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Screening and Characterization of Triazophos Tolerant Bacteria from Soil

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Triazophos (Hostathion 40% EC) an organophosphate is widely used pesticide to combat insect pests on various crops of vegetables and cotton. Its overuse has led to the contamination of environment which is of great concern. In the present study efforts were being made to isolate, screen and characterize bacterial strains which could tolerate triazophos at higher doses and utilize it as sole carbon source. Two bacterial isolates out of forty tolerated Hostathion 40% EC. These two bacterial isolates were characterized morphologically, biochemically and identified using 16S rRNA sequencing technique as *Staphylococcus lentus* and *Lysinibacillus fusiformis.* These two strains were reported to tolerate triazophos as sole carbon source suggesting their utilization further in bioremediation process.

Keywords: *Lysinibacillus fusiformis,* organophosphate*, Staphylococcus lentus,* triazophos.

Current agricultural practices largely rely on the extensive application of agrochemicals including inorganic fertilizers and pesticides. Pesticides play an inevitable role both in increasing agricultural production and in ensuring supply of high quality food. Indian pesticide market is second largest in Asia and ranks twelth globally with a net production of 82,000-85,000 million tons. Out of which 43,000-85,000 tons of pesticides are being used to control various crop pests (Subramanyam *et al*., 2012). Various groups of pesticides are being used worldwide, out of them organophosphates (OPs) form a major and most widely used group after the cessation of use of organochlorine pesticides in 1970s. But their unrestricted use in agriculture has led to pollution of ecosystem as well as neurotoxicity in humans (Kanekar *et al*., 2004).

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Triazophos [0,0-diethyl 0-1-phenyl-1H-1,2,4-triazole-3-yl phosphorothioate] an organophosphate is moderately toxic, broad spectrum and non-systemic in nature which is extensively sprayed to control pests on various crops like cotton, tea, maize, paddy and vegetables (Lin and Yuan 2005). This pesticide is in great use in different areas of Punjab on various crops especially on cotton and vegetables. According to the International Union of Pure and Applied Chemistry (IUPAC), triazophos may harm metabolism of non-target organisms as an acetyl cholinesterase inhibitor and neurotoxicant. It is accumulating in the environment and high levels of triazophos residues have been found in a wide variety of foods according to several US and European food monitoring schemes causing threats to human health through food chains (Wang *et al*., 2005). Its misuse as a fungicide in intertidal aquaculture to protect farming shellfish such as *Sinonovacula constricta* and *Tegillarca granosa* from diseases resulted in various fish kill

accidences. Thus, the evaluation of environmental safety for triazophos is of great concern. Keeping these points in view, the present study was undertaken to isolate, screen and characterize triazophos tolerant bacteria from soil so that tolerant isolate can be exploited for use in bioremediation.

MATERIALS AND METHODS

Soil sampling

Soil samples were collected from agricultural fields of cotton and vegetable of different districts of Punjab viz Fazilka, Bathinda Jalandhar, Patiala which had history of ten years exposure to triazophos. Soil samples were also collected from bank of Buddha Nullah. These soil samples were brought to the laboratory, air dried, sieved through 2mm mesh to remove coarse soil particles and then stored at 4°C for further processing.

Chemicals and Media

A commercial preparation of Triazophos i.e. Hostathion (40% EC) obtained from Cheminova India Ltd., Panoli, Distt. Bharuch was used in the present study. All other chemicals (AR grade) were procured from Hi-Media Laboratories Pvt. Ltd., Mumbai.

For isolation of Triazophos tolerant microorganisms : Dorn's medium was used [Media composition (g/l): $Na_2HPO_4.12H_2O$ 3.0; KH_2PO_4 1.0; $(NH_4)_2SO_4$ 1.0; Ammonium ferric citrate 0.01; Yeast Extract 0.1; Trace Element Solution 10ml; Distilled Water 1000ml; Agar 20.0. The composition of trace element solution was in (g/l) $MgSO_4.7H_2O$ 10.0; $CaCl_2.2H_2O$ 2.0; $MnSO_4.H_2O$ 3.0 and $FeSO_4.7H_2O$ 0.2; pH 7.2±0.2.] Dorn's medium was autoclaved and then supplemented with 50mg/ml triazophos as a sole carbon source.

Methodology for isolation and screening

Triazophos tolerant bacteria were isolated from soil as per the method of Yang *et al*., (2011) with slight modifications. Soil samples (10.0 g) were added in 250 ml Erlenmeyer flasks containing 100 ml of Dorn's medium supplemented with 50 mg/ml triazophos. The flasks were incubated on a rotary shaker at 250 rpm for 7 days at 30^{0}C. At periodic intervals, a loopful of sample from the flasks was streaked on plates containing Dorn's medium supplemented with triazophos (50 mg/ml) and were incubated at 30^{0}C for 72 hrs. The individual bacterial colonies that grew on the medium were sub-cultured on Dorn's medium containing triazophos of the same concentration. These bacterial isolates were further tested and screened for their ability to grow at higher concentrations of triazophos (100mg/ml, 200mg/ml, 500mg/ml and upto 1000mg/ml) as their only carbon source in the Dorn's broth. Finally, those strains which could tolerate 1000 mg/ml triazophos concentration were selected and used for further morphological, biochemical and molecular studies.

Identification of the isolate

The morphological and biochemical tests for the selected bacterial isolates were performed by using standard methods. The bacterial strains were taxonomically identified from Bergey's Manual of Determinative Bacteriology (1994) Edition IX and further confirmed by DNA isolation and 16S rRNA sequencing technique by out sourcing from Xcelris labs Ltd. Premchand Nagar Road, Bodakdev, Ahmedabad 380054, India.

RESULTS

Isolation of triazophos tolerant bacteria from soil

Soil samples were collected from various regions of Punjab and out of them forty bacterial strains were isolated which could tolerate 50 mg/ml triazophos. These isolates were further screened at higher concentrations (100mg/ml, 200mg/ml, 500mg/ml and upto 1000mg/ml). Only two bacterial strains V1 and V4 exhibited maximum tolerance to 1000mg/ml concentration (*Table 1*). These strains were selected and used for further studies.

Morphological and biochemical characterization of bacterial isolates

These isolates were further tested morphologically based on their shape, cell arrangement, fluorescence and Gram reaction (*Table 2*). Biochemical characterization based on various biochemical tests like catalase production, H_2S production, IMViC test, oxidase production and carbohydrate fermentation was done (*Table 3*). The optimal pH and temperature for the isolates was found to be 6.4-8 and 30^{0}C respectively.

Identification of the bacterial strains was done by consulting Bergey's Manual of Determinative Bacteriology (1994) Edition IX. Accordingly, bacterial isolate V1 and V4

Table 1. Selected bacterial isolates and their source

Source of soil collection	Village/ Location	District	Total number of bacterial isolates	Selected bacterial isolates at higher conc. (1000ppm)
Buddha Nullah	Haebowal	Ludhiana	9 (H1-H9)	
Cotton field	Karni Khera	Fazilka	6 (F1-F6)	
Cotton field	Romana	Bathinda	8 (K1-K8)	
Vegetable field	Bhogpur	Jalandhar	5 (V1-V5)	V1 and V4
Vegetable field	Kheri Gujran	Patiala	6 (B1-B6)	
Vegetable field	Chudpur	Ludhiana	6 (C1-C6)	

Table 2. Morphological characterization of triazophos tolerant bacterial isolates

Characteristics	Bacterial isolates	
	V1	V4
Cell shape	Coccus	Rod shaped
Fluorescence	Negative	Negative
Cell arrangement	Single, in pairs and/or tetrads	Single and in chains
Cell surface	Opaque	Opaque
Gram reaction	Gram positive	Gram positive
Endospore formation	Negative	Positive (central)
Motility	Non-Motile	Motile

Table 3. Biochemical characterization of triazophos tolerant bacterial isolates

Characteristics	Bacterial isolates	
	V1	V4
Catalase production	+	+
Oxidase production	+	+
Urea hydrolysis	-	+
H_2S production	-	-
Esculin hydrolysis	+	-
Starch hydrolysis	+	-
Casein hydrolysis	+	+
Gelatin hydrolysis	+	+
Indole test	-	-
Voges-proskauer test	-	-
Methyl red test	-	-
Citate utilisation	-	+
Nitrate reduction test	-	-
Sucrose utilisation	+	-
Fructose utilisation	+	-
Lactose utilisation	-	-
Glucose utilisation	+	-

+ = positive reaction; - = negative reaction

presumptively identified as a member of *Staphylococcus* and *Bacillus* respectively.

Molecular characterization of bacterial isolates

Fragment of 16S rRNA gene was amplified by PCR using gene specific primers. A single discrete PCR amplicon band of 1500 bp was observed when resolved on Agarose Gel (*Figure 1*). On the basis of 16S rRNA gene similarity, isolate V1 showed 99% sequence homology with *Staphylococcus lentus* strain (EF528296.1) and isolate V4 showed 99% sequence homology with *Lysinibacillus fusiformis* strain (GU125642.1). Phylogenetic tree was constructed for both the isolates using MEGA 4 (*Figure 2, 3*). Based on nucleotide homology and phylogenetic analysis, the bacterial isolates V1 and V4 were identified as *Staphylococcus lentus* and *Lysinibacillus fusiformis.* Several workers have also reported characterization of bacteria based on 16S rRNA gene sequence analysis (Zhu *et al*., 2010; Guo *et al*., 2009).

DISCUSSIONS

Isolation of different bacterial strains tolerant to pesticides has been extensively studied by various scientists . Several bacterial strains belonging to genus *Klebsiella* (Wang *et al*., 2005); *Diaphorobacter* (Guo *et al*., 2009); *Bacillus* (Tang and You 2011) have been reported to utilize triazophos as their sole carbon and nitrogen source. Results obtained in the present study were in agreement with the findings of earlier scientists like Yang *et al*., (2011) who isolated three triazophos tolerant bacterial strains from the triazophos polluted soil samples in China using Luria-Bertani (LB) medium and mineral salt medium supplemented with triazophos (50 mg/ml) as sole carbon source for growth at 30^0 C. After the screening process, a strain designated as *Diaphorobacter* sp., TPD-1 was selected. Similarly, Wang *et al*., (2005) isolated twenty-eight different microorganisms which had the ability to tolerate triazophos using basal salt medium (BSM) supplemented with triazophos (100 mg/ml) as sole carbon and nitrogen source.

Two strains V1 and V4 identified as *Staphylococcus lentus* and *Lysinibacillus fusiformis* using 16S rRNA sequencing technique were the firstly identified strains against triazophos tolerance. Mohan and Naveena (2015) also reported the role of *Lysinibacillus fusiformis* in acephate (an organophosphate) degradation in paddy soil. Various strains of *Lysinibacillus fusiformis* have been widely employed in biodegradation of environmental contaminants

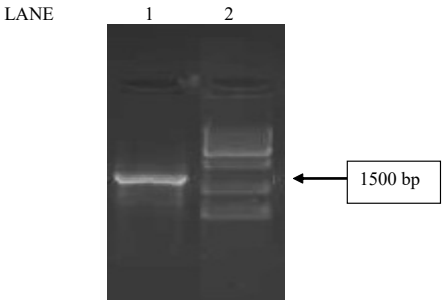

Fig. 1. Gel Image of 16S rRNA amplicon (LANE 1); DNA Marker (LANE 2)

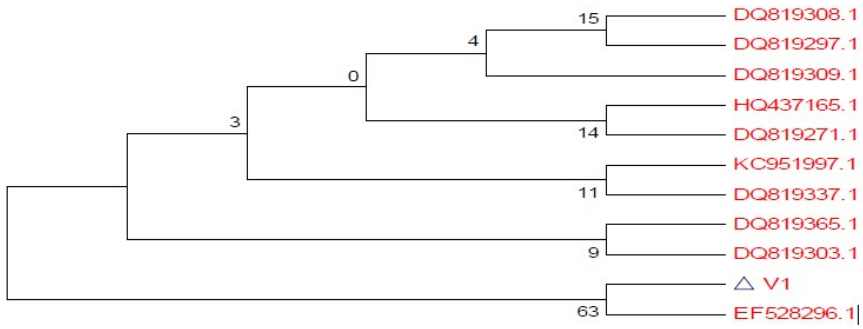

Fig. 2. Phylogenetic tree for V1

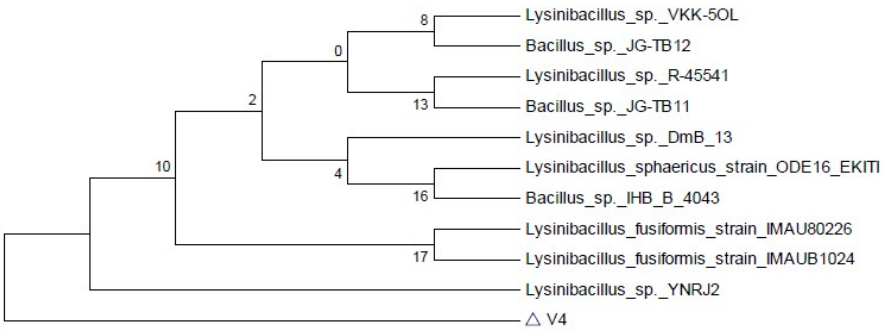

Fig. 3. Phylogenetic tree for V4

such as hydrocarbon (Dongfeng *et al*., 2011), sulfonated azo dyes (Saratale *et al*., 2013) etc. *Lysinibacillus sp.* isolated from agricultural land has biodegrading activity on herbicide fomesafen and could degrade upto 81.32% within a week of incubation (Liang *et al*., 2009). On the other hand Baishya and Sharma (2014) reported the role of *Staphylococcus sp.* in degradation of malathion and quinalphos when isolation was carried out using Mineral Salts Medium (MSM).

Morphological and biochemical characterization results were also found to be in corroboration with the findings of Tang and You (2011) and Li *et al*., (2008) who studied the morphological and cultural features of organophosphate degrading *Bacillus* sp. (TAP-1) and (Dsp-6) respectively; isolated from pesticide contaminated soil.

Lysinibacillus sp. is widely employed for the detoxification of factory wastewaters to remove chromium contamination. Thus these two strains can be widely employed for removal of contaminants from environment and ecosystem can be made pollution free.

CONCLUSION

The aim of this work was identifying new strains of bacteria which could grow easily even in the presence of highest concentration (1000 mg/ml) of triazophos. The purpose behind this study was to isolate such strains so that they could be bioaugmented and used in bioremediation of pesticide contaminated sites thus help reducing chemical pesticides from soil and maintaining pesticide free environment.

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REFERENCES

1. Baishya, K., Sharma, H.P. Isolation and characterization of organophosphorus pesticide degrading bacterial isolates. *Arch. Appl. Sci. Res.,* 2014; **6**:144-9.
2. Bergey, D.H., Holt, J.G., Krieg, N.R., Sneath, P.H.A. Bergey's Manual of Determinative Bacteriology (9th ed.). Lippincott Williams & Wilkins. 1994; ISBN 0-683 00603-7.
3. Berkhoff, H.A., Vinal, A.C. Congo red medium to distinguish between invasive and non invasive *Escherichia coli* pathogenic for poultry. *Avian Diseases*. 1986; **30**: 117-21.
4. Dongfeng, Z., Weilin, W., Yunbo, Z., Qiyou, L., Haibin, Y., Chaocheng, Z. Study on isolation, identification of a petroleum hydrocarbon degrading bacterium *Bacillus fusiformis sp.* and influence of environmental factors on degradation efficiency. *Environ. Prot.* 2011; **13**: 74-82.
5. Guo, X.Q., Li, R., Lin, D.Q., Zhu, B., Li, S.P., Jiang, J.D. Isolation and characterization of triazophos degrading strain GS-1 and its degrading characteristics. *Microbiol*. 2009; **36**: 1143-9.
6. Kanekar, P.P., Bhadbhade, B., Deshpande, N.M., Sarnaik, S.S. Biodegradation of organophosphorus pesticides. *Proc. Indian National Sci. Acad.* 2004; **70**: 57-70.
7. Li, X., Jiang, J., Gu, L., Waseem, A.S., He, J., Li, S. Diversity of chlorpyrifos-degrading bacteria isolated from chlorpyrifos contaminated samples. *Inter. Biodeterior. Biodegrad.* 2008; **62**: 331-5.
8. Liang, B., Lu, P., Li, H., Li, R., Li, S., Huang, X., Biodegradation of fomesafen by strain *Lysinibacillus sp*. ZB-1 isolated from soil. *Chemosphere*. 2009; **77**: 1614-9.
9. Lin, K.D., Yuan, D.X. Degradation kinetics and products of triazophos in intertidal sediment. *J. Environ. Sci*. 2005; **17**: 933-6.
10. Mohan, N., Naveena, L. Isolation and Determination of Efficacy of Acephate Degrading Bacteria from Agricultural Soil. *J. Environ. Sci. Toxicol. Food Technol.* 2015; **9**: 10-20.
11. Saratale, R.G., Gandhi, S.S., Purankar, M.V, Kurade, M.B., Govindwar, S.P., Oh, S.E., Saratale, G.D. Decolorization and detoxification of sulfonated azo dye CI Remazol Red and textile effluent by isolated *Lysinibacillus sp*. *J. biosci. bioeng*. 2013; **115**: 658-67.
12. Subramanyam, D., Lokanatha, V., Rajendra, W., Vijayalakshmi, B. Biomanagement of pesticides for sustainable environment: An Indian Scenario. *Int. J. Biol. Pharm. Res.* 2012; **3**: 826-34.
13. Tang, M., You, M. Isolation, identification and characterization of a novel triazophos degrading *Bacillus* sp. (TAP-1). *Microbiol. Res*. 2011; **49**: 144-51.
14. Velazquez, A., Xamena, N., Creus, A., Marcos,

R. Mutagenic evaluation of the organophosphorus insecticides methyl parathion and triazophos in Drosophila melanogaster. *J. Toxicol. Environ. Health*. 1990; **31**: 313-25.

15. Wang, L., Zhang, L., Chen, H., Tian, Q., Zhu, G. Isolation of a triazophos degrading strain *Klebsiella* sp. E6 effectively utilizing triazophos as sole nitrogen source. *FEMS Microbiol. Lett*. 2005; **253**: 259-65.
16. Yang, C., Li, R., Song, Y., Chen, K., Li, S., Jiang, J. Identification of the biochemical degradation pathway of triazophos and its intermediate in *Diaphorobacter* sp. TPD-1. *Curr. Microbiol.* 2011; **62**: 1294-301.
17. Zhu, J., Zhao, Y., Qiu, J. Isolation and application of a chlorpyrifos degrading *Bacillus licheniformis* ZHU-1. *Afr. J. Microbiol. Res.*2010; **4**: 2410-3.