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BIODEGRADATION OF MALATHION BY *BACILLUS LICHENIFORMIS* STRAIN ML-1

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Abstract: Malathion, a well-known organophosphate pesticide, has been used in agriculture over the last two decades for controlling pests of economically important crops. In the present study, a single bacterium, ML-1, was isolated by soil-enrichment technique and identified as *Bacillus licheniformis* on the basis of the 16S rRNA technique. The bacterium was grown in carbon-free minimal salt medium (MSM) and was found to be very efficient in utilizing malathion as the sole source of carbon. Biodegradation experiments were performed in MSM without carbon source to determine the malathion degradation by the selected strain, and the residues of malathion were determined quantitatively using HPLC techniques. *Bacillus licheniformis* showed very promising results and efficiently consumed malathion as the sole carbon source via malathion carboxylesterase (MCE), and about 78% malathion was degraded within 5 days. The carboxylesterase activity was determined by using crude extract while using malathion as substrate, and the residues were determined by HPLC. It has been found that the MCE hydrolyzed 87% malathion within 96 h of incubation. Characterization of crude MCE revealed that the enzyme is robust in nature in terms of organic solvents, as it was found to be stable in various concentrations of ethanol and acetonitrile. Similarly, and it can work in a wide pH and temperature range. The results of this study highlighted the potential of *Bacillus licheniformis* strain ML-1 as a biodegrader that can be used for the bioremediation of malathion-contaminated soil.

Key words: Biodegradation; malathion; *Bacillus licheniformis*; carboxylesterase; organophosphorus pesticides

INTRODUCTION

Currently, among the diverse groups of pesticides used worldwide, organophosphorus (OP) pesticides form the major and most extensively used class of agricultural pesticides that account for more than 38% of the total world market (Singh 2009; Theriot and Grunden 2011). OPs are neurotoxic due to their ability to inhibit the acetylcholinesterase (AChE), a key enzyme for normal nerve signal transmission (Cherian et al., 2005; Singh and Walker 2006; Theriot and Grunden 2011). Most OPs have the same general structure, where a phosphate group or phosphate derivative is part of an organic molecule (Singh and Walker 2006; Ortiz-Hernández and Sánchez-Salinas 2010). Malathion [S-(1, 2-dicarbethoxyethyl)-O, O-dimethyldithiophosphate] is a non-systemic, wide-spectrum, phosphorodithioate organophosphorus compound (Xie et al., 2013; Goda et al., 2010). It is commonly used as a general purpose insecticide for the control of agricultural soil-dwelling insects, household insects, flies and animal parasites (Singh et al., 2012). Despite its high toxicity, it is still extensively used throughout the world. Along with harmful insects, malathion is toxic to many beneficial insects. It is highly injurious to some aquatic non-target species, such as aquatic stages of amphibians and some aquatic invertebrates. The toxic effects of malathion are well known on a wide range of animals that include fishes, annelids, crustaceans, echinoderms, insects, molluscs, nematodes, flatworms and many zooplanktons. The effects on mammals, especially human beings, include

leukemia, kidney damage, brain damage, lung damage, etc., while its carcinogenic effects include chromosomal aberrations in human blood cells and gene loss from human DNA (Kanade et al., 2012).

Microbial degradation is generally regarded as the safest, least disruptive and most cost-effective technology. Microorganisms are considered to be the major factors that determine the fate of xenobiotics including OP insecticides in the environment (Singh and Walker, 2006). Numerous bacterial strains belonging to different taxonomic groups have a great degradation potential for organophosphorus insecticides, including malathion (Rosenberg and Alexander, 1979; Kamal et al., 2008; Mohamed et al., 2010; Kanade et al., 2012; Hamouda et al., 2013).

Carboxylesterases (CEs) or carboxylic ester hydrolases are among the major enzymes used in pesticide bioremediation and in recent years several CEs of animal, plant and bacterial origin have been identified and studied (Morana et al., 2002; Mnisi et al., 2005; Silvia et al., 2009). CEs hydrolyze organophosphorus pesticides, carbamates, pyrethroids and even some organochlorinated pesticides (Goda et al., 2010). They hydrolyze the ester bonds of carboxyl ester substrate molecules, such as those present in malathion to form alcohols and carboxylic acids (Bornscheuer 2002). In many studies, microbial carboxylesterase genes involved in malathion degradation have been cloned and the recombinant proteins have been expressed as well.

The purpose of the present study was to isolate the bacterial strain that has the potential to utilize malathion as a sole carbon source. To ascertain these abilities, the utilization of malathion as the sole carbon source in liquid medium by bacteria, as well as the effect of an additional carbon source on the rate of malathion degradation were also determined.

MATERIALS AND METHODS

Reagents and chemicals

All analytical grade reagents and chemicals were purchased from Merck, Germany and Sigma-Aldrich, USA. Analytical grade malathion of purity > 99% was obtained from Sigma-Aldrich, USA. Synthetic oligonucleotides used in the study were obtained from Integrated DNA Technologies, USA.

Soil sampling and enrichment

Soil samples were collected from agricultural fields in Pakistan where commercial crops like cotton and tobacco were grown and have a history of pesticide application, including malathion. Soil samples were collected from the contaminated areas in sterile polythene bags, which were air dried and stored at 4°C to maintain the biological activity of the soil microflora. An enrichment culture technique was used to isolate bacterial strains responsible for the biodegradation of malathion. Soil samples were first enriched with commercial grade malathion to increase the probability of finding malathion-degrading native microflora. Approximately 15 g of soil was added to 10 mL commercial grade malathion and was incubated in the dark at room temperature for 1 month (Goda et al., 2010). The soil samples were then further enriched by initiating a shake-flask enrichment culture where previously enriched soil was suspended in minimal salt medium (MSM) containing analytical grade malathion at 220 rpm for 14 days (Naqvi et al., 2013)

Isolation of malathion-degrading microbes

Bacteria capable of degrading malathion were isolated from previously enriched soil on minimal salt medium (MSM). The MSM composition (w/v) was 0.1% NH_4NO_3, 0.1% NaCl, 0.15% K_2HPO_4, 0.05% KH_2PO_4, 0.01% $MgSO_4.7H_2O$, 0.0025% $FeSO_4$, 1% glucose and 1.5% agar per 100 mL of deionized water. Malathion-degrading bacteria were isolated from this enrichment culture by spreading on MSM agar plates where malathion (350 µg/mL) was added as either carbon or phosphorus source, or both as carbon and phosphorus sources; negative controls were always used to check any contamination of said sources in the media. The plates were incubated at 32°C for two days to get prominent bacterial colonies. The resulting colonies were selected on the basis of their mor-

phological difference and further purified on LB agar plates. The efficiency was measured by growing them separately in MSM containing malathion as either a carbon or phosphorus source, or as a source of both. The isolate that presented the highest growing ability was selected for further study. An axenic strain was maintained on minimal salt medium at 30°C and also cryopreserved for long-term storage.

Taxonomic identification of the bacterial strain

Molecular taxonomic identification was carried out by sequencing and analyzing the 16S rRNA gene fragment. Genomic DNA extraction from strain ML-1 was carried out using the method described by Sambrook et al. (1989). The 16S rRNA gene of the bacterial isolate was amplified by a set of universal primers, 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). PCR amplification was performed in a total volume of 25 µL by using a 2X PCR master mix (Fermentas, Life Sciences) in a 2720 Thermal Cycler of Applied Biosystems. The thermal cycler conditions for amplification were: initial denaturation of 95°C for 5 min, followed by 35 cycles of denaturation at 90°C for 30 s, primer annealing at 50°C for 30 s, extension at 72°C for 2 min; and final polymerization at 72°C for 2 min. The amplified product was resolved electrophoretically using 1% (w/v) agarose gel prepared in TBE buffer and compared to a 2 kb DNA ladder. An Affymetrix PrepEase® Gel Extraction Kit from USB Corporation was used to purify DNA from agarose gel. The amplified products were sequenced and compared to other 16S rRNA gene sequences available in the National Center for Biotechnology Information (NCBI) public database by basic local alignment search tool (BLAST) searching.

Biodegradation of malathion in liquid media

Malathion degradation experiments were carried out in MSM containing a single initial concentration (25 ppm). In one of the treatments, i.e. T1, malathion was added as the sole source of carbon. The effect of stimulants, i.e. the addition of carbon (0.1% glucose) source in T2 and both carbon and phosphorus (0.1%) sources in T3 was also monitored. All experiments were conducted in triplicate and a negative control without microbial inoculation was also carried out under the same conditions. All cultures were incubated at 32°C on an orbital shaker at 250 rpm. Sampling was done by drawing the 3-mL aliquot aseptically after every 48 h for 5 days. Growth of the bacterial strain was monitored by measuring the optical density at 600 nm (OD_{600}) and samples were placed at 4°C till extraction.

Isolation of malathion residues and analysis

The residual malathion and its possible metabolites were extracted from the culture media. Malathion was extracted twice by adding an equal volume of ethyl acetate to each aliquot. After vigorous shaking for 5 min, the organic layer was separated. The water contents, if present, were completely removed by passing the organic layer through anhydrous Na_2SO_4. The solvent was then allowed to evaporate completely at room temperature and residual malathion was reconstituted in 500 µL acetonitrile (Goda et al., 2010). The samples were then stored at 4°C until analysis by HPLC technique. The residues of malathion were determined quantitatively by using an HPLC system (Perkin Elmer, USA) equipped with a UV/VIS detector set at a wavelength of 215 nm. A C-18 reversed-phase analytical column with a suitable guard column was used and the mobile phase was a mixture of acetonitrile and water (50:50 v/v). The flow rate was 1.0 mL/min with an injection volume of 20 µL. Different concentrations of malathion were run for making a standard curve (Singh et al., 2012). For quantification, the retention indices of samples taken after different days were matched and their peak areas were compared. Statistical analysis was performed using the SPSS package and the mean, average, ANOVA and Duncan's tests were performed to observe the significant difference between various treatments and within the treatments with the passage of time.

Enzyme extraction

The bacterial strain was grown in LB broth to the late logarithmic phase; cells were harvested by centrifuga-

tion at 10000 rpm at 4°C. The cell pellet was washed and resuspended in 1 mL of 0.1 M phosphate buffer (pH 7.5). The cells were disrupted by sonication for 10 min while bursting them for 30 s, allowing a 15-s cooling time between each burst. The disrupted cells were centrifuged at a speed of 12000 rpm at 4°C for 30 min to remove all cell debris and the cell-free extract (CFE) was used as enzyme source for the enzyme activity (Chaudhry et al., 1988). Total cellular protein concentration was determined by the method of Lowry et al. (1951), using bovine serum albumin (BSA) as standard. To confirm the protein extraction, SDS-PAGE was performed stained with Coomassie brilliant blue in 40% (v/v) methanol plus 7% (v/v) glacial acetic acid and destained with 5% (v/v) methanol, 7% (v/v) glacial acetic acid and water (Mohamed et al., 2010; Xie et al., 2013).

Enzyme assay

A substrate-based enzyme assay was performed to determine malathion degradation (Xie et al., 2013). Malathion was dissolved in methanol (1 mM) and 100 µL was mixed with 700 µL of 0.1 M phosphate buffer (pH 7.5) and 200 µL CFE in a total reaction volume of 1 mL. Malathion solution without CFE served as a control. The reaction mixtures were incubated at 37°C and constant aliquots were collected at regular intervals. Residual malathion was extracted and analyzed by HPLC. Different concentrations of standard malathion solution were also injected to construct a standard curve.

Characterization of crude enzyme

The stability of malathion carboxylesterase (MCE) was observed at various pH (3-10) and temperatures (20-70°C) using malathion as a substrate. Additionally, the effect of different organic solvents, i.e. ethanol and acetonitrile, in various concentrations from 0-10% was observed to determine the stability of MCE against them. All treatments and blank samples were incubated at 28°C for 4 h. In the enzyme activity assay, a decline in the optical density measured at 240 nm served as the measure of increasing hydrolysis.

RESULTS

Isolation and identification of the candidate strain

Standard isolation and enrichment techniques yielded a bacterial isolate (ML-1) capable of degrading malathion. The strain showed visible growth on MSM supplemented with 350 µg/mL of malathion. The colonies grew well at 37°C within 48 h of incubation and utilized malathion as the sole carbon source. The genomic DNA of the isolate was used for amplification of 16S rDNA by using universal primers 27F and 1492R by PCR. The resulting bands were cut and eluted; the DNA thus obtained was subjected to sequencing. The amplified 16S rRNA of the bacterial isolate was sequenced and analyzed by BLAST search in the NCBI public database. The sequence of approximately 1562 base pairs of the 16S rRNA gene of the isolate was 99% identical to that of the 16S rRNA gene of *Bacillus licheniformis* (GenBank accession no. KF840408). Based on the sequence similarity, the strain was designated as *Bacillus licheniformis* ML-1 and its 16S rRNA sequence was deposited in the GenBank database with accession no. KM893455.

Biodegradation experiments

Degradation and residual determination of malathion

The results indicate the promising ability of the bacterial strain to use malathion as a carbon source. The residual malathion was monitored by HPLC for a period of 120 h in liquid culture. The data showed a considerable removal of malathion with elapsed time in inoculated media. The residual malathion was quantitatively measured by using HPLC technique. The results revealed that the *Bacillus licheniformis* strain ML-1 is highly efficient in degrading malathion and about 78% degradation of malathion was obtained within 5 days of incubation. The degradation pattern of malathion in MSM is shown in Fig. 1. Degradation of malathion was negligible in all non-inoculated controls, which indicates the degradation is only because of the consumption of malathion as

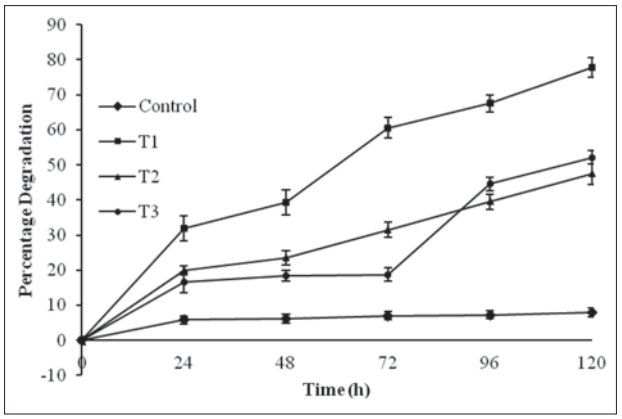

Fig. 1. Percent degradation of malathion in liquid culture media after different treatments T1 – without the stimulant malathion as the sole source of carbon; T2 – containing glucose as stimulant); T3 – containing phosphorus as stimulant. Error bars represent the standard deviation which was within 5% of the mean.

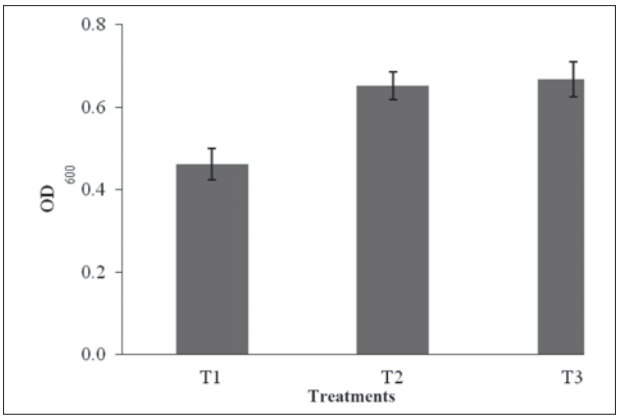

Fig. 2. Growth of *Bacillus licheniformis* in different treatments T1 – without the stimulant malathion as the sole source of carbon; T2 – containing glucose as stimulant; T3 – containing phosphorus as stimulant. Error bars represent the standard deviation which was within 5% of the mean.

carbon source by *Bacillus licheniformis* ML-1. Statistical analysis revealed that treatments have significant differences between themselves and treatment T1 was shown to be highly efficient in terms of malathion consumption or degradation as seen in Table 1 and 2. Malathion consumption (μM/h) was also measured, and it was found that the consumption of malathion was significantly higher in treatment T1 as compared to T2 and T3, as seen in Table 1. The rate of degradation of malathion in all treatments compared to the control increased significantly with time (Table 2), with the most significant increase in degradation observed in T1 after 120 h of treatment.

Table 1. Malathion consumption calculated with elapsed time of inoculated media.

Treatments	Substrate Consumption (μM/h)	Substrate Consumption (μM/day)	Substrate Consumption (μM/year)
T1	0.17252083	4.1405	1511.2825
T2	0.121875	2.925	1067.625
T3	0.125	3	1095

Table 2. Comparison of the percentage of degradation between different treatments with time.

Time (h)	Treatments			
	Control	T1	T2	T3
0	0.0K	0.00K	0.00K	0.00K
24	5.8J	31.88G	19.92HI	16.57I
48	6.2J	39.25F	23.50H	18.37I
72	7.0J	60.53C	31.60G	18.77I
96	7.3J	67.54B	39.5F	44.58E
120	8.0J	77.87A	47.37E	52.17D

Different letters indicate significant differences between the rates of degradation with time; values that share the same letters do not display significant differences.

Growth of bacterial isolate in liquid culture supplemented with malathion

The OD_{600} showed a steady increase in the bacterial mass in MSM supplemented with malathion as a sole source of carbon within 5 days of incubation. Glucose and phosphorus, in the MSM significantly stimulated the bacterial growth and was approximately two times faster than growth of the isolate in MSM with malathion as the sole carbon source (Fig. 2 but had no significant effect on malathion degradation.

Enzyme assay

Malathion degradation was monitored by HPLC analysis. Malathion was eluted at retention time of 11 min. The results revealed that there was a substantial reduction in the levels of malathion initially added to the reaction mixture. MCE hydrolyzed 87% of malathion within 72 h of incubation. The enzyme ac-

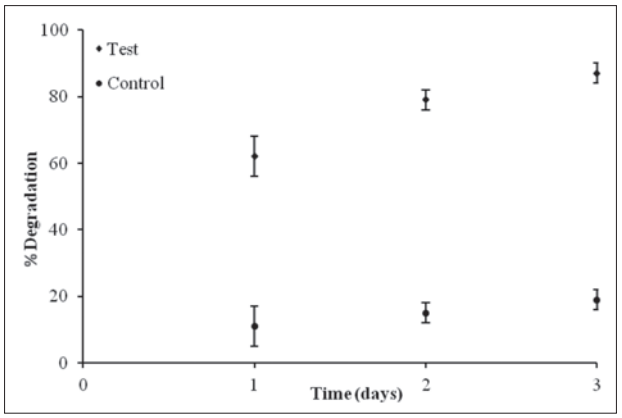

Fig. 3. Degradation of malathion in reaction mixture incubated at room temperature with crude extract as source of MCE. The error bars represent the standard deviation which was within 5% of the mean.

tivity increased with increasing incubation time and maximum activity was observed after 3 days of incubation, whereas no significant change in malathion concentration was observed in the blank sample incubated without MCE (Fig. 3).

Characterization of the crude enzyme

The optimum activity of MCE was observed at pH 7.5. At very low pH (3.0), the MCE had no activity but at a pH of 9.0 it was stable enough and showed activity when compared to the lower pH (3.0). These results revealed that the MCE is stable at wide range of pH and able to metabolize malathion, except at pH 3.0 where it shows no activity (Fig. 4). The activity of the enzyme was observed at different temperatures to determine how effective MCE is at various temperatures. In the present research, it was found that the MCE isolated from *Bacillus licheniformis* strain ML-1 was able to degrade malathion in wide range of temperatures. MCE has no activity at very low temperature, while from 30-40°C it showed high activity against malathion. Similarly, as the temperature increased above 50°C, the activity of the enzyme was reduced (Fig. 4). The activity of the enzyme in the presence of denaturing agents like organic solvents was also studied. Organic solvents including acetonitrile and ethanol were used in varying concentrations from

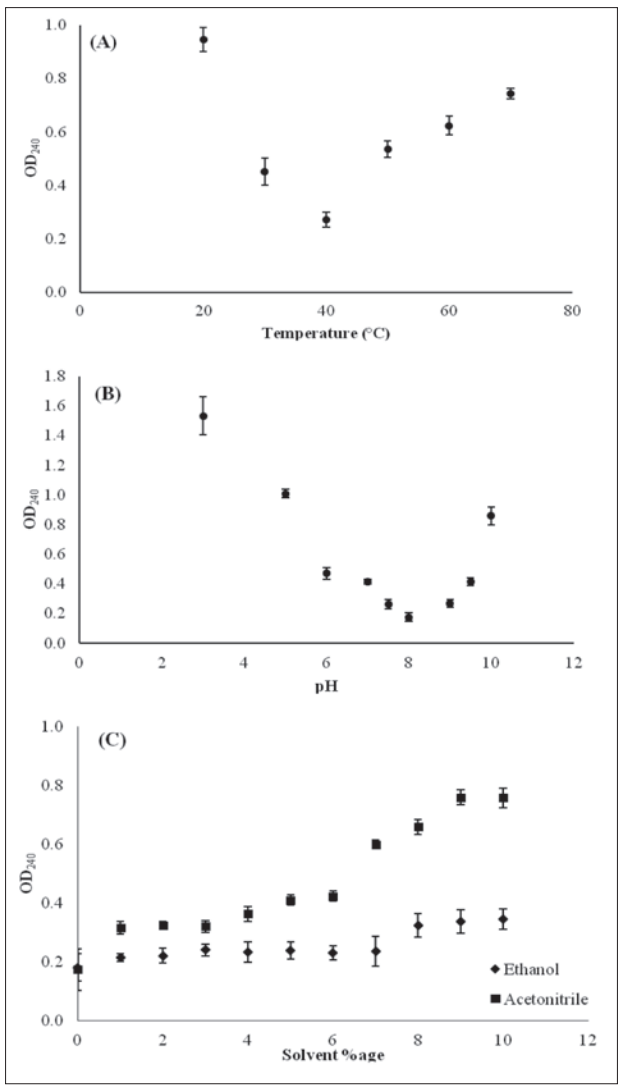

Fig. 4. Activity of MCE while monitoring the absorbance. Lower absorbance indicates that the parent compound (malathion) decreases due to enzyme activity. A – pH (3-10); B – temperature (20-70°C); C – acetonitrile and ethanol concentration (0-10%). The data are presented as averages from three independent experiments; the error bars represent the standard deviation which was within 5% of the mean.

0-10% to determine enzyme activity. All treatments and blank sample without the enzyme were incubated at 28°C for 4 h. Our results revealed that the enzyme was resistant to the presence of solvents as the addition of 6% (v/v) ethanol and 7% (v/v) acetonitrile had little effect on enzyme activity (Fig. 4).

DISCUSSION

Organophosphorus insecticides like malathion are considered to be potentially hazardous and have been known to cause several adverse effects on humans and other non-target organisms (Goda et al., 2010; Xie et al., 2013). Therefore, remediation of sites contaminated with these OPs is of general interest. Bioremediation provides an efficient and cheap alternative for the removal of such toxic pollutants from the environment (Sutherland et al., 2004). It is very important to find new microbial strains for the effective degradation of organophosphorus insecticides. Several bacterial strains capable of degrading organophosphorus compounds have been isolated from pesticide-contaminated soils (Singh and Walker 2006).

In the present study, a bacterium (ML-1) was isolated from contaminated soil with a history of pesticide application. The strain showed visible growth on MSM supplemented with 350 µg/mL malathion which it utilized it as the sole source of carbon. Bacterial degradation and utilization of malathion and similar compounds have been reported by Bhaskaran et al. (1973), Rosenberg and Alexander (1979), Nelson (1982), Kamel and Al-Awadi (1987), Kamal et al. (2008), Mohamed et al. (2010), Kanade et al. (2012) and Hamouda et al. (2013).

The selected isolate was identified through the amplification and sequencing of the 16S rRNA gene. Based upon percentage homology with the known database, the malathion-degrading strain (ML-1) was identified as *Bacillus licheniformis*. Several *Bacillus* species have already been reported to degrade different OP compounds, including malathion. Mohamed et al. (2010) isolated a bacterial strain identified as *Bacillus thuringiensis* was able to utilize malathion as sole carbon and phosphorus source. Similarly, Hamouda et al. (2013) reported the isolation of nine malathion-degrading bacterial strains belonging to *Acinetobacter* and *Bacillus* species. In another study, two aerobic malathion-degrading bacterial strains, *Brevibacillus* sp. and *Bacillus cereus*, were isolated from an agricultural soil sample by an enrichment culture technique (Singh et al., 2012).

An enzyme assay was carried out to determine malathion carboxylesterase (MCE) activity. Results revealed a substantial reduction in the levels of malathion with the passage of time. MCE hydrolyzed 87% malathion within 72 h of incubation. In accordance with the above results, a carboxylesterase gene was heterologously expressed from a thermophilic bacterium, *Alicyclobacillus tengchongensis*, to determine its malathion-degradation potential. The recombinant enzyme hydrolyzed 50% malathion (5 mg/L) within 25 min and 89% malathion within 100 min (Xie et al., 2013).

In the current study, the malathion-degradation potential of the isolate ML-1 was examined during 120 h of incubation in liquid culture, and the residual malathion was quantitatively measured by using the HPLC technique. The results revealed that 78% of the malathion initially added to the medium was hydrolyzed by the strain ML-1 within 5 days of incubation time. In previous reports, the degradation of malathion in soil by *Bacillus* sp. has also been reported. Residual malathion was monitored by HPLC analysis and it was found that 74.11% of malaoxon (an analog of malathion) was degraded by the strain (Singh et al., 2013). Similarly, Singh et al. (2012) reported the degradation of malathion by *Brevibacillus* sp. and *Bacillus cereus* in liquid media. Reverse phase HPLC-UV analysis indicated that the strains were able to degrade 36.22% and 49.31% of malathion, respectively, after 7 days of incubation.

The degradation pattern of the isolate was also monitored in the presence of carbon and phosphorus stimulants. Malathion degradation in the presence of stimulants was comparatively slow compared to the medium where malathion was present as the only carbon source. This can be explained by the preferential use of glucose and phosphorus by the isolate instead of malathion.

The stability of malathion carboxylesterase (MCE) was also tested under different parameters, namely pH, temperature and in presence of organic solvents such as ethanol and acetonitrile. Results revealed that MCE is stable in a wide range of pH and

able to metabolize malathion, except at pH 3.0 where it shows no activity. The activity of the enzyme was observed at different temperatures to determine how effective MCE is at various temperatures. As in environmental applications, temperature is the most important factor that may alter enzyme activity. MCE seems to be robust in nature in terms of temperature and can work at various temperatures up to 40°C. The enzyme was also fairly resistant to the presence of organic solvents where the addition of 6% (v/v) ethanol and 7% (v/v) acetonitrile had little effect on its activity. This finding was also observed by Xie et al. (2013), who reported the heterologous expression of a carboxylesterase from *Alicyclobacillus* sp. to gain insight into the characteristics and malathion degradation of the recombinant enzyme. The optimum temperature for the enzyme activity was 60°C. The purified enzyme was optimally active at pH 7; in contrast, the enzyme was not stable at pH 5.4 or 8. Similarly, a methyl carbamate-degrading hydrolase (MCD) of *Achromobacter* WM-111 was heterologously expressed. The purified enzyme catalyzed the hydrolysis of carbamate, carboxyl ester and a phophotriester, and was found to be relatively resistant to thermal and solvent-mediated denaturation (Naqvi et al., 2009). The results of this study highlighted the potential of *Bacillus licheniformis* strain ML-1 as a biodegrader that can be used for the bioremediation of malathion-contaminated soil.

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