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BIODEGRADATION OF MALATHION USING CUTINASE FROM A SOIL ISOLATE OF *FUSARIUM* SP.

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ABSTRACT: Malathion, an organophosphate insecticide, has been extensively used for household and agricultural pests. A major amount of malathion and its remains, exposed to the environment, are rapidly absorbed by all routes including the gastrointestinal tract, skin, mucous membranes, and lungs. From animal testing, the toxic effect of malathion has been shown to affect the central nervous system of invertebrates, immune system of higher vertebrate wildlife, and adrenal glands, liver and blood of fish. Malathion also causes detectable mutations and as per IARC, malathion is a probable carcinogen. Cutinases are hydrolytic enzymes that catalyze the cleavage of ester bonds that make them highly potential industrial biocatalyst. In recent years, the cutinases have been exploited for many biotechnological applications. In the present investigation, fungi showing cutinase activity were isolated from soil samples. Strain showing maximum cutinase activity was tentatively identified as *Fusarium* sp. and was selected for further studies. The optimum temperature and pH for cutinase activity were 50°C and 6.0, respectively. GC-MS results revealed that application of cutinase on malathion for 3 days resulted in 53% reduction of malathion. Current study describes cutinase as an attractive alternative for the remediation of malathion contaminated soil residues.

Keywords: Malathion, Biodegradation, Cutinase, Insecticide, *Fusarium*

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1.INTRODUCTION

Agricultural productivity depends on land, water, fertilizers, seed and insecticides. Every yearapproximately, 35–45% crop production is lost due to insects, weeds and several diseases.

Organophosphorous insecticides are mostly preferred in agriculture due to their low persistence in the environment, but its use affects animals and human beings [1]. Organophosphorous insecticides accounts for more than 36% of the total world market. Most organophosphorous insecticides have the same general structure, where a phosphate group or phosphate derivative is part of an organic molecule [2]. Organophosphorus compounds have a covalent carbon to phosphorus (C-P) bond that is resistant to chemical hydrolysis, thermal decomposition and photolysis [3]. Many authors stated that the enzymes belonging to the different groups like esterase, phosphatasehave a great degradation potential of the organophosphorus insecticides and other pesticides [4].Studies on capabilities of enzymatic degradation are important in the development of new remediation strategies for the detoxification of different pesticides. This remediation technique can act as reliable and cost-effective method to clean up polluted environments. Some organophosphorus insecticides such as chlorpyrifos, parathion, malathionetc are commonly used for over 50 years. Malathion is one of the intensively used organophosphorous insecticide [5]. Malathion, also known as S-(1,2-dicarbethoxyethyl)-O,O-dimethyldithiophosphate, has also been used as a DDT substitute for the control of field crop pests, household insects, flies and animal parasites. It has been known as the first organophosphorous insecticide with high selective toxicity [6]. It has been extensively used for household and agricultural pests. A major amount of malathion and its remains, exposed to the environment, are rapidly absorbed by all routes including the gastrointestinal tract, skin, mucous membranes, and lungs [7]. From animal testing, the toxic effect of malathion has been shown to affect the central nervous system of invertebrates, immune system of higher vertebrate wildlife, and adrenal glands, liver and blood of fish [8]. Malathion also causes detectable mutations and as per IARC, malathion is a probable carcinogen [9]. Malathion binds to the acetylcholinesterase at nerve endings throughout the bodies of insects and other organisms which results in over stimulation of the nervous system which ultimately causes death [10]. There are some methods which are used to detoxify organophosphate insecticides. These methods mainly rely on chemical treatment, incineration and landfills [11]. Some investigators found that soil contaminated with insecticides could be possibly decontaminated by inoculation with specifically adapted microorganisms [12]. Despite having many advantages, use of microbes has some disadvantages like time intensiveness, operational hurdles. Microbial enzymes show higher efficiency than the microbes because it is ultimately the enzyme which would be playing role in degradation even in role of microbial degradation [13]. Enzymes can be used in remediation of environmental pollutants. Use of enzyme is a natural process, very effective as compared to the other methods and is applicable for in-situ bioremediation [14]. Cutinases are among the major enzymes used in insecticide bioremediation and in recent years several cutinase or esterases of bacterial and fungal origin have been identified and studied [9,15,16]. Cutinase hydrolyze the ester bonds of carboxyl ester substrate molecules, such as those present in malathion to form alcohols and carboxylic acids [17]. As a lipolytic enzyme,

cutinase has been reported as a versatile enzyme showing many interesting properties for applications in industrial products and processes [9]. The purpose of the present study was to evaluate the capabilities of cutinase enzyme, isolated from soil fungus to biodegrade malathion. Biodegradation by-products were detected by GC-MS.

2. MATERIALS AND METHODS

A. Isolation and screening of cutinase-producing fungi

1) Isolation of fungal samples from soil

Soil samples were collected from different parts of Chandigarh, India. The samples were homogenized, serially diluted in the sterile physiological saline, spread on potato dextrose agar plates and were incubated at 30^{O}C for 48 h. The fungal species were purified through repeated subculture and used for further studies.

2) Screening for cutinase activity

Isolated fungal samples were cultivated in 20 ml of mineral medium as per Pio and Macedo (2008), having the following composition: 0.06% $NaNO_3$, 0.06% K_2HPO_4, 0.02% $MgSO_4$, 0.02% KCl, 0.01% $FeSO_4.7H_2O$, pH 7 in the presence of a cutinase inducer (1% orange cutin), in 250 ml Erlenmeyer flasks, incubated at 30^0C for 2 days at 100 rpm in a rotary shaker [18]. After incubation, the fungal supernatants were collected by centrifugation (10,000 rpm for 15 min) and were analyzed for cutinase activity. Cutinase enzyme assay was done as per Pio and Macedo (2008) [18]. In this, 0.070 ml of fungal supernatant was mixed with 3.43 ml of substrate solution. Stock solution of substrate consisted of 1.12 mM paranitrophenyl butyrate dissolved in 50 mM phosphate buffer, pH 7.2, also containing 0.2% Triton X-100 and 0.43 M tetrahydrofuran. Hydrolysis of paranitrophenyl butyrate into paranitrophenol was determined spectrophotometrically at 405nm against a control reaction. One unit of cutinase activity was defined as the amount of cutinase required to release one micromole of p-nitrophenol per min under the specified conditions. Fungal isolate showing maximum cutinase activity was tentatively identified as per Navi et al (1999) [19] and was selected for further studies.

A. Effect of temperature and pH on cutinase activity

The optimum temperature for cutinase activity was determined by incubating the reaction mixture at different temperature ranging from 30^0C to 60^0C. pH optima was evaluated by incubating the reaction mixture for 30 min in the presence of appropriate buffers: 50mM Citrate buffer (pH 3-5), 50mM sodium phosphate buffer (pH 6-7), 50mM TrisHCl (pH 8-9). Activity of each sample was determined by method given above.

B. Determination of biodegradation efficiency of malathion

Biodegradation of malathion by cutinase was carried out as per Kim et al (2005) [9]. Solution of malathion was prepared by dissolving malathion (50%, purchased from local market) in pure methanol (99%, Sigma, India). Enzymatic degradation of malathion began by adding 50 ml of

enzyme to 50 ml of malathion solution (500g/l) in 250ml Erlenmeyer flask. Control flask was prepared by adding 50ml of inactivated enzyme and 50 ml of malathion solution. Incubation was done for 3 days in dark shaking incubator (50^0C, 100rpm). % biodegradation of malathion was determined by calculating the peak area difference of control and test [14].

C. Analysis of malathion biodegradation

Analysis of malathion biodegradation was done as per Kim et al (2005) [9]. 500 µl of sample was taken each from control and the test flask, mixed with 500 µl of *n*-hexane (>99%, Sigma) and incubated for 3 hours at room temperature. After incubation, complete phase separation occurred and non-polar compounds including malathion and several degradation products were successfully extracted into the upper organic phase. All chemical compounds in *n*-hexane were analyzed using GC-MS facility available at Central Instrumentation Laboratory, Panjab University, Chandigarh.

3. RESULTS AND DISCUSSION

A. Fungal Screening and identification

Isolation of fungi from soil samples was done on Potato Dextrose Agar medium. 30 fungal organisms were isolated from soil. Out of 30 isolated fungal samples, AR8 showed the maximum cutinase activity and was tentatively identified as *Fusarium* sp. as per Navi et al (1999) [19] (Figure 1).

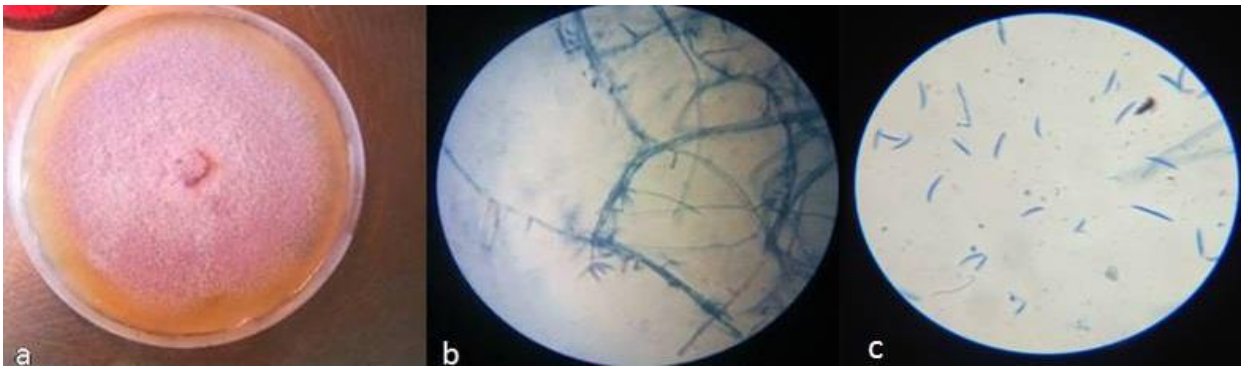


Figure 1: a) pure culture of AR8 identified as *Fusarium* sp. on potato dextrose agar (PDA) plate; b) lactophenol cotton blue mount of AR8 (*Fusarium* sp.); c) microconidia and macroconidia indicate respective structures (45x).

Table 1: Macroscopic And Micrcoscopic Characteristics Of AR8 *Fusarium* Sp.

Colony features on Potato Dextrose Agar (PDA) medium	Colony color	White to cream
	Pigmentation	Pink to Purple
	Reverse	Dark Brown
	Elevation	Slightly raised
	Form	Circular
	Margin	Entire
Phalide	Monophalide and polyphalide	
Chlamydospores	Absent	
Microconidia	Chain	Absent
	Shape	Curved and thick
	Size	5-6µm x 1-1.5µm
	Septation	Non septate
Macroconidia	Chain	Absent
	Shape	Straight to slightly curved
	Size	20-25µm x 2-3µm
	Septation	2-3 Septate
Hyphae	Septate and hyaline	

B. Effect of temperature and pH on cutinase activity

The effect of incubation temperature on enzyme production was studied in the temperature range of 30^0C to 60^0C under submerged fermentation (Figure 2a). Variation in temperature was observed for cutinase production by the test fungus. However, the optimum temperature was found to be 50°C (163 U/ml) thereafter it decreased slowly (Figure 2a). Similar results were reported by Adiguzel et al (2017), Yang et al (2013), Maeda et al (2005), Kazenwadel et al (2012) [20,21,22,23]. The effect of pH on enzyme production revealed a continuous increase in enzyme activity from pH 3 to 6 with the maximum at pH 6. Further increase in pH declined the activity (Figure 2b). Some reports have shown that many bacterial/ fungal strains achieved maximum enzyme production at neutral and alkali pH values [24,25,26]. This pH optimum is very similar to the optima reported for purified cutinase from *Venturia inaequalis* [27]. Similarly maximum cutinase activity has been reported at pH 6 from *Aspergillus niger* and *Trichoderma reesei* [28,29]. The optimal hydrolyzing activity of cutinase at acidic pH may be correlated to the environmental conditions encountered by the penetrating pathogen on a moist leaf surface where leaf pathogens are mostly active at acidic pH [27].

(a) (b)

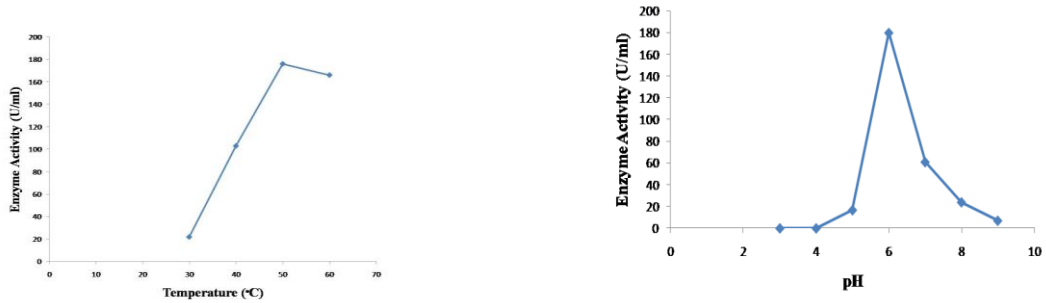


Figure 2: Characterization of cutinase

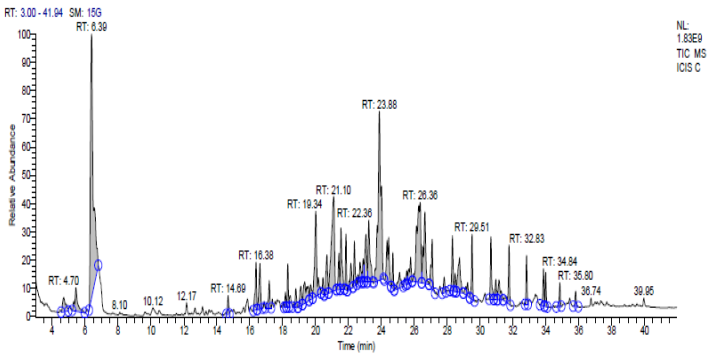
(a) Temperature profile

(b) pH profile

C. Biodegradation efficiency of malathion

The recovery efficiency of malathion and its degradation products were estimated by GC-MS. The GC-MS analysis of control sample (malathion with inactivated cutinase) and the test sample (malathion treated separately with cutinase for 3 days) showed the presence of malathion (Retention Time of 23.88 minutes). During the degradation of malathion by the cutinase, the non-degraded residual malathion and the degraded products were monitored through GC/MS analysis (Figure 3). Using fungal cutinase, almost 53% of initial malathion was degraded within 3 days of treatment. Malathion was detected as parent compound at 23.88 min, molecular formula $C_{10}H_{19}O_6PS_2$ and molecular weight 330.360. In the present study, the malathion biodegradation capability was studied using 50 ml of cutinase enzyme with 50 ml of malathion (500 g/L). The experimental system was incubated at 50^0C for 3 days. The percentage degradation of malathion was 53%, according to the equation was mentioned in material and method. The spectral peak of malathion was found to be clearly higher in the case of control (Figure 3). The results of GC-MS show the fragmented pattern of malathion and its degraded products.

(a)



(b)

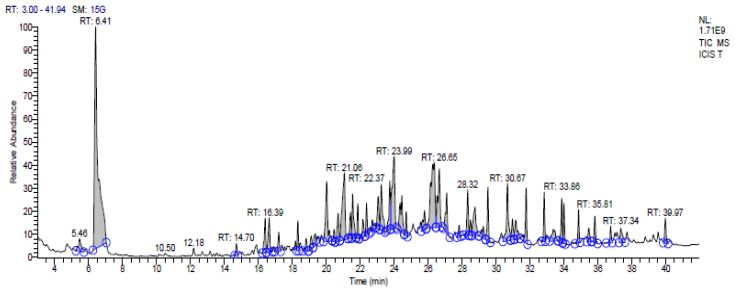


Figure 3: GC-MS analysis of

(a) Control (malathion+ inactivated enzyme)

(b) Test (malathion + activated enzyme)

The GC-MS results of test sample showed the number of metabolic intermediates formed during the degradation of malathion by cutinase (Table 2). The mass spectra of malathion containing samples treated enzyme showed no known toxic intermediates. The results suggested that the enzyme was not forming any toxic intermediates during the degradation of malathion and thus could be utilized in the bioremediation process of malathion contaminated soil.

Table 2: The Number Of Metabolic Intermediates Formed During The Degradation Of Malathion By Cutinase (Results Were Obtained By GC-MS)

Peak #	Retention Time	Peak Area	Area %	Peak Height	Name
1	27.82	504416708.77	0.45	87523447.84	Dodecane, 2- methyl-6-propyl
2	23.06	1668045633.38	1.47	239989516.25	4,6-di-tert-butyl-m-cresol
3	23.99	6086247784.80	5.37	533789342.66	n- Hexadecanoic acid
4	27.82	504416708.77	0.45	87523447.84	Phthalic acid,3,5-difluorophenyl 6-ethyloct-3-yl ester
5	37.34	1125275275.57	0.99	104002425.54	Fumaric acid, hexadecyloctyl ester
6	21.42	1073799388.36	0.95	194220377.81	2- Hexadecanol
7	22.15	913507713.92	0.81	131206677.64	Nonane, 3- methyl-5-propyl
8	24.70	788165631.13	0.70	180440200.16	Palmitic anhydride

Organophosphorous insecticides like malathion are potential carcinogen. It affects nerve fibres by irreversibly inhibiting the acetylcholinesterase [30]. Hence, it is important to find novel biocatalyst

that is effective in the degradation of malathion in environment. There are some reports about the use of cutinase for bioremediation of pollutants [31,32,33,34]. According to our results, during the degradation of malathion, many by products appeared. Eventually, at the end of the reaction (72 hours), almost 53% malathion was degraded by the enzyme as shown in Figure 3. The exact amount of malathion was measured by GC-MS based on retention time. It is to be noted that chromatogram shows a specific peak in reaction time and the area under the peak is considered as the amount of the compound (malathion). In control (Figure 3a), we used malathion with inactivated enzyme as a standard to obtain specific retention time and the peak of malathion. As shown in Figure 3b, the peak height of malathion decreased with time, which indicated the degradation of malathion by cutinase.

4. CONCLUSION

In conclusion, our results revealed the significant capability of fungal cutinase to remediate malathion. In the present study, considerable removal of malathion in liquid medium after three days incubation was observed. In inoculated medium, more than 50% of malathion was degraded to other harmless compounds which are not currently classified as an Endocrine Disrupting Chemical. Hence, work in this regard should continue to characterize the enzymatic components responsible for the utilization of malathion and other organophosphorus insecticides in order to evaluate its efficiency for the bioremediation of environmental pollutants.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The author confirms that the data supporting the findings of this research are available within the article.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests.

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