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Isolation, characterization and growth response study of chlorpyrifos utilizing soil bacterium *Pseudomonas putida* JR16

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ABSTRACT

Agricultural pesticides are one of the indispensable materials used to meet increasing demand of food crops. Consequently, pesticides find their way to various ecosystems resulting in detrimental pollution problems. Chlorpyrifos is a predominantly used organophosphorus pesticide leading to harmful effects including abnormal cell division. Scientific attempt to eliminate these contaminants from the environment using biodegradation approach has been appreciated. Soil bacteria capable of utilizing chlorpyrifos are potential bioremediation candidates. In context to the same, the present study isolated six bacteria growing in presence of chlorpyrifos as sole carbon source from agricultural soils. The obtained bacterial isolates characterized based on their morphological characteristics, biochemical reactions were found to be *Pseudomonas spp. JR16, Pseudomonas spp. J1, Pseudomonas spp. J2, Pantoea spp. J3, Enterobacter spp. J4 and Kocuria spp. J5.* Among these, the most potential of them viz., *Pseudomonas spp. JR16*, was further proceeded for molecular identification and was observed to be the *Pseudomonas putida* JR16. The influence of different chlorpyrifos concentration, various carbon sources, metals sources, temperature and pH on the growth of *Pseudomonas putida* JR16 was assessed for developing optimum conditions for on field application of the strain for biodegradation of chlorpyrifos.

Key words: Chlorpyrifos, Growth response, Pesticides, *Pseudomonas putida* JR16.

INTRODUCTION

A wide range of agrochemicals have been used in developing countries to sustain the food productivity essential for feeding the ever growing population. These agrochemicals, mostly pesticides are used to control pest attack (Ramanathan and Lalithakumari, 1999). In many cases, these compounds are observed to be persistent in nature. On the whole it has been observed that the increased use of these chemicals has caused consider-able environmental pollution (Abo-Amer, 2011; Gupta *et al.* 2017).

Organophosphate pesticides are an assembly of widely used, highly heterogeneous compounds that share a phosphoric acid derivative chemical structure (Singh *et al.* 2003). Chlorpyrifos [(O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate] is an extensively used agricultural insecticide, belonging to the class II pesticides with moderate toxicity according to the WHO classification. The reported half-life of chlorpyrifos in soil varies from 10 to 120 days, with 3,5,6-trichloro-2-pyridinol (TCP) as the major degradation product. Several attempts have been made to isolate organophosphate degrading bacteria from agricultural soil (Kurhade *et al*. 2016). Reports from the Environmental Protection Agency (1997) suggest that a wide range of water and terrestrial ecosystems may be contaminated with chlorpyrifos, finally causing adverse effects on the biological systems (Surekha *et al.* 2008). The contamination has been found up to about 24 km away from the point of use. If the pesticide is not degraded or detoxified quickly enough, the risk of its off-site migration may pose health risk to humans (Fulekar and Geetha, 2008). The manufacturing and formulation process of chlorpyrifos also generate waste which needs to be treated (Patel *et al.* 2014). Catabolism and detoxification metabolism occur when a soil microorganism uses the pesticide as a carbon and energy source.

Bioremediation is an environmental clean-up technique involving the use of naturally occurring microorganisms in the decontamination process (Agu *et al.* 2014; Kunadia *et al.* 2016). The biodegradation and bioremediation of organophosphorus pesticides by soil microorganisms has been reported by many workers (Visalakshi *et al.* 1980; Kumar, 2011; Singh *et al.* 2004) who have isolated *Enterobacter* B-14 strain which could degrade chlorpyrifos. Yang *et al.* (2006) and Li *et al.* (2007) isolated *Stenotrophomonas* species and *Sphingomonas*

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species respectively. Use of pesticide-degrading microbial systems for removal of pollutants requires an understanding of ecological, physiological, and biochemical requirements of the degrading microorganisms. Hence, the pesticide degradation process in different ecosystems is one of the major area of interest due to the unique core community of microbes in the specific niche.

In context to the same, the objective of present study was to isolate, characterize and identify native strains of bacteria capable of degrading chlorpyrifos using an enrichment culture technique, from selected agro ecosystem which included agricultural soil cultivating cotton crop and contaminated with the said pesticide since a decade. We investigated the influence of chlorpyrifos concentration, sources of carbon, and metal ions, temperature and pH on growth response of the most potentially screened isolate under shaking culture condition.

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 from Rajkot district of Saurashtra region of gujarat state 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): KH_2PO_4, 4.8; K_2HPO_4, 1.2; NH_4NO_3, 1.0; $MgSO_4.7H_2O$. 0.2; Ca $(NO_3)_2.4H_2O$, 0.04; and Fe $(SO_4)_3$, 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.

Table 1: Physical parameters of chlorpyrifos

Molecular formula	$C_9H_{11}C_{l3}NO_3PS$
Molecular weight	350.59 g/mol
Melting point	42°C
Water solubility	2mg/l
Density	1.4 g/cm³
Boiling point	160°C
Color	Colorless crystals
Classification	Organophosphate(Crystalline-Insecticide)

Characterization and identification of bacterial isolates: Pure bacterial isolates grown were 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, whole genome sequencing (WGS) of the most potential bacterial isolate JR16 was performed using Illumina Hiseq platform at Eurofins Genomics, Bangalore, India. The assembly was perfomed using MyPro and compared against NCBI 16s database to identify 16s sequence and assign taxonomy. The sequence was also classified with RDP Naive Bayesian rRNA Classifier Version 2.11 (Shafiani and Malik, 2003) using RDP 16S rRNA training set 16. The WGS was submitted to NCBI under accession PZKR00000000.

Optimization of culture condition: The growth response of JR16 isolate in presence of various concentrations of chlorpyrifos was analyzed under shaking culture conditions. The inoculum was prepared by growing bacterial isolate in 250 ml EM flask containing 100ml of MSM. The flask was incubated at 37°C and 120 RPM for 24 hours. 1ml of 24 hours young culture (*ca.* $1.163x10^5$ cells.ml^{-1}, determined by viable count method) was used for further experiments. The growth response of bacterial isolate was determined as optical density (OD) using UV-VIS Spectrophotometer (model SPECORD® 200 PLUS of Analytikjena) at 600nm.

Influence of chlorpyrifos concentration on the JR16 growth (Plate Assay): The tolerance to various concentrations of chlorpyrifos by JR16 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 (Akbar and Sultan, 2016).

Influence of temperature on growth response of JR16: 250ml EM flask consisting 100ml MSM supplemented with 100ppm chlorpyrifos and inoculated with 1ml of actively growing culture of JR16 were incubated at various temperature (15°C, 25°C, 30°C, 37°C and 45°C) for 72 hours under shaking culture condition at 120 rpm. The increase in bacterial growth was measured at an interval of 24 hours. Un-inoculated control was taken as a blank in all experiments.

Influence of pH on growth response of JR16: 250ml EM flask consisting 100ml MSM supplemented with 100 ppm chlorpyrifos at different pH values were inoculated with 1ml of actively growing culture of JR16. The flasks were incubated at 37°C for 72 hours under shaking culture condition at 120 rpm. Increase in bacterial growth was measured at the interval of 24 hours.

Influence of carbon sources on growth response of JR16: 250ml EM flasks consisting 100ml MSM supplemented with 100ppm chlorpyrifos and 0.25% of various carbon sources

Table 2(a): Biochemical tests for gram negative bacterial isolates (VITEK® 2).

Biochemical tests	JR16	J1	J2	J3	J4
APPA[ala-phe–pro ARYLEMIDASE]	-	-	-	-	-
ADO[ADONITOL]	-	-	-	-	-
PyrA[l- Pyrrolydonyl ARYLEMIDASE]	-	-	-	-	-
IARL[ARABITOL]	-	-	-	-	-
dCELCELLOBIOSE]	-	-	-	+	+
BGAL[BETA GELACTOSODASE]	-	-	-	+	+
H2Sproduction	-	-	-	-	-
BNAG[BETA –N-ACETYL GLUCOSAMINIDASE]	-	-	-	-	+
AGLTp[Glutamyl arylimidasepNA]	-	-	-	-	-
dGLU[D glucose]	+	-	+	+	+
GGT[GAMMA GLUTAMYL TRANSFERASE]	+	-	+	+	+
OFF[FERMENTATION/GLUCOSE]	-	-	-	-	+
BGLU[BETA- GLUCOSIDASE]	-	-	-	+	-
dMAL[D –MALTOSE]	-	-	-	+	+
dMAN[D- MANNITOL]]	-	-	-	+	+
dMNE[D- MANNOSE]	+	-	+	+	+
BXYL[BETA XYLOSIDASE]	-	-	-	-	+
BAlap[BETA Alanine arylimidasepNA]	-	-	-	-	-
ProA[Ll-proline ARYLEMIDASE]	+	+	+	-	-
LIPLIPASE]	-	-	-	-	-
PLE[PALATINOSE]	-	-	-	-	+
TyrA[TYROSINE ARYLEMIDASE]	+	-	+	+	+
URE[UREASE]	+	-	-	-	-
dSOR[D- SORBITOL]	-	-	-	+	+
SAC[SACCHEROSE/SUCROSE]	+	-	-	+	+
dTAG[D- TAGATOSE	-	-	-	-	-
dTRE[D-TRIHALOSE]	-	-	-	+	+
CITCITRATE SODIUM]	+	-	+	-	+
MNT[MALONATE]	-	+	-	-	+
5KG[5-KETO GLUCONATE]	-	-	-	-	-
ILATk[L- LACTATE Alkalinization]	+	-	+	-	+
AGLU[ALPHA GLUCOSIDASE]	-	-	-	-	-
SUCT [SUCCINATE Alkalinization]	+	-	+	-	+
NAGA[Beta N- ACETYL –GALACTOSAMINIDASE]	-	-	-	-	+
AGAL [[ALPHA GALACTOCIDASE]	-	-	-	+	+
PHOS[PHOSPHATSE]	-	-	-	-	+
GlyA [GLYCINE ARYLEMIDASE]	-	-	-	-	-
ODC[ORNITHIN DECAROXYLASE]	-	-	-	-	+
LDC[LYSINE DECARBOXYLASE]	-	-	-	-	-
0DEC[DECARBOXYLASE BASE]				-	-
IHISaL-HISTIDINE Assimilation]	+	-	+	-	-
CMT[COUMARATE]	+	-	+	+	-
BGUR[BETA GLUCORONIDASE	-	-	-	-	-
0129R[0/129 RESISTANCE]	+	-	+	-	+
GGAA[Glu-Gly-Arg ARYLEMIDASE]	-	-	-	-	-
IMLTa[L- MALATE assimilation]	+	-	-	-	-
ELLMAN	-	-	-	-	-
ILATa[[L- LACTATE assimilation]	+	(-)	-	-	-

Test negative; +; Test positive; JR16:*Pseudomonas spp*- JR16; J1 : *Pseudomonas* spp-J1;J2: *Pseudomonas* spp- J2; J3 *Pentoea spp*/ J3; J4: *Enterobac spp.*J4

(xylose, sorbitol, arabinose, mannitol, glucose and sucrose) were inoculated with the 1ml of actively growing culture of JR16. The flasks were incubated at 37°C for 72 hours under shaking culture condition at 120 rpm. Increase in bacterial growth was measured at an interval of 24 hours.

Influence of metal ions on growth response of JR16: 250ml EM flasks consisting 100 ml MSM (pH 8.0) and 100 ppm chlorpyrifos was amended with 5ppm of Mn(II), Fe(II), Hg(II), Cu(II)and Zn(II), respectively. MSM was inoculated with actively growing culture of JR16 (OD=1 at λ_{600}nm). The flasks were incubated at 37°C for 72 hours under shaking culture condition at 120 rpm. Increase in bacterial growth was measured at an interval of 24 hours. MSM without these metal ions served as control.

Table 2(b): Biochemical tests for gram negative bacterial isolates (VITEK® 2).

Biochemical tests	J5
AMY(D-AMYGDALIN)	-
PIPLC (PHOSPHOCITYLINOCITOL PHOSPHOLIPASE C)	-
Dxyl (D-XYLOSE)	-
ADH1(ARGININE DEHYDROLASE)	+
BGAL (BETA GALCTOCODASE)	-
AGLU (ALPHA GLUCOCIDASE)	-
APPA (ALA-PHE PRO ARYLAMIDASE)	-
CDEX (CYCLODEXTRIN)	-
AspA (L-aspartate ARYLAMIDASE)	-
BGAR (BETAGALCTOPYRANOSIDASE)	-
AMAN(ALPHA MANNOCIDASE)	-
PHOS (PHOSPHATASE)	-
LeuA (Leucine ARYLAMIDASE)	+
ProA(L- Proline ARYLAMIDASE)	+
BGURr (BETA GLUCORONIDASE)	-
AGAL(Alanine ARYLAMIDASE)	-
PyrA (L-Pyrrolidonyl- ARYLAMIDASE)	-
BGUR (BETA GLUCORONIDASE)	-
AlaA (alanine ARYLAMIDASE)	+
TyrA (Tyrosine ARYLAMIDASE)	+
Dsor (D- SORBITOL)	-
URE(UREASE)	-
POLYB (POLYMIXIN B RESISTANCE)	-
dGAL(D-GALCTOSE)	-
Drib (D-RIBOSE)	-
ILATk (L-LACTATE alkalization)	+
LAC(LACTOSE)	-
NAG(N ACETYLE D GLUCOSEAMINE)	-
dMAL(D-MALTOSE)	-
BACI(BACTERIACINE RESISTANCE)	+
NOVO(NOVO BIOCINE RESISTANCE)	+
NC6.5 (Growth in 6.5 % NaCl)	-
dMAN(D-MANNITOL)	-
dMNE (D- MANNOSE)	+
MBdG (METHYL – B-D GLUCOPYRNOSIDE)	-
PUL(PULLULAN)	-
dRAF(D-RAFFINOSE)	-
O129R(O/129 RESISTANCE compo.vibrio)	+
SAL (SALICIN)	-
SAC(SACCHROSE /SUCROSE)	+
dTRE (D-TREHALOSE)	-
ADH2s (ARGININE DIHYDROLASE 2)	-
OPTO(OPTOCHINE RESISTANCE)	+

Test negative; +; Test positive; J5: Kocuriapp. J5

Chlorpyrifos degradation study using HPLC: 250ml Erlenmeyer flasks (in tripliacte) containing 100 ml BH broth supplemented with chlorpyrifos (100 ppm) were inoculated with 18h old culture of JR16. The flasks were incubated at 37°C. Samples were harvested after 0, 3, 6, 9,12, 15, 18 and 21 days. Chlorpyrifos from medium was extracted using acetonitrile in equal volume followed by vigorous shaking on a reciprocating shaker at 150 rpm/min. After centrifugation the samples were filtered through 0.2µn nylon membrane filter and samples were analysed through HPLC. Sunfire C_{18} column with 3.4µm, 4.6×100mm was used. Equilibration was carried out with 0.01% glacial acetic 3.7 pH, Acetonitrile (10:90) equilibration was done by passing 10 column volumes of the same buffer. 20µl of sample was loaded in injector. For the standardization of technique, 0.55ml/min flow rates was maintained.

RESULTS AND DISCUSSION

Pesticides have been widely used for protection of the food and crop. These pesticides are not only harmful for the target pests, but also unintentionally cause toxicity in non-target organisms including humans and hence there is an urgent need to eliminate these pollutants from the environment.

Bacterial identification: Bioremediation is an effective strategy in which degradation potential of microorganisms is used to eliminate pollutants from the environment. In context to same, we isolated and identified chlorpyrifos degrading bacteria and based on morphological, Gram's reactions and biochemical characteristics, the bacterial isolates were identified as *Pseudomonas spp*. JR16, *Pseudomonas spp*. J1, *Pseudomonas spp*. J2, *Pentoea spp*. J3, *Enterobacter spp*. J4 and *Kocuria spp*. J5 (Table 2a and 2b). WGS inferred 16s rRNA homology placed JR16 close to *Pseudomonas putida spp*. with 99% identity. Akbar and Sultan (2016) reported the potential of two bacterial strains, *Achromobacter xylosoxidans* JCp4 and *Ochrobactrum* sp. FCp1 to degrade chlorpyrifos.

Influence of chlorpyrifos concentration: *P. putida* JR16 and *Pseudomonas spp*. J2 showed maximum tolerance to chlorpyrifos (1000 ppm and 800 ppm, respectively). While *Pseudomonas spp*. J1 and *Kokuria spp*. J5 had a similar tolerance to chlorpyrifos (700ppm) and the least tolerance was noted for *Pentoea spp*. J3 and *Enterobacter spp*. J4 (500ppm) (Fig 1). A report revealed that a *Pseudomonas* sp. isolated from soil could tolerate up to 800 µg/ml of endosulfan, 1,600 µg/ml of carbofuran and 1,600 µg/ml of malathion (Yadav *et al.* 2014). The possible reason for decline in growth at higher chlorpyrifos concentration could be substrate inhibition (Olson *et al.* 2003). This might be due to inhibition of anionic transportation, cell acidification and undesirable substrate binding to cell parts (Chu *et al.* 2006).

Influence of temperature on growth response of *P. putida* JR16: Current findings showed that the *P. putida* JR16 grew maximum at 37°C during 72 hours of incubation. *P. putida* JR16 also achieved sufficient growth at 28°C, 35°C and 40°C of temperature. Minimum growth of *P. putida* JR16 was observed at 25°C (Fig 2). Microbial growth is temperature specific and temperature is an important physical parameter that affects the cell metabolism directly or indirectly. Those processes, which are catalyzed by enzyme, are affected by incubation temperature. Scientific literature shows that the maximum growth and activity of microorganisms in the soil take place in the temperature range of 25°C to 35°C. A previous similar study reported that the isolate B-14 degraded chlorpyrifos rapidly at 15 to 35°C (Yang *et al.* 2006). Li *et al*. (2007) optimized the temperature 30°C for chlorpyrifos degradation by the isolate *Stenotrophomonas*.

Influence of pH on growth response of *P. putida* JR16: Maximum growth was observed at pH 7 and 8. A decline in growth of *P. putida* JR16 was observed at pH < 6 and >8 (Fig 3). *P. putida* JR16 was able to tolerate a wide range of pH (5-9). A previous study investigated that *Enterobacter* sp. increased degradation with increase in pH from 4.7 to 6.7 but as pH increased from 6.7 to 8.4; there was non-significant difference in rate of degradation (Cho *et al.* 2002).

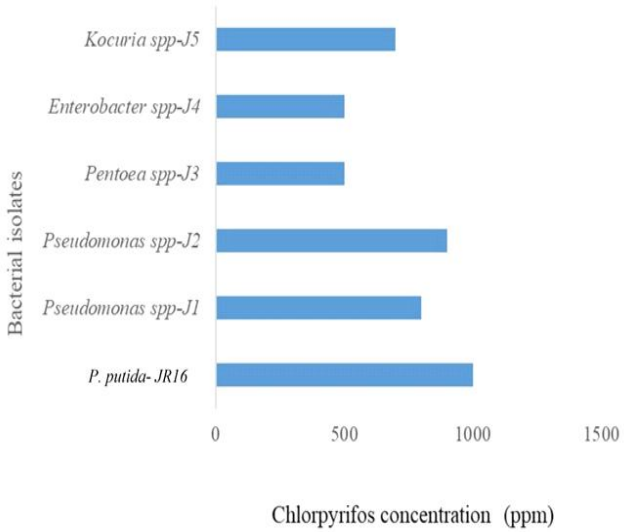

Fig 1: Growth response of bacterial isolates on MSA plates consisting various concentrations (100-1000ppm) of chlorpyrifos.

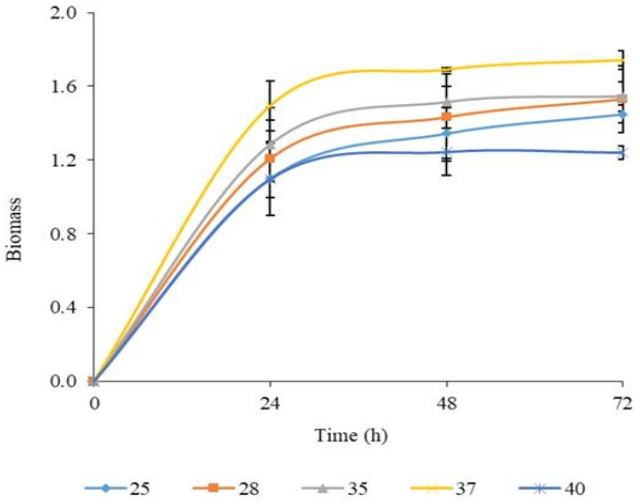

Fig 2: Growth rate of *P. putida JR16* along a range of incubation temperature.

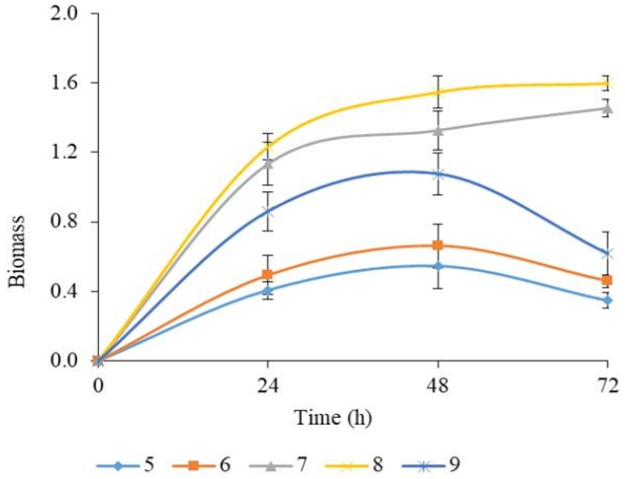

Fig 3: Effect of different pH on *P. putida JR16* growth.

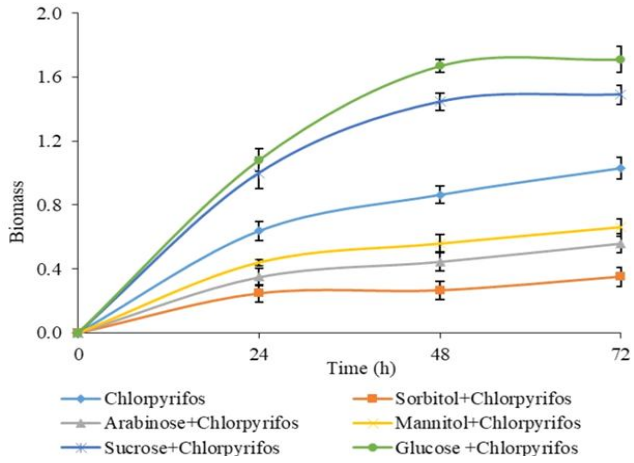

Fig 4: Growth response of *P. putida JR16* in presence various carbon sources.

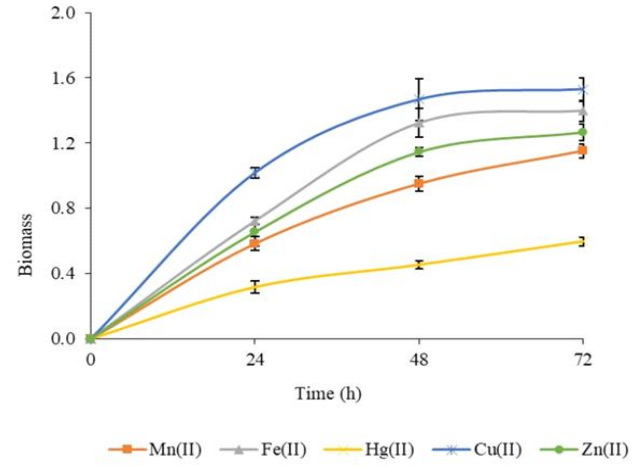

Fig 5: Effect of metal ions on *P. putida JR16* growth.

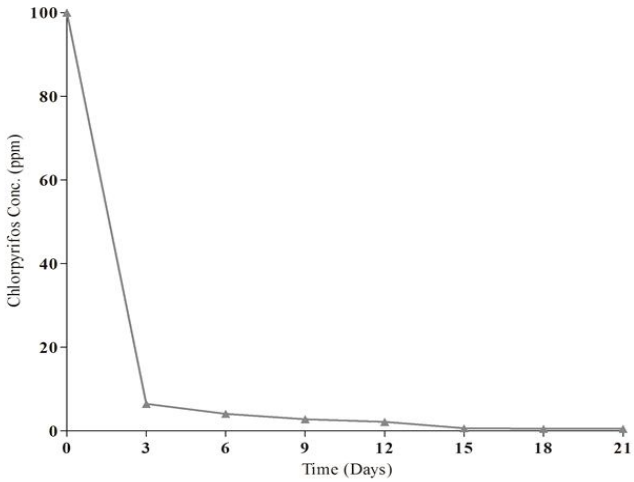

Fig 6: Degradation dynamics of chlorpyrifos inoculated with *P. putida* JR16 at an initial chlorpyrifos concentration of 100 ppm.

Ionic strength (pH) of the medium is of prime importance for almost all physiological processes. The soil pH may affect adsorption, abiotic and biotic degradation of chlorpyrifos. It may be due to the fact that very alkaline or acidic pH has inhibitory influence on bacterial activities (Chu *et al.* 2006).

Influence of carbon sources on growth response of *P. putida* JR16: Presence of easily degradable carbon sources in the medium accelerated growth of *P. putida* JR16 (Fig 4). The growth of *P. putida* JR16 achieved in presence of glucose was maximum after 72 hours. The minimum growth response was observed in presence of sorbitol, arabinose and mannitol.

Influence of metal ions on growth response of *P. putida* JR16: Substantial amount of trace metals is added in pesticide formulation without any scientific reason. Hence, effect of presence of various metal ions like Mn (II), Fe (II), Hg (II), Cu (II) and Zn(II) may affect their biodegradation and subsequently the growth response *P. putida* JR16 was examined (Fig 5). Results showed that copper ion was found to increase the growth of *P. putida* JR16 after 72 hours of incubation. Minimum growth of *P. putida* JR16 was achieved in presence of Hg (II). Biodegradation of chlorpyrifos is a biochemical reaction involving electrons, and metals act as electron acceptor affecting the reaction pathway. Cu (II) and Fe (II) may be necessary for bacterial growth and act as cofactor for chlorpyrifos degrading enzyme in low concentration (Xu *et al.* 2008).

Chlorpyrifos degradation study using HPLC: We found that the isolate was able to degrade more than 98% chlorpyrifos after 21 days of incubation (Fig 6). The retention time for chlorpyrifos was observed at ~4.57, after 21 days the peak for pesticide was reduced to 0.95% with an unknown peak of 99.05% area at 1.521. Pesticides contaminated agricultural soil can be remediated successfully by augmenting it with potential bacterial isolates and the *Pseudomonas* isolated can be a potential candidate for the same.

CONCLUSION

Overall, the present study reports isolation and characterization of potential bacterial strains growing in presence of chlorpyrifos as a sole source of carbon from a cotton cultivated agricultural field soil samples. *P. putida* JR16 was able to grow in the presence of different concentrations of pesticide and also showed degradation of more ~99% after 21 days of incubation. This provides important information on optimization of critical parameters to enhance chlorpyrifos degradation by the isolated bacterial strain. Results from the present study confirm that the chlorpyrifos-degrading bacterial isolates could be used successfully for the removal of chlorpyrifos from contaminated agricultural soil.

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