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UTILIZATION OF AN ORGANO PHOSPHATE PESTICIDE BY A NEWLY ISOLATED BACTERIAL ISOLATE BACILLUS SPP. JR5

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ABSTRACT
Extensive use of agrochemicals including various pesticides is the major nonpoint source of pollution of water and soil. A variety of pesticides have been used in agriculture as well as non-agricultural purposes. Chlorpyrifos, an organophosphate pesticide is widely used in agricultural fields. It is harmful to a variety of organisms that comprise living soil biota along with useful arthropods, fish, birds, human, animal, and plant. In present study, the ability of a bacterium *Bacillus subtilis* (identified based on morphological, biochemical and 16S rRNA characterization) was used for the degradation of an organophosphate pesticide (chlorpyrifos). *Bacillus subtilis* grow in presence of 600ppm chlorpyrifos as a sole source of carbon. The ability of this isolate to utilize chlorpyrifos as sole source of carbon can be exploit to remediate agro pesticides contaminated soils and water.

KEYWORD: Extensive use of agrochemicals pesticides contaminated soils and water.

INTRODUCTION
Pesticides have been used worldwide to control pests and pathogens for sustainable food productivity that is necessary to support the world population. The earliest pesticides that were used included either inorganic or plant-derived products. However, in the past few years the use of chemical control has increased tremendously.[1] The wide use of organophosphorus pesticides has created numerous problems, including the pollution of the environment.[2] Organophosphates generally include organic compounds with a phosphorous atom. First ever organophosphorous insecticide manufactured and used was tetraethyl pyrophosphate in 1937.[3] Subgroups of organophosphorous compounds have about 100 different types of organophosphates, many of them have been banned due to high toxicity. Quinalphos, monocrotophos, chlorpyrifos, malathion, and parathion are some of the widely used organophosphorouspesticides. Among these, chlorpyrifos (O, OdiethylO-3,5,6 trichloropyridin-2-yl phosphorothioate) is extensively used in agriculture as a commercial insecticide.[4] According to the WHO classification, chlorpyrifos belongs to class II pesticides with moderate toxicity.[5]

It was introduced in 1965 by Dow Chemical Company India.[6] It has large blights on public health and environment resulting from its long residual period in soil and water.[7] Extensive use of chlorpyrifos contaminates air, ground water, rivers and lakes. The contamination has been found up to about 24 km away from the point of use, Chlorpyrifos-oxon and 3,5,6-trichloro-2-pyridinol (TCP) are the two potent transformation products of chlorpyrifos, have been found in groundwater. Such contamination can led to the insecticides and their transformation products are being transported long distances.[8]

Catabolism and detoxificationmetabolism occur when a soil microorganism usesthe pesticide as a carbon and energy source. Many studies have employed pure cultures of soil isolates or agar plate counts of soil populations. The biodegradation of organophosphorus pesticides by soil microorganisms has been reported by many workers.[2,9,10] Microbial degradation process todetoxify pesticide contaminants can be effectively used to overcome the pollution problems. Soil bacteria with the ability to degrade several pesticides have been isolated from soil showing enhanced biodegradation. It has been suggested that cultures of bacteria with the ability to degrade specific compounds can be used for bioremediation of pesticide polluted sites.[11]

The objective of this study was to isolate, characterize and identify native strains of bacteria that are capable of degrading chlorpyrifos.

MATERIALS AND METHODS

Pesticides and chemicals

A technical grade chlorpyrifos (95% E.C.) was procured from pesticide synthesizing industrial unit of Ankleshwar, Gujarat, India (Table 1).

Soil sample collection

Agricultural land used for cotton crop cultivation in which chlorpyrifos was sprayed since last 8-10 years was used as sample collection site. Soil samples were collected using standard method from 12-15cm below the soil surface. Collected soil samples were air-dried, sieved (<2 mm) and stored at 4°C till further analysis.

Isolation of chlorpyrifos utilizing bacteria

2 gm of soil was added to 250ml Erlenmeyer (EM) flasks containing 100ml of sterile MSM [(g/l): KH2PO4, 4.8; K2HPO4, 1.2; NH4NO3, 1.0; MgSO4.7H2O. 0.2; Ca (NO3)2.4H2O, 0.04; and Fe (SO4)3, 0.001] supplemented with chlorpyrifos (100 ppm). The flasks were incubated on a rotary shaker for enrichment at 120 rpm for 10 days at 37°C. Chlorpyrifos (100 ppm) consisting mineral salt agar (MSA) plates were spread over with 10µl of the former enriched samples and incubated at 37°C for 48 hrs. Individual colonies were sub-cultured onto MSA containing chlorpyrifos (100ppm) until pure cultures were obtained. Bacterial isolates grown on Chlorpyrifos containing media were subjected to morphological, cultural and biochemical analysis. The pure cultures were stored in chlorpyrifos mineral salt agar slant (CMSA) at 4°C and 40% glycerol stock at -20°C.

Characterization and identification of bacterial isolates

Pure bacterial isolate JR5 grown was characterized based on morphological and physiological characteristics. These isolates were also identified based on VITEK®2 bacterial identification system version 07.01- based biochemical reactions performed by bacteria.

Further, 16s rRNA gene of a potential bacterial isolates JR5 was sequenced using universal primers 27F and 1492R on ABI 3730xl Genetic analyzer. The obtained sequences were manually inspected and trimmed and forward and reverse strands were assembled using Codon Code Aligner v6.0.2. The determined sequence was matched with NCBI database using BLASTn and top ten hits based on maximum score were taken further for phylogenetic analysis using MEGA7. Sequences were aligned using Clustal W and then the evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model, and a bootstrap analysis for analysis of the phylogenetic topology. Sequence of bacterial isolates JR5was submitted to NCBI under accession SUB2609138.

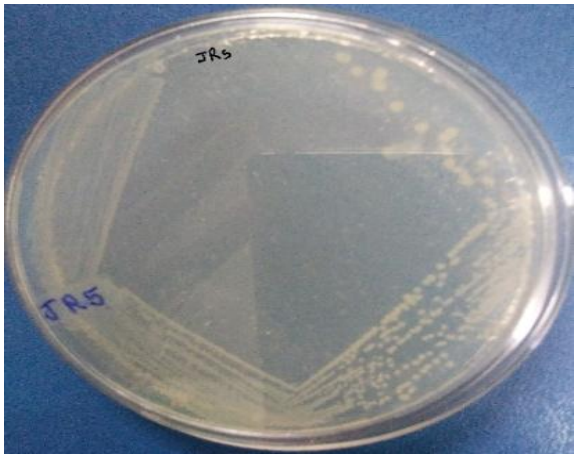
Influence of chlorpyrifos concentration on growth of JR5 growth (Plate Assay)

The tolerance to various concentrations of chlorpyrifos by JR5 was determined by streaking the bacterial isolates on MSA plates consisting various concentrations of chlorpyrifos 100-1000 ppm. All the plates were incubated for 37°C for 48 hours to get visible growth.[12]

RESULTS

Isolation

Bacterial isolate JR5 is gram positive, it was found that colony was opaque A characteristic pigmentation of colony is white, with entire undulate margin and circular, forms were observed. Size of colony was moderate to large having rough surface and elevation was umbonate.



Isolates	Shape	Margin	Elevation	Size	Texture	Appearance	Pigmentation	Optical properties	Gram staining
JR5	circular	Undulate	umbonate	large	Dry	dull	White	Opaque	Gram positive

Identification

No.	Test	Code	Isolate
			JR5
1	BETA-XYLOSIDASE	BXYL	+
3	L-Lysine-ARYLAMIDASE	LysA	-
4	L-Aspartate ARYLAMIDASE	AspA	-
5	Leucine ARYLAMIDASE	LeuA	-
7	Phenylalanine ARYLAMIDASE	PheA	+
8	L-Proline ARYLAMIDASE	ProA	-
9	BETA-GALACTOSIDASE	BGAL	+
10	L-Pyrrolidonyl-ARYLAMIDASE	PyrA	-

11	ALPHA-GALACTOSIDASE	AGAL	+
12	Alanine ARYLAMIDASE	AlaA	-
13	Tyrosine ARYLAMIDASE	TyrA	-
14	BETA-N-ACETYL-GLUCOSAMINIDASE	BNAG	-
15	Ala-Phe-Pro ARYLAMIDASE	APPA	+
18	CYCLODEXTRINE	CDEX	-
19	D-GALACTOSE	dGAL	-
21	GLYCOGENE	GLYG	-
22	myo-INOSITOL	INO	-
24	METHYL-A-D-GLUCOPYRANOSIDE acidification	MdG	+
25	ELLMAN	ELLM	-
26	METHYL-D-XYLOSIDE	MdX	-
27	ALPHA-MANNOSIDASE	AMAN	+
29	MALTOTRIOSE	MTE	-
30	Glycine ARYLAMIDASE	GlyA	-
31	D-MANNITOL	dMAN	+
32	D-MANNOSE	dMNE	+
34	D-MELEZITOSE	dMLZ	-
36	N-ACETYL-D-GLUCOSAMINE	NAG	-
37	PALATINOSE	PLE	-
39	L-RHAMNOSE	IRHA	-
41	BETA-GLUCOSIDASE	BGLU	+
43	BETA-MANNOSIDASE	BMAN	-
44	PHOSPHORYL CHOLINE	PHC	-
45	PYRUVATE	PVATE	+
46	ALPHA-GLUCOSIDASE	AGLU	-
47	D-TAGATOSE	dTAG	+
48	D-TREHALOSE	dTRE	+
50	INULIN	INU	-
53	D-GLUCOSE	dGLU	+
54	D-RIBOSE	dRIB	+
56	PUTRESCINE assimilation	PSCNa	-
58	GROWTH IN 6.5% NaCl	NaCl 6.5%	+
59	KANAMYCIN RESISTANCE	KAN	-
60	OLEANDOMYCIN RESISTANCE	OLD	-
61	ESCULIN hydrolyse	ESC	+
62	TETRAZOLIUM RED	TTZ	-
63	POLYMYXIN_B RESISTANCE	POLYB_R	-

Isolate	**Identification**
JR5	*Bacillus subtilis*

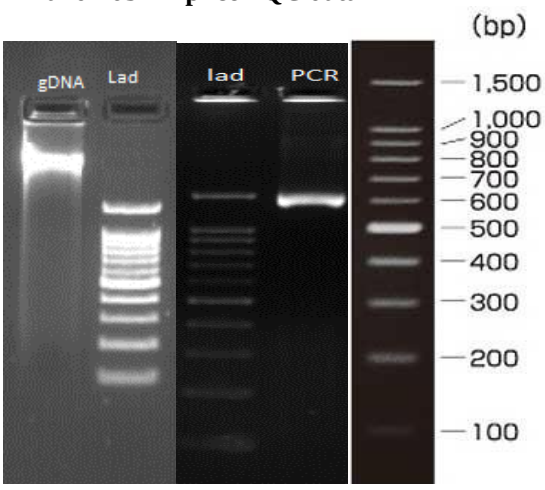
16. S rDNA sequencing of isolates

To conform biochemical test result further 16S rDNA sequencing was carried out to identify bacterial isolates.

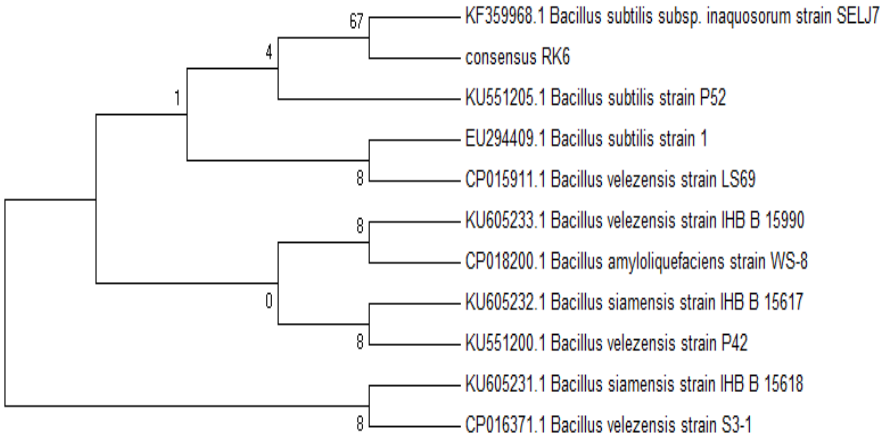
On the basis of 16S rDNA nucleotides homology and phylogenetic analysis; It was found out to be novel strain isolates. Hence, the sequence was submitted to Gen Bankunder below accession number.

ID	**NAME**	**GenBank Accession Number**
RK6	*Bacillus subtilis JR5*	SUB2609138

gDNA and 16S Amplicon QC data



The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model.[13] The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed.[14] Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches[14] Initial tree(s)for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1188 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.[15]



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