponding to which maximum degradation rate was observed.

2.4. Analysis of residual malathion

Malathion concentrations were analyzed using a Thermo-Fisher 7610 gas chromatograph (GC). The GC is equipped with a flame-ionization detector (FID) and BP-5 capillary column (25 m × 0.32 mm). The initial temperature was maintained at 80 °C for 30 seconds and then raised to 150 °C at a rate of 10 °C and maintained for 30 seconds then raised to 180 °C at a rate 10 °C. The total GC sampling time was 11 min. The retention times were 3 min 34 seconds for malathion. Injector and detector temperature was 220 °C and 230 °C in the detection system. Nitrogen served as the carrier gas, and oxygen and hydrogen served as the fuel gasses for the FID.

2.5. Bacterial morphology

The bacterium *P. putida* morphology was analyzed using scanning electron microscopy; a strip of the filter paper size (0.22 µm) was used to filter the bacterial sample. Further, the filter paper dried overnight at 30 °C in the oven. The resolution of the bacterium was good without any coating of the gold and carbon particles. The morphology of the bacteria was examined

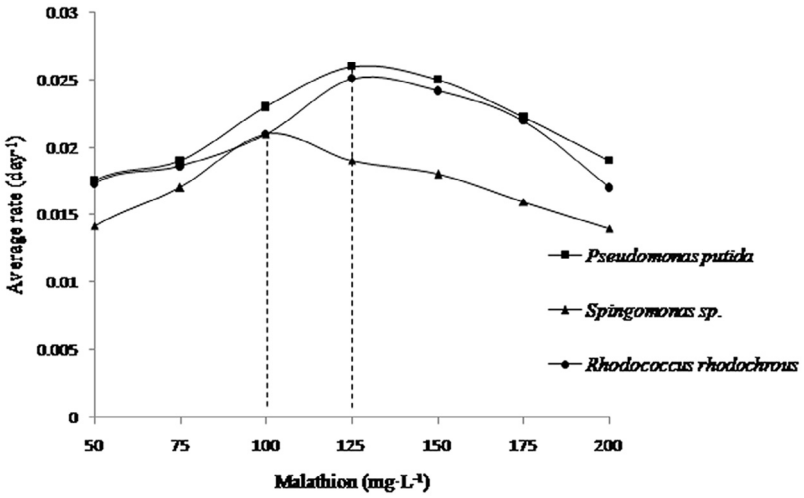

Fig. 1. Effect of average rate and optimum concentration of malathion on different bacterial species.

using a low-vacuum in order to minimize the harm to the bacterium sample (Model: SEM QUANTA 200F).

2.6. Functional group analysis

FT-IR analysis was carried out of bacterial liquid culture sample taken after 10 days, with model no. NICOLET 5700 FT-IR. Functional groups and the bands were recorded in the spectrum in terms of percentage transmission and wave number from 4000 to 400 cm^{-1}.

2.7. Metabolites analysis

Metabolites formed during malathion degradation were determined using GC-MS [30]. The sample was prepared by extracting residual malathion with the addition of an equal volume of chloroform and degraded sample, and after vigorous shaking, the organic layer was separated. The extracted samples were analyzed using GC-MS-QP2010 Ultra (JNU New Delhi). The gas was at 1.21 $mL \cdot min^{-1}$ with split ratio 100, injection temperature at 260 °C. MS measurements were done at a temperature of 230 °C for MS ion source, 270 °C for MS interference, with total time of 20 min and solvent delay of 3 min.

3. Results and discussion

3.1. Batch degradation experiments

Batch experiment was conducted for all the three organisms parallel with the concentration range of 25–200 $mg \cdot L^{-1}$ of malathion. The average degradation rate (0.026 day^{-1}) for *P. putida* and (0.0251 day^{-1}) for *R. rhodochrous* species increases up to 125 $mg \cdot L^{-1}$ and at above concentration, there is a gradual decrease in rate as shown in Fig. 1. Similarly, the rate of degradation (0.021 day^{-1}) increased up to 100 $mg \cdot L^{-1}$ for *Sphingomonas sp*. and further increase in the concentration of malathion resulted in a decrease of rate. This may be because of inhibitory effects of metabolites [32]. Fig. 1 shows the concentration of malathion that was optimized with respect to each bacteria based on its tolerable limit and was found to be 125 $mg \cdot L^{-1}$ for *P. putida* and *R. rhodochrous*, 100 $mg \cdot L^{-1}$ for *Sphingomonas sp*. From Fig. 2 it is seen that *P. putida* and *R. rhodochrous* degraded malathion to 72% of 125 $mg \cdot L^{-1}$ in the 7th day and 70% in the 8th day, respectively, while *Sphingomonas sp*. degraded to 64% of 100 $mg \cdot L^{-1}$ in the 9th day. The results exhibited that *P. putida* was found to be more efficient for malathion degradation compared with the other bacterial species. This could be due to facts that carboxylesterase gene is responsible for degradation of malathion. The results are inconsistent with reported studies of Kim et al. and Goda et al. [25,37].

The bacterial growth was studied by taking the dry cell mass of organisms and it was observed that it increased in cell mass

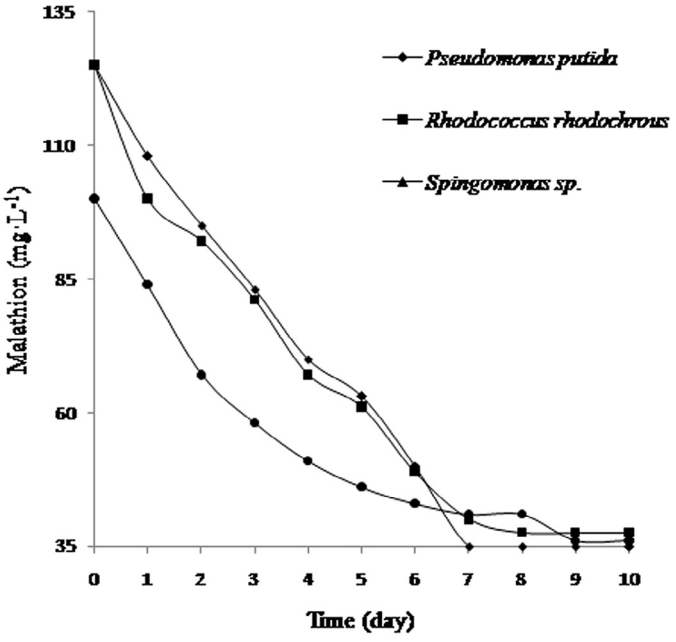

Fig. 2. The study of biodegradation of malathion concentration with respect to time (day).

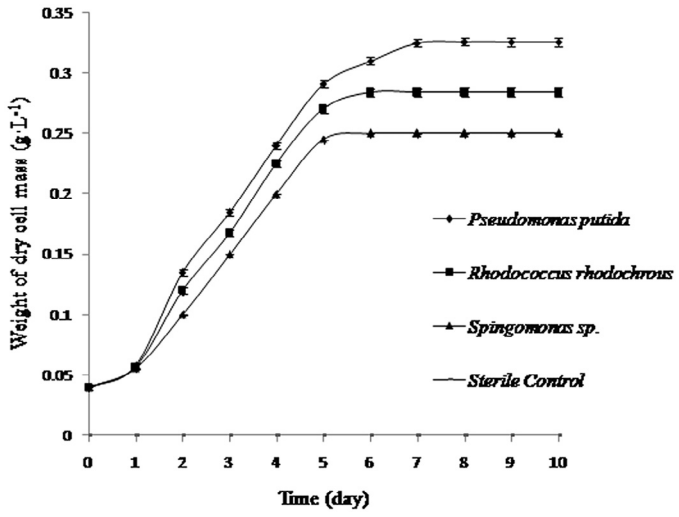

Fig. 3. The measurement of bacterial weight of dry cell mass ($g \cdot L^{-1}$) with respect to time (day).

up to 7 days. After 7 days there is a stationary phase (Fig. 3) due to deficiency of nutrient that may also be oxygen limitations. This is in agreement with the primary work [25]. A typical plot for degradation of malathion by *P. putida* is shown in Fig. 4. Results show the percentage degradation of malathion. From Fig. 4 it is seen that the bacterial growth increases up to the 7th day and simultaneously the concentration of malathion is decreasing continuously up to the 7th day. Consequently after the 7th-day growth, degradation was found to be in steady state. The results reveal that growth and degradation were inhibited due to metabolite and nutrient deficiency [31]. Similar observations were made for the other two species.

3.2. Kinetics for biodegradation of malathion

The kinetic model parameters are estimated by various factors such as inoculum size, substrate concentrations, time, and biomass. The Monod model has been selected to study the biodegradation of malathion in the present study. The Monod model used is as follows:

$$\mu = \frac{1}{X}\frac{dx}{dt} = \frac{\mu_{max} s}{K_s + S} \tag{2}$$

Where μ is specific growth rate (day^{-1}), μ_{max} is maximum specific growth rate (day^{-1}), K_s is half-saturation constant ($mg \cdot L^{-1}$), X, S, and t are microbial cell, initial substrate concentrations ($mg \cdot L^{-1}$), and time, respectively. Linear form of the above equation was

$$\frac{1}{\mu} = \frac{K_s}{\mu_{max} S} + \frac{1}{\mu_{max}} \tag{3}$$

The value of $\frac{1}{\mu}$ and $\frac{1}{S}$ were calculated and plotted to obtain μ_{max} and K_s as shown in Fig. 5 and Table 3 lists the value of K_s, μ_{max} and $\frac{\mu_{max}}{K_s}$. The Monod model was fitted with the observed and experimental data as shown in Fig. 6. In the present study the values of specific growth rates (μ_{max}) and half rate constants (K_s) were found to be in the range of 0.299–0.312 day^{-1} and 80.69–83.67 $mg \cdot L^{-1}$, respectively. In the biodegradation lower value of K_s (80.69 $mg \cdot L^{-1}$) and higher value of μ_{max} (0.312 day^{-1}) are desirable because of the higher affinity of bacterial species (*P. putida*) for degrading malathion. Therefore, it is better to combine the two kinetic constants into a single parameter $\frac{\mu_{max}}{K_s}$ and consider this parameter as a useful index for

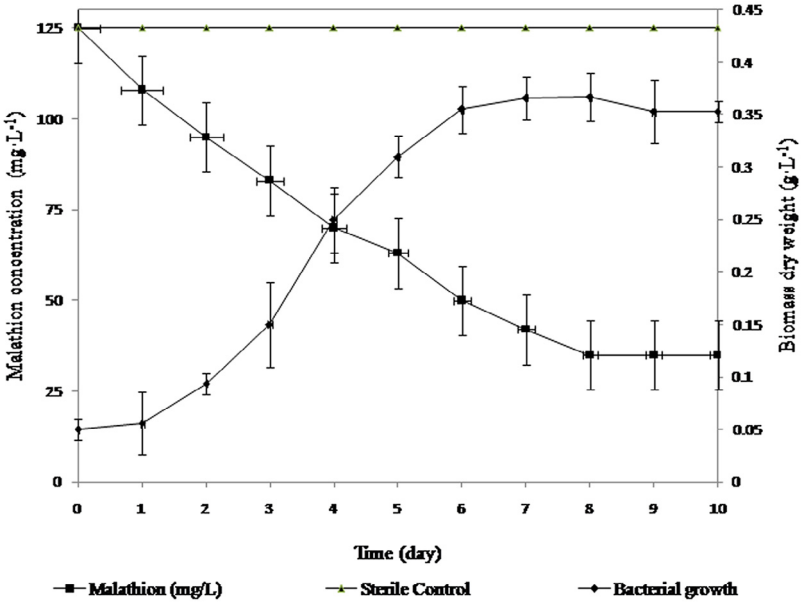

Fig. 4. The study of degradation of malathion by *Pseudomonas putida.*

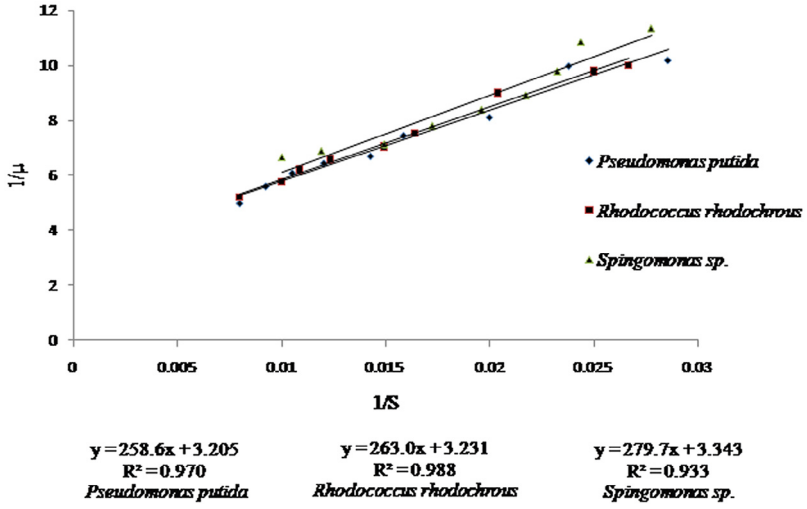

Fig. 5. The kinetic study of calculation of *Ks* and μ_{max} by Monod model best fitted plot.

biological degradation [38–40]. The isolate with a higher value of $\frac{\mu_{max}}{K_s}$ will have more degradation potential and growth in biochemical processes [41]. In the present case the value of $\frac{\mu_{max}}{K_s}$ (0.00387 L · mg^{-1} · day^{-1}) was found to be maximum for *P. putida* as compared to other bacterial species. The results clearly indicate that *P. putida* is more efficient than other species. The result can't be compared due to unavailability of data for malathion degradation.

3.3. Bacterial morphology

The resolution of the bacterium was good without any coating of the gold and platinum particles. The morphology of

Table 3
Growth kinetic parameters of different bacterial species by Monod model.

Sl. no.	Microorganisms	μ_{max} (day^{-1})	K_S (mg·L^{-1})	$\frac{\mu_{max}}{K_S}$ (L · mg^{-1} · day^{-1})	R^2
1	*Pseudomonas putida*	0.312	80.69	0.00387	0.970
2	*Rhodococcus rhodochrous*	0.309	81.34	0.00379	0.988
3	*Sphingomonas sp.*	0.299	83.67	0.00357	0.933

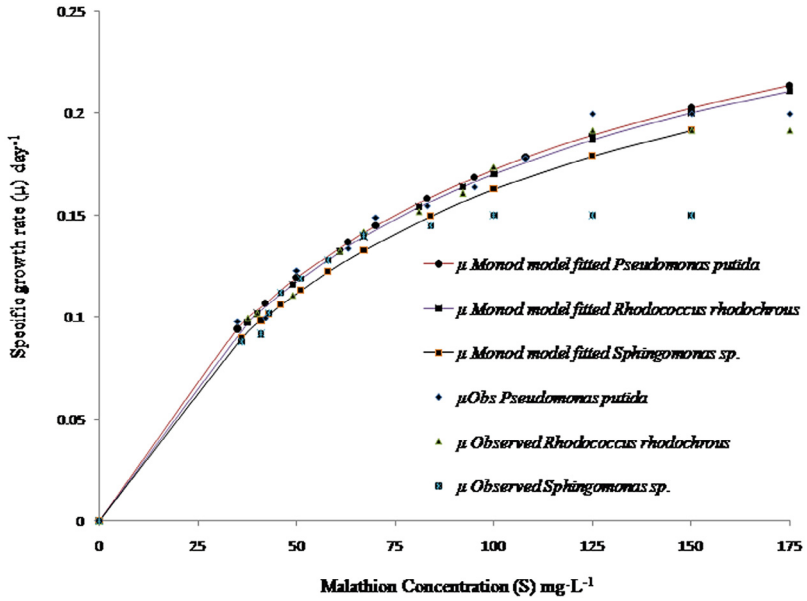

Fig. 6. The study of growth kinetic Monod model for different bacterial species with observed data and experimental data.

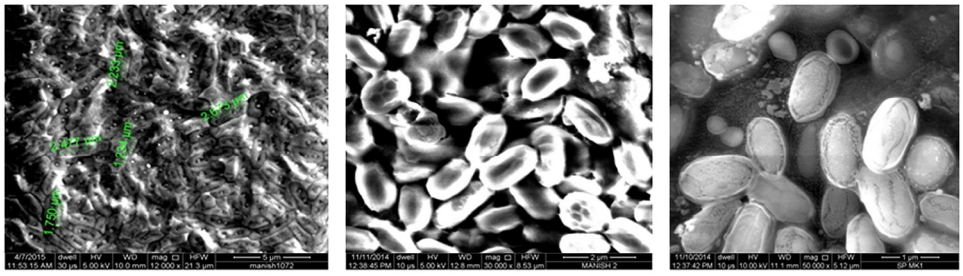

Fig. 7. The morphological study of *Pseudomonas putida* by SEM analysis.

the bacteria was examined using a low-resolution in order to minimize the harm to the bacterium using scanning electron microscope. The bacteria morphology was studied as available in the literature [42]. It was observed that scanning electron micrographs of *P. putida* have shown a long, rod morphology as depicted in Fig. 7. Scanning electron micrographs corresponding to a free-biofilm and a ten-day biofilm development in the presence of malathion as a carbon source are shown in Fig. 7. It was observed that there was no change found in the morphology of *P. putida.* It could be confirmed that the bacterium has the potential to withstand the pollutant load. Similar results were also observed by Tazdait et al. [32] for malathion degradation with acclimated activated sludge.

3.4. Functional group analysis

The result exhibits that malathion was degraded in liquid culture. From Fig. 8a it is seen that the spectrum ranges between 3271 and 3361 cm^{-1} and is typical for hydroxyl groups (O-H). The peak at band position 1636 cm^{-1} corresponding to the carbonyl groups (C = O) indicates that malathion was degraded by the *P. putida* during the experiment. In the control sample, the band spectrum of malathion was found in the range of 400–3280 cm^{-1} as shown in Fig. 8b.

3.5. Metabolites analysis

The results of GC-MS show the fragmented pattern of malathion, maloxon, malathion monocarboxylic acid and 2-mercaptosuccinic acid. The details are given in Table 4. Three types of products were detected in MSM medium by GC-MS. The spectrum pattern of malathion (without inoculums) shows a parent ion peak at m/z 330 molecular formula of malathion and was not strong (Fig. 9a). The parent ion undergoes a change in the form of cleavage of S–CH bond to form two highly abundant fragments, at m/z 173 and 125. The mass spectrum

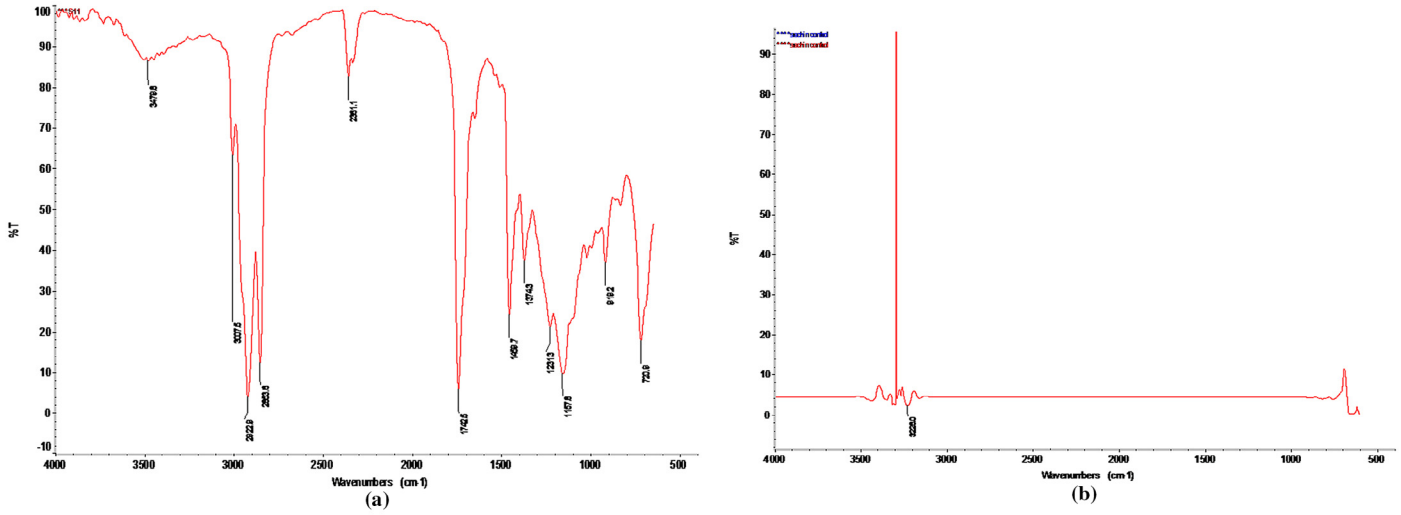

Fig. 8. The study of functional group analysis by FT-IR of (a) biodegraded intermediates of malathion and (b) control malathion.

Table 4
Intermediate metabolites formed during biodegradation of malathion by *Pseudomonas putida.*

Sl. no.	Compound	Formula	CAS no.	Molecular weight
1	Malathion	$C_{10}H_{19}O_6PS_2$	CAS:121-75-5	330
2	Malaoxon	$C_{10}H_{19}O_7PS$	CAS:1634-78-2	314
3	Malathion monocarboxylic acid	$C_8H_{15}O_6PS_2$	CAS:1190-29-0	302
4	2-mercaptosuccinic acid	$C_4H_6O_4S$	CAS:70-49-5	150

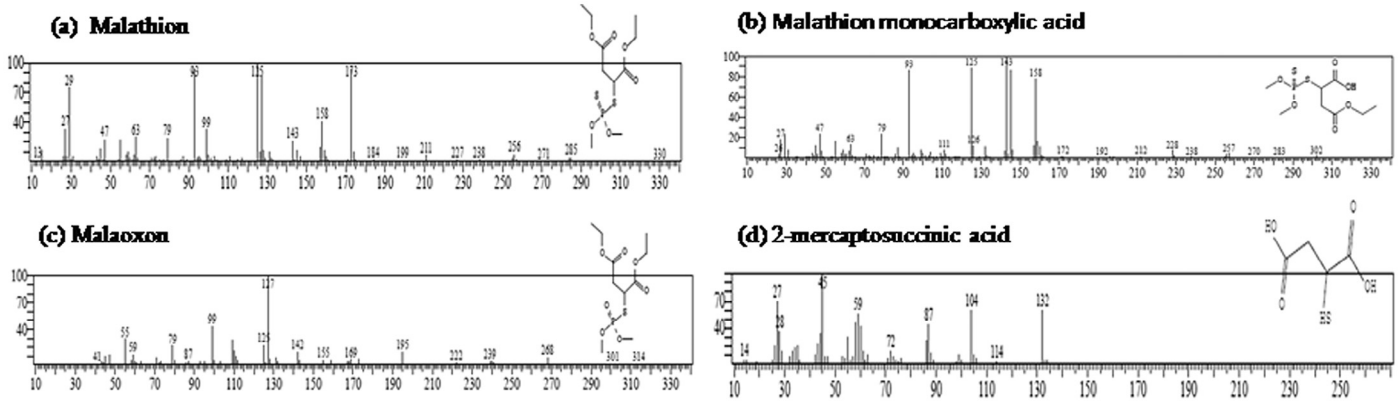

Fig. 9. GCMS analysis of (a) control malathion and (b) malathion monocarboxylic acid, (c) malaxion, (d) 2-mercaptosuccinic acid metabolites formed during malathion biodegradation.

shows two additional peaks at m/z 256 and 184 which correspond to loss of one and two $–COOC_2H_5$ fragments, respectively, from the parent ion. Another peak observed at m/z 285 is due to the evolution of CO_2 from the parent ion. Mass spectrum shows the peak at m/z 314 which was for malaoxon (Fig. 9b). Spectrum in Fig. 9c shows common features of malathion monocarboxylic acid, m/z 302 and Fig. 9d shows the fragments of 2-mercaptosuccinic acid. These results confirmed that the malathion was degraded during biodegradation process and converted into three metabolites in the present work, which is in agreement with previous works [25,29,37,43–45].

4. Conclusion

In the present study biodegradation efficacy of three bacterial species has been investigated. *P. putida* exhibited greater potential for malathion degradation than other bacterial species. Malathion degradation was found to be 72% at a concentration of 125 $mg{\cdot}L^{-1}$ by *P. putida*. The value of kinetic constants K_s was observed in the ranges of 80.69−83.67 $mg{\cdot}L^{-1}$ and μ_{max} varies from 0.312 to 0.299 day^{-1}. This study would be helpful in the practical application of *P. putida* for removal of malathion from the contaminated environment.

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