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From Toxins to Safety: *Alternaria alstromeriae's* High-Efficiency Malathion Biodegradation Unveiled

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Abstract

This study investigated the biodegradation of the pesticide malathion by soil fungi, focusing on *Alternaria alstromeriae KRMY EMIC001* (GenBank: PQ764546). The fungus demonstrated high degradation efficiency, achieving 89.5% degradation at 30ppm malathion under optimized conditions (25°C, pH 6, 21 days). Efficiency remained robust (86.5%) even at higher concentrations (150ppm). The study also examined the impact of different nitrogen and carbon sources on the degradation process. Additionally, the level of phosphatase enzyme in *Alternaria alstromeriae* KRMY EMIC001 was measured in the presence of malathion, showing a gradual decrease as malathion concentrations declined, highlighting the enzyme's role in the biodegradation process.

Keywords: Biodegradation; Malathion; Pollution; *Alternaria alstromeriae*; Phosphatase enzyme

Introduction

Malathion ($C_{10}H_{19}O_6PS_2$), a broad-spectrum organophosphorus insecticide, inhibits acetylcholinesterase and is extensively used in agriculture and public health programs. Despite its efficacy, malathion and its degradation byproducts (e.g., malaoxon) exhibit heightened neurotoxicity and environmental persistence, threatening ecosystems and human health—particularly in densely treated regions [1]. In Egypt, its affordability has exacerbated contamination of soil and water systems [2], necessitating sustainable remediation strategies. Conventional malathion removal via chemical oxidation or adsorption is costly and generates secondary pollutants [3]. In contrast, microbial degradation leverages enzymatic pathways (e.g., phosphotriesterases) to mineralize malathion into non-toxic intermediates [4]. While bacterial degraders (e.g., *Pseudomonas* spp.) are well-characterized [5]. Pesticides, including malathion, represent a diverse group of chemical compounds with varying physicochemical properties, necessitating the development of specialized analytical methods and equipment. This diversity can pose challenges for many laboratories, particularly in resource-limited settings [6]. Human exposure to pesticides occurs primarily through ingestion, inhalation, or dermal contact, with ingestion being the most significant route of contamination, followed by inhalation [7,8]. Fungal systems—particularly soil-inhabiting genera like *Alternaria*—remain overlooked. Existing research prioritizes white-rot fungi (e.g., *Phanerochaete chrysosporium*), despite evidence that *Alternaria* spp. thrive in pesticide-contaminated soils and secrete hydrolytic enzymes (e.g., phosphatases) with untapped bioremediation potential. We report the isolation and optimization of *Alternaria alstromeriae* KRMY EMIC001 (GenBank: PQ764546), a newly documented fungal strain capable of degrading 89.5% of malathion (30ppm) within 21 days. Fungal systems offer distinct advantages over bacteria, including: Environmental resilience: Tolerance to pH/temperature fluctuations (optimal at pH 6, 25°C). Enzymatic efficiency: Extracellular phosphatase production correlates with degradation rates. Scalability: Cost-effective growth in low-nutrient conditions, ideal for resource-limited settings. This study bridges a critical gap in fungal bioremediation, positioning *A. alstromeriae* as a viable agent for large-scale malathion detoxification.

Materials and Methods

Collection of soil and water samples for fungal isolation

Soil and water samples were collected from various locations in Kafr El-Zayat City, Gharbia Governorate, Egypt. Soil samples

were collected from pesticide-contaminated sites at a depth of 5-10cm, air-dried, and sieved through a 2mm mesh. The sieved samples were stored in sterile polyethylene bags and transported to the laboratory, where they were kept at 3°C until further processing. Water samples were collected in sterilized glass bottles and processed within 24 hours of collection [9].

Cultural medium

Czapek-Dox agar medium was used for the isolation, screening, and cultivation of fungi. The medium was prepared by dissolving the following components in 1000 mL of distilled water (g/L): sucrose (30.0), sodium nitrate (2.0), potassium dihydrogen phosphate (1.0), potassium chloride (0.5), magnesium sulfate (0.5), ferrous sulfate (0.01), and agar (15.0). The pH was adjusted to 7.3 ± 0.2, and chloramphenicol (0.05g/L) was added as a bacteriostatic agent. The medium was autoclaved at 121°C for 15 minutes before use [10].

Fungal isolation and identification

Fungi were isolated from soil and water samples using the serial dilution method [11]. Aliquots of the diluted samples were inoculated onto Czapek-Dox agar plates and incubated at 28 ± 2°C. Fungal colonies were subcultured onto fresh medium and purified using either the hyphal tip or single spore technique. Purified isolates were transferred to Czapek-Dox agar slants for further identification and characterization.

Inoculum preparation

Fungal inoculum was prepared by growing the isolates on sterilized potato dextrose agar (PDA) slants for 5 days at 30°C. Spore suspensions were prepared by adding 0.9% NaCl solution containing a few drops of Tween 80 to each slant and gently brushing the surface with an inoculating loop under aseptic conditions. The spore suspension was diluted, and clumps were dispersed by stirring. Serial dilutions were prepared in 0.1% Tween 80, and spore counts were determined using a hemocytometer. A spore suspension containing 1×10^6 spores/mL was used as the inoculum [12].

Screening for pesticide degradation by isolated fungi

Screening on solid medium

The degradation capability of the fungal isolates was assessed using Czapek-Dox agar plates supplemented with malathion at concentrations of 0, 30, 60, 90, and 150ppm. Chloramphenicol (0.05% w/v) was added to prevent bacterial contamination, and the pH was adjusted to 7.3 using 1N HCl. Plates were inoculated with a 9mm fungal disc and incubated at 28°C for 7 days. Fungal growth and tolerance to malathion were evaluated by measuring colony diameter and comparing it to control plates without malathion.

Screening in liquid medium

screening was performed in 250mL Erlenmeyer flasks containing 100mL of Czapek-Dox broth supplemented with varying concentrations of malathion. The pH of the medium was adjusted to 7.0, and chloramphenicol was added after autoclaving. Each flask was inoculated with 1×10^7 spores/mL and incubated in a rotary shaker at 28°C and 130rpm for 7 days. After incubation, the culture was filtered through Whatman No. 2 filter paper, and the fungal biomass was determined by dry weight measurement. The filtrate was centrifuged at 10,000rpm for 10 minutes, and the supernatant was collected for spectrophotometric analysis of malathion degradation.

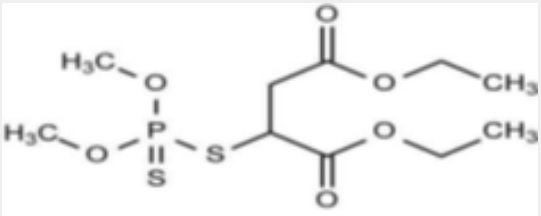

Figure 1: Chemical structure of malathion: -Diethyl 2- dimethoxyphosphinothioylsulfanylbutanedioate ($C_{10}H_{19}O_6PS_2$).

Determination of malathion degradation by spectrophotometry

The maximum absorbance wavelength for malathion was determined to be 520nm. A standard curve was constructed using known concentrations of malathion to quantify its degradation. The percentage of malathion degradation was calculated based on the reduction in absorbance at 520nm after fungal treatment (Figure 2).

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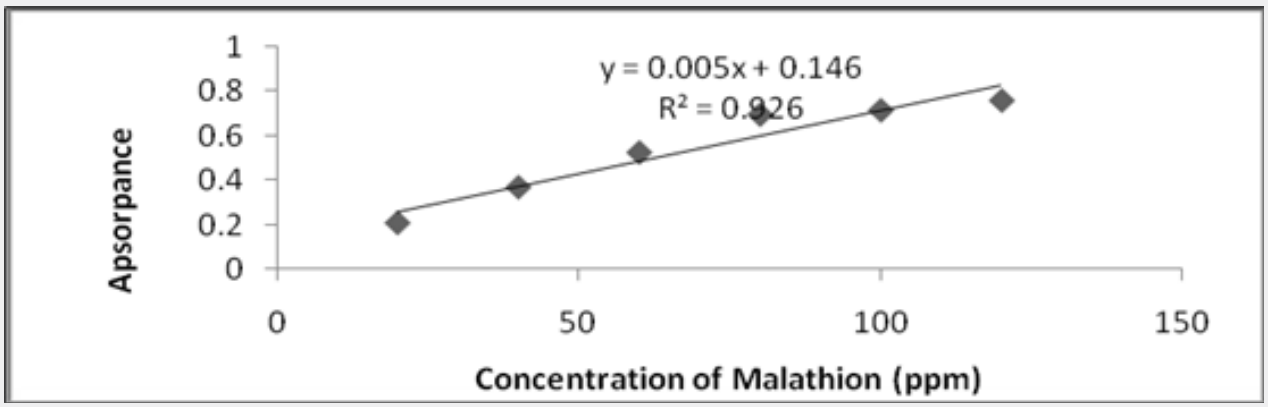

Figure 2: Standard curve of Malathion (ppm).

Identification of the isolated fungus with high degradation ability (isolate no. 2)

Fungal isolate No. 2 demonstrated the highest malathion degradation capability and was selected for further characterization. Pure cultures of the isolate were prepared and identified using light microscopy. Morphological identification was based on colony characteristics, cultural features, and the structure of sporulating organs, following standard mycological keys [13,14].

Molecular identification of fungal strains

The fungal strain (isolate No. 2) was further identified by 18S r RNA sequence analysis. DNA extraction was performed using a modified Chelex protocol: 20µL Chelex, 40µL TE buffer, and fungal mycelia were heated to 95°C for 2 minutes, followed by centrifugation at 13,000 × g for 2 minutes. The supernatant containing DNA was transferred to clean tubes and stored at -18°C.

The internal transcribed spacer (ITS) region was amplified using PCR with the following conditions: 35 cycles of denaturation at 94°C for 35 seconds, annealing at 55°C for 55 seconds, and extension at 72°C for 45 seconds, with a final extension of 7 minutes at 72°C. The primers used were ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [15,16]. PCR products were sequenced by Macrogen (Animal Health Research Institute (AHRI), and sequences were aligned using MEGA version 5. A BLAST search in Gen Bank identified the most similar sequences. Phylogenetic analysis was performed using the Maximum Likelihood method in MEGA version 5, with bootstrap support based on 1,000 replicates. The isolate was identified as *Alternaria alstromeriae* KRMY EMIC001.

Optimization of Growth Conditions for Malathion Biodegradation by Selected Isolates.

The growth conditions for isolate No. 2 were optimized in Czapek-Dox liquid medium under varying physiological parameters:

pH: 2, 4, 6, and 8

Temperature: 20, 25, 27, and 30°C

Incubation time: 7, 14, 21, and 30 days

Carbon sources: Zero carbon, glucose, fructose, sucrose, maltose, lactose, and starch

Nitrogen sources: Zero nitrogen, sodium nitrate, ammonium nitrate, urea, asparagine, and peptone

Cultures were incubated under shaking conditions at 130rpm.

Assay of phosphatase activity

Phosphatase activity was measured alongside malathion degradation. Samples were centrifuged at 10,000× g for 15 minutes at 4°C, and fungal cells were washed three times with 10mmol L^{-1} sodium carbonate/bicarbonate buffer (pH 10.5). Cells were resuspended in 5mL of the same buffer and sonicated for 10 seconds (10 cycles with 15-second intervals) to release phosphatase enzymes. The supernatant was collected after centrifugation to remove cell debris.

Phosphatase activity was determined using an alkaline phosphatase kit (Spectrum, Egypt). The working solution was prepared by mixing 900µL buffer (R1) with 100µL substrate (R2). A 10µL sample was added to 1mL of the working solution, and absorbance was measured at 405nm using a DTN-405 semi-automated chemistry analyzer. The mean absorbance change per minute (ΔA/min) was calculated [17].

Gas chromatography-mass spectrometry (GC-MS) analysis

After the incubation period, 100mL of growth medium containing malathion was mixed with 50mL dichloromethane and 40mL of 20% sodium chloride solution in a 500mL separatory funnel. The mixture was partitioned three times with dichloromethane, filtered through anhydrous sodium sulfate, and evaporated to dryness using a rotary evaporator at 30°C. The residue was dissolved in 1mL acetone, filtered through a 0.45µm syringe filter, and 200µL was injected into the GC-MS system.

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GC-MS analysis was performed using a Trace GC Ultra-TSQ Quantum mass spectrometer (Thermo Scientific, USA) equipped with a TG-5MS capillary column (30m × 0.25mm × 0.25µm). The oven temperature was held at 70°C for 5 minutes, then increased to 280°C at 5°C/min and held for 5 minutes. The injector and MS transfer line temperatures were set at 250°C. Helium was used as the carrier gas at a flow rate of 1 =mL/min. The system operated in SIM/SCAN mode, with a solvent delay of 4 minutes [18].

Statistical analysis

All experiments were conducted in triplicate. Mean values and standard deviations were calculated using Microsoft Excel 2010.

Results and Discussion

Isolation and screening of malathion-degrading fungal strains

Table 1: Fungi isolates from soil and water samples were collected around Kafer El Zayate Company (KEC) for pesticides.

Number of Isolate	Site	Type of Sample
Isolate: 1	Around the company	Soil(S1)
Isolate: 2	Around the company	Soil(S2)
Isolates: 3, 4, 5, 6	Around the company	Soil(S3)
Isolates: 10, 11	Around the company	Soil(S4)
Isolates: 12, 13, 14, 15	Around the company	Soil(S5)
Isolates: 7, 8, 9	Wastewater beside the company	Water (W1)

Table 2: Effect of different concentration of malathion (ppm) on redial growth on different isolates.

	Redial Growth (cm)					
	Different Concentration of Malathion (ppm)					
Isolate	Control (0ppm)	30ppm	60ppm	90ppm	120ppm	150ppm
1	7.7 ± 0.01	2.6 ± 0.01	1.7 ± 0.01	0.8 ± 0.02	0.8 ± 0.02	0.8 ± 0.02
2	7.7 ± 0.02	5.7 ± 0.01	4.2 ± 0.01	3.8 ± 0.01	2.4 ± 0.01	1.9 ± 0.01
3	7.31 ± 0.02	2.72 ± 0.01	1.8 ± 0.01	1.3 ± 0.01	1.0 ± 0.01	0.8 ± 0.01
4	6.63 ± 0.01	2.7 ± 0.01	1.6 ± 0.01	1.1 ± 0.01	1.0 ± 0.01	0.8 ± 0.01
5	6.8 ± 0.02	2.2 ± 0.02	1.5 ± 0.01	0.9 ± 0.01	1.0 ± 0.01	0.8 ± 0.01
6	7.7 ± 0.01	2.6 ± 0.01	1.7 ± 0.02	0.8 ± 0.02	0.8 ± 0.01	0.8 ± 0.01
7	7.31 ± 0.01	2.8 ± 0.01	1.9 ± 0.01	1.2 ± 0.02	1.0 ± 0.01	0.8 ± 0.01
8	7.31 ± 0.01	2.72 ± 0.01	1.8 ± 0.01	1.3 ± 0.01	1.0 ± 0.01	0.8 ± 0.01
9	6.63 ± 0.01	2.7 ± 0.02	1.6 ± 0.01	1.1 ± 0.01	1.0 ± 0.01	0.8 ± 0.01
10	6.8 ± 0.01	2.2 ± 0.01	1.5 ± 0.01	0.9 ± 0.02	1.0 ± 0.01	0.8 ± 0.01
11	7.7 ± 0.01	2.6 ± 0.02	1.7 ± 0.01	0.8 ± 0.01	0.8 ± 0.03	0.8 ± 0.01
12	7.31 ± 0.01	2.8 ± 0.02	1.9 ± 0.01	1.2 ± 0.01	1.0 ± 0.01	0.8 ± 0.01
13	7.31 ± 0.02	2.72 ± 0.01	1.8 ± 0.01	1.3 ± 0.01	1.0 ± 0.01	0.8 ± 0.01
14	6.63 ± 0.03	2.7 ± 0.01	1.6 ± 0.02	1.1 ± 0.01	1.0 ± 0.01	0.8 ± 0.01
15	6.8 ± 0.01	2.2 ± 0.01	1.5 ± 0.01	1.1 ± 0.01	1.0 ± 0.01	0.8 ± 0.01

Fifteen fungal strains were isolated from soil samples and three from wastewater adjacent to Kafr El-Zayat Pesticides Company (KEC), Egypt (Table 1). Initial screening under varying malathion concentrations (30-150ppm) identified six isolates (1–6) capable of degrading the pesticide. Isolate 2 (*Alternaria alstromeriae* KRMY EMIC001) exhibited superior radial growth (7.7 ± 0.02cm in control; 1.9 ± 0.01cm at 150ppm) and degradation efficiency (Table 2), outperforming other strains. This aligns with reports of *Alternaria* spp.'s bioremediation potential [3] and enzymatic organophosphate degradation mechanisms observed in *Fusarium oxysporum* [4].

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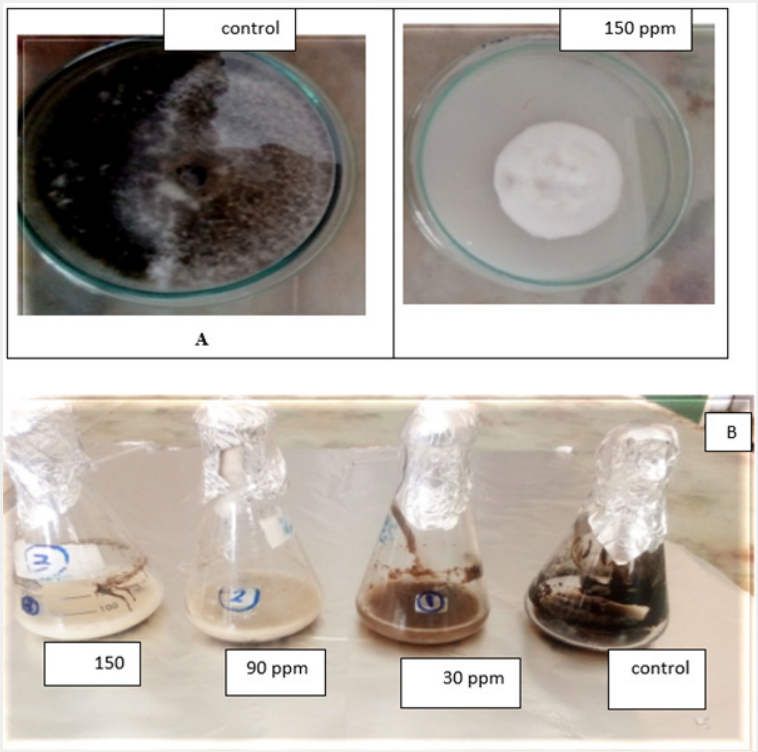

Figure 3: Linear growth and degradation percent of (Alternaria alstroemeriae KRMY EMIC001) under different concentration of malathion (ppm) on solid media (A) and liquid media (B), after 7-day isolate No. 2.

Molecular identification and phylogenetic analysis

ITS sequencing (1,391bp) of isolate 2 revealed 96% nucleotide identity with *Alternaria alstromeriae* KRMY EMIC001 (NCBI: PQ764546). Phylogenetic analysis (MEGA 5, Maximum Likelihood, 1,000 bootstrap replicates) confirmed its taxonomic position within the *Alternaria* clade (Figure 4), consistent with ITS-based fungal identification [19].

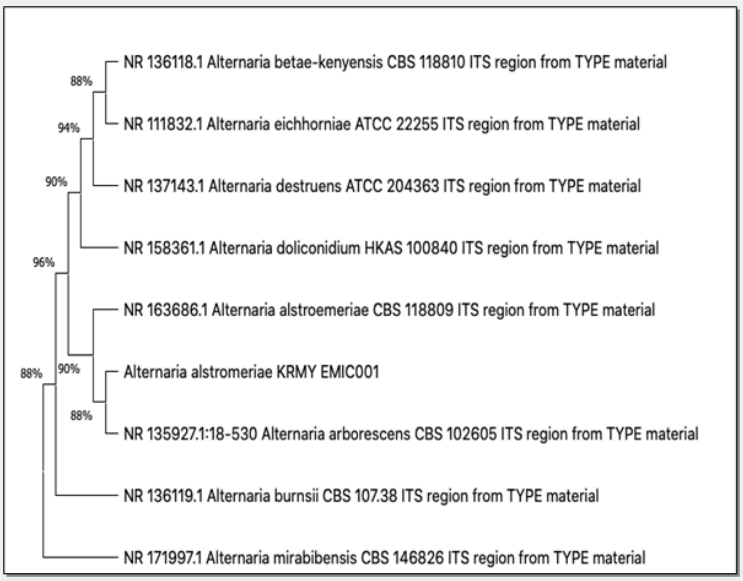

Figure 4: A maximum likelihood phylogenetic tree with 1000 bootstrap replicates is constructed.

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Optimization of malathion degradation

Optimal degradation by *A. alstromeriae* occurred at pH 6, 25°C, with sucrose (67.0 ± 0.01% at 30ppm) and sodium nitrate (77.1 ± 0.02% at 30ppm) as carbon and nitrogen sources, respectively (Table 4). Prolonged incubation (21 days) enhanced degradation to 85.8 ± 0.05% (30 ppm) and 79.7 ± 0.01% (150ppm), mirroring enzymatic adaptation kinetics in *Pleurotus ostreatus* [20]. The pH and temperature optima correlate with fungal phosphatase activity [21].

Table 3: These findings highlight the potential of *Alternaria alstromeriae* KRMY EMIC001 as a promising candidate for the bioremediation of malathion-contaminated environments Isolate from 7 to 15 have no degradation effect on malathion.

Isolate	Degradation of Malathion (ppm) after Different Days at a Different Concentration(ppm)				
	Concentration (ppm)	7 days	14 days	21 days	30 days
1	30	15.1±0.01	25.8±0.02	37.5±0.02	40.1±0.01
	90	42.3±0.02	48.9±0.02	50.5±0.03	51.2±0.03
	150	45.6±0.01	48.6±0.02	54.6±0.02	53.7±0.02
2	30	67.1±0.03	77.8±0.01	89.5±0.01	81.2±0.01
	90	74.8±0.02	74.9±0.01	83.0±0.02	79.2±0.01
	150	77.8±0.02	80.8±0.01	86.8±0.02	82.5±0.01
3	30	12.8±0.01	22.0±0.01	27.8±0.01	28.8±0.03
	90	16.5±0.01	27.3±0.03	26.8±0.01	27.2±0.05
	150	10.3±0.02	19.57±0.01	32.6±0.01	29.7±0.04
4	30	5.7±0.03	6.0±0.01	7.3±0.01	8.1±0.03
	90	2.8±0.03	8.0±0.01	17.2±0.01	19.3±0.04
	150	4.7±0.02	5.3±0.01	18.4±0.01	23.1±0.01
5	30	0.6±0.01	6.0±0.01	6.6±0.01	7.3±0.01
	90	0.9±0.01	1.9±0.01	3.84±0.02	2.9±0.01
	150	0.4±0.01	0.9±0.01	2.3±0.03	4.0±0.03
6	30	0.2±0.01	0.2±0.01	0.6±0.04	0.7±0.02
	90	0	1.0±0.01	0.84±0.01	0.72±0.02
	150	0	0.4	0.5	0.61

Table 4: Growth factors Optimizing conditions for malathion degradation percentage with *Alternaria alstroemeriae* KRMY EMIC001.

Growth Factors	Optimum Factor	Malathion (ppm)	
		30	150
		Degradation Percentage	
Carbon source	Sucrose	67.0±0.01	63.2±0.02
pH	6	74.7±0.02	71.2±0.02
Nitrogen source	Sodium nitrate	77.1±0.02	71.4±0.02
Time course	21 days	85.8±0.05	79.7±0.01
Temperature	25oC	77.1±0.02	68.2±0.01

Phosphatase activity and enzymatic degradation

Phosphatase activity peaked at 318.6 ± 0.04U/L (150ppm, 30 days), correlating with malathion degradation (Table 5). This suggests phosphatase-mediated hydrolysis of P–O–C bonds, yielding less toxic metabolites (e.g., dimethyl thiophosphate), consistent with organophosphate degradation pathways [22].

Table 5: Determination of phosphates enzyme activity.

Determination of Phosphates Enzyme Activity(U/L)		
Concentration (ppm)		Incubation Period
150	30	(day)
not detected	not detected	zero
248.1 ±0.02	42.2 ±0.02	7

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297.3 ±0.02	67.1± 0.01	14
314 .1±0.01	71.8± 0.05	21
318 .6±0.04	63.6 ±0.03	30

GC-MS analysis of degradation products

GC-MS confirmed an 89.5% reduction in malathion after 21 days, with dimethyl 3-thiaadipate (13.962% abundance) as a primary metabolite (Figure 5, Tables 6-7). The absence of toxic intermediates (e.g., phosphorodithioic acid) and the prevalence of alkanes/esters indicate detoxification via P–S bond cleavage, a mechanism observed in *Fusarium* spp. [2,23].

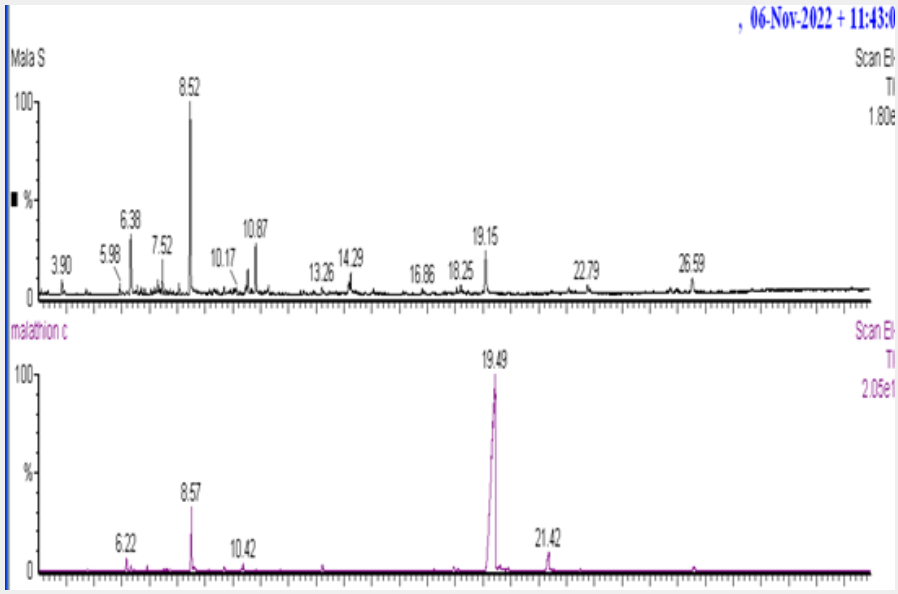

Figure 5: GC mass peak of sample (after degradation) and control (before degradation).

Table 6: Chemichal sturacture of controle(malathion + media) compound and its area% with number of peak.

Chemical Structure	Toxicity	Compound of Control	Area% (N: Normalized%)	N. of Peak	Reference Number
$C_3H_9O_2PS_2$	Fatal if ingested, inhaled, or absorbed through skin	Phosphorodithioic acid, OO, S-Trimethyl ester	1.251 (N:1.57%)	6.22	[20]
$C_7H_{12}O_4S$	Useful in organic synthesis	Dimethyl 3-thiaadipate	5.306 (N:6.68%)	8.57	[25]
-C(=NH) NH_2	May cause skin/eye irritation	Ethanimidamide,2-chloro-N^2-(2-chlorobenzenesulfonic acid)	0.901 (N:1.13%)	10.42	[15]
$C_{10}H_{19}O_6PS_2$ (Malathion)	Toxic and carcinogenic	Malathion (Diethyl 2-dimethoxyphosphinothioylsulfanylbutanedioate)	79.473 (N:100%)	19.49	[24]
$(CH_2)_2(CO_2H)_2$ derivative	Toxic if swallowed; suspected genetic defects; organ damage (CNS, PNS)	Succinic acid, mercapto-, diethyl ester, S-ester with O, S-dimethylphosphorodithioate	3.748 (N:4.71%)	21.42	[2]

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Table 7: Chemichal sturacture of sample (fungi + malathion+ media) compound and its area % with number of peak.

Chemical Structure	Toxicity Description	Compound of Sample	Area% (N: Normalized%)	No. of Peak	Reference
$C_6H_{12}O$	Specific toxicity varies	2,2,3,3-tetraethyl oxirane	0.486 (N:3.48%)	3.9	[2]
$C_9H_{20}O_2Si$	Causes skin irritation	Silane, cyclohexyldimethoxymethyl	0.706 (N:5.05%)	5.98	[7]
$C_8H_{12}O_4$	May cause allergic reactions	2-butenedioic (E), diethyl ester	5.454 (N:39.06%)	6.38	[16]
$C_{15}H_{32}$	Alkanes generally low toxicity	Dodecane, 2,7,10-trimethyl	1.678 (N:12.02%)	7.52	[16]
$C_7H_{12}O_4S$	Useful in organic synthesis	Dimethyl 3-thiaadipate	13.962 (N:100%)	8.52	[25]
$C_{12}H_{26}$	Alkanes generally low toxicity	Decane, 2,4-dimethyl	0.492 (N:3.52%)	10.17	[16]
$C_{17}H_{30}OSi$	Causes skin/eye irritation	Phenol, 2,4-bis(1,1-dimethylethyl)	3.647 (N:26.12%)	10.84	[26]
$Si(CH_3)_3$	Limited toxicity data	2-(1-methylsilacyclobutyl) benzoic acid TMS ester	0.704 (N:5.05%)	13.26	[17]
$C_{12}H_{25}I$	Toxic if ingested/inhaled	1-iodo-2-methylundecane	1.759 (N:12.60%)	14.29	[17]
$C_{20}H_{38}O_2$	Specific toxicity varies	2-8-Octadecen-1-ol acetate	0.552 (N:3.96%)	16.86	[26]
$C_{21}H_{44}$	Alkanes low toxicity	Eicosane, 2-methyl	0.704 (N:5.04%)	18.25	[26]
$C_{10}H_{19}O_6PS_2$	Toxic and carcinogenic	Malathion	6.571 (N:47.07%)	19.15	[23]
$C_{18}H_{36}O_2$	Stearic acid low toxicity	Octadecanoic acid	0.671 (N:4.81%)	22.79	[26]
$C_6H_8O_3S$	Limited toxicity data	3-thiophenecarboxylic acid, tetrahydro-4-oxo-, methyl ester	2.486 (N:17.80%)	26.59	[17]

Chemical composition of control and sample

The chemical structures and relative abundances of compounds in the control and sample were analyzed (Tables 6 & 7). The control contained a high concentration of malathion (79.473%), along with other toxic compounds such as phosphorodithioic acid, OO, S-trimethyl ester. In contrast, the sample treated with *Alternaria alstroemeriae* KRMY EMIC001 showed a significant reduction in malathion concentration (6.571%) and the presence of less toxic degradation products. These findings highlight the potential of *Alternaria alstroemeriae* Table 6. Chemichal sturacture of controle(malathion + media) compound and its area% with number of peak. KRMY EMIC001 to mitigate environmental pollution by converting toxic pesticides into less harmful compounds [27-40].

Conclusion

Alstromeriae KRMY EMIC001 demonstrates significant potential for malathion bioremediation, achieving >85% degradation under optimized conditions. ITS-based phylogenetic analysis confirmed its identity, while enzymatic and GC-MS data elucidated a phosphatase-driven degradation pathway. This aligns with sustainable strategies for mitigating pesticide pollution, emphasizing the strain's applicability in contaminated environments

Conflict of Interest Statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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