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Presence of Organophosphate Insecticide Degrading Bacteria in Agricultural Soils of Burdwan, West Bengal, India

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ABSTRACT

At present organophosphate (OP) insecticides are applied extensively and indiscriminately throughout the world during cultivation. These neurotoxic compounds cause adverse effect on non-target organisms like human and other mammals. Tons of OP compounds are synthesized yearly to control the insect-pest from crops infestation during cultivation and crops storage. Not only that these OPs are also used as house hold insect repellent. During cultivation of crops farmers are applied these OPs in non-systematic way. This indiscriminate application of OPs may destabilize the normal ecosystem by deteriorating of normal microbiota of soil and ultimately soil fertility is deteriorated day by day. In this situation urgently cost effective, eco-friendly strategies are required to biodegrade/bioremediate these toxic OPs from the environment to restore the soil fertility, normal ecosystem for very green future. To keep this point of view in mind the present study reports for the isolation of OP degrading bacterial strains from soil samples collected from three agricultural fields (Paddy fields) of Burdwan and adjoining area. My findings also suggests immediate need to design strategies so that restoration activities may be carried out to restore aboriginal microbial community that is responsible for well known fertile soil of Burdwan. If not take care, in near future, we may face situation of decreased soil fertility and crop yield. These strains are biotechnologically important and might find application in bioremediation of OPs contaminated agricultural fields and water bodies, in Burdwan.

Keywords Organophosphate, Screening of OP degrading bacteria, Chlorpyrifos, Soil fertility..

INTRODUCTION

Organophosphate (OP) insecticides were introduced during Second World War and in India these are primarily used for controlling cereal, vegetable, fruit insect pests and medically important insect vectors (mainly mosquitos) (Firozjaei *et al.* 2015, Fernández-López *et al.* 2017). Chlorpyrifos, Parathion, Malathion, Profenofos and Acephate are the most commonly used insecticides. Although, their application has led to better plant protection and crop yield but their extensive use has resulted in pollution. Both neurotoxic OP compounds and their hydrolysis intermediates are known to be hazardous and are listed

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as priority pollutants by EPA, USA (Alvarenga *et al.* 2014, Pailan *et al.* 2015, Pailan and Saha 2015, Foong *et al.* 2020). Their long term presence and persistence in ecological niches has resulted in extensive damage to non-target animals, including humans (Ortiz-Hernandez and Sanchez-Salinas 2010, Shen *et al.* 2010). Few years ago, in India, death of 22 school children was reported by accidental contamination of food by OP insecticides (http://www.cbsnews.com/news/organophosphate-pesticides-eyed-as-cause-of-india-poisonings-how-toxic/). Burdwan district is well known for cultivation of paddy, potato, mustard and vegetables and is popularly known as granary of West Bengal and India. In order to protect these crops from insect pests and better crop yield, farmers are indiscriminately using various OP insecticides over past one decade which ultimately may lead to acidification and deterioration of soil fertility due to perturbation in complex microbial community structure. Extensive and indiscriminate use of insecticides at farm yard and their accidental release into environment may affect the normal biogeochemical cycle by destabilization of soil microbial community structure (Aktar *et al.* 2009, Latif *et al.* 2012, Akbar and Sultan 2016). The latter is very important component for maintenance of soil fertility and productivity. Under these circumstances, bioremediation of toxic OPs and their toxic hydrolysis intermediates must be carried out for restoration of normal ecosystem (Singh 2009). The bioremediation of OP contaminated sites may be carried out by intact microorganisms and/or their enzymes, under aerobic and anaerobic conditions (Shen *et al.* 2010, Wang *et al.* 2012) and offers environmental friendly cost effectives methods of restoring the ecosystems.

In present study I report for the isolation of OP degrading bacterial strains from soil samples collected from three agricultural fields (Paddy fields) of Burdwan and adjoining area. My findings suggests immediate need to design strategies so that restoration activities may be carried out to restore aboriginal microbial community that is responsible for well known fertile soil of Burdwan. If not take care, in near future, we may face situation of decreased soil fertility and crop yield. These strains are biotechnologically important and might find application in bioremediation of OP contaminated agricultural fields and water bodies, in Burdwan.

Table 1. Collection sites for soil samples.

Site 1	Site 2	Site 3
Collected from paddy field of Memari of Burdwan district. Five samples collected	Collected from paddy and pulse field of Burdwan University farm field. Five samples from each field were collected.	Collected from paddy field of Narigram of Burdwan district. Five samples collected.

MATERIALS AND METHODS

Chemicals

Analytical grade Chlorpyrifos (98.5% purity) was obtained from Dr Ehrenstorfer GmbH, Augsburg, Germany and analytical grade Parathion (99.5% purity), Trichloro pyridinol (TCP) and 4- nitro phenol (4-NP) from Sigma-Aldrich, Germany. Other chemicals, media and solvents were purchased from reputed manufacturers (Himedia, SRL and Spectrochem).

Sample collection

Soil samples were collected from three different agricultural fields of Burdwan, West Bengal, India (Table 1).

Determination of viable count of bacteria of soil samples

Viable count of soil samples were determined by dilution platting method using Tryptic soy agar (TSA) plate, at 37°C.

Isolation and enrichment culture technique

The mineral medium (MM; components in (g/L) K_2HPO_4, 0.2, KH_2PO_4, 0.8, $MgSO_4$. $7H_2O$, 0.2, $CaSO_4$. H_2O, 0.1, $NaMoO_4.2H_2O$, 0.003, $FeSO_4.7H_2O$, 0.005, $(NH_4)_2SO_4$, 1.0) used in this study was supplemented with 200 ppm of chlorpyrifos for enrichment culture of OP degrading bacteria. For culturing pure stable OP degrading bacterial isolates, agar (1.5%) plates were used that had the same MM with respective OP compound.

Screening technique

BTB agar plate assay

Bromo thymol blue (BTB) plate based screening assay technique was carried out to screen chlorpyrifos degrading microorganisms qualitatively. All the bacterial isolates were patched on MM + chlorpyrifos (200 ppm) agar medium containing BTB indicator and incubated at 37ºC for 24-48 hrs. Yellow colored colonies were recorded positive for chlorpyrifos degrading strains while non-degrading strains did not grow at all. Whenever a bacterium utilizes and degrades chlorpyrifos as sole carbon source, TCP (acidic in nature) is generated, thereby turning the color from green to yellow.

Tetrazolium chloride agar plate assay

Another suitable qualitative plate based technique was carried out to screen the chlorpyrifos degrading microorganisms. All the isolates were patched on MM+ chlorpyrifos (200 ppm) medium containing tetrazolium chloride (TZC) (0.02%) as artificial electron acceptor. While microorganisms mineralize chlorpyrifos as a sole source of carbon and complete its electron transport chain, TZC is reduced and results in formation of red color formazon. As a result of this reaction bacterial colonies that can utilize chlorpyrifos as sole carbon source appeared as red on the agar plate.

The isolates having better activity were sub-cultured repeatedly for 5 times to fresh MM-agar plate containing chlorpyrifos as sole source of carbon to obtain culture with stable phenotype of OP degradation.

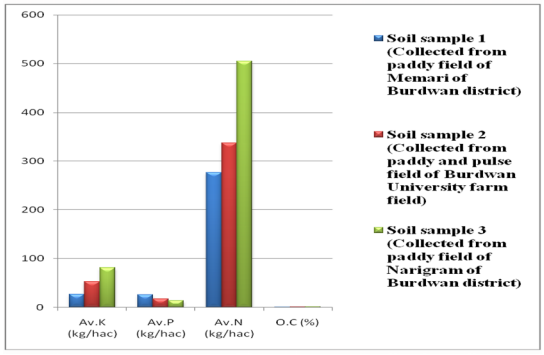

Fig. 1. Physico-chemical analysis of soil sample.

Tolerance to different OP compounds and their toxic hydrolysis intermediates

All the isolates with stable phenotype of chlorpyrifos utilization and growth, so obtained from enrichment culture technique, were next tested for their ability to tolerate different OP compounds (chlorpyrifos and parathion) and some of their toxic hydrolysis intermediate products (4-nitro phenol and 3,5,6 trichloro pyridinol).

Identification of OP degrading bacteria

Identification of OP degrading bacteria was based on 16S rRNA gene. For each isolate, genomic DNA was isolated, 16S rRNA gene was amplified and the amplicons were sequenced following Saha and Chakrabarti (2006). The partial 16S rRNA gene was used as query against various online tools available at RDP site (http://rdp.cme.msu.edu/classifier/classifier.jsp) based on this analyses, the isolates were assigned to respective genus.

RESULTS AND DISCUSSION

Soil sample analysis

Soil samples were analyzed for its physico-chemical properties such as average Potassium (K), Phosphorous (P), Nitrogen (N) and Organic content (Fig. 1).

Viable count

Viable count of soil samples were determined by dilution platting method using tryptic soy agar (TSA) plate, at 37°C (Table 2).

Isolation and screening of OP utilizing bacterial isolates

After a two successive (BTB and TZC; Fig. 2) plate based screening assay techniques and five rounds of repeated transfers on OP supplemented agar plates, 17 stable bacterial cultures were selected (Table 3). Out of 17 strains, 4 were from Memari, 10 from Burdwan University agricultural field and 4 from Narigram, Burdwan (Fig. 3).

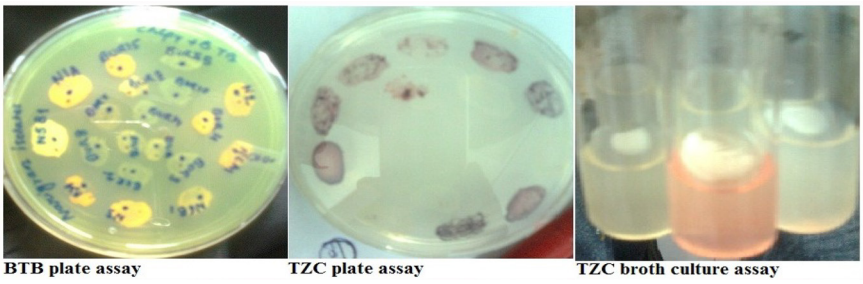

Fig. 2. BTB and TZC screening assay for isolation of chlorpyrifos degrading isolates.

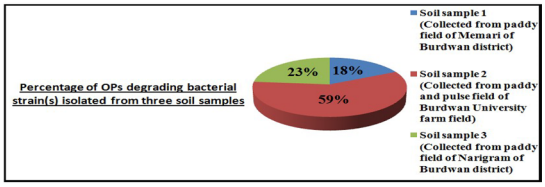

Fig. 3. Percentage of OPs degrading bacterial isolate from soil sample.

Tolerance studies

Though fifteen isolates among seventeen could degrade and utilize 200-300 ppm of chlorpyrifos optimally as sole source of carbon but they can tolerate up to 800 ppm (Table 4). Moreover, most of the cultures, could utilize parathion and 4-nitro phenol (4-NP or p-nitrophenol) as sole source of carbon and four of them can utilize TCP (Table 5). Degradation of 4-NP was indicated by decolorization of the yellow color of 4-NP.

Identification of OP degrading isolates

Based on partial 16S rRNA gene sequence analyses, isolates were affiliated to different genera (Table 6). The species status is not clear as more analyses as per polyphasic taxonomic approach needs to be carried out.

Major genera involved in degradation were recorded as *Acinetobacter, Bacillus, Proteus, Pseudomonas, Staphylococcus* and *Streptomyces.* Most of bacterial strains belonging to these generally have been reported to be involved in degradation of OP compounds. It may be noted that several scientific investigations has recently indicated the role of *Acinetobacter* spp. in degradation of xenobiotic compounds, including OP compounds (Fang –Yao *et al.* 2007). My analyses, strongly suggest, existence of *Acinetobacter* in Memari soil samples.

Table 2. Viable count of soil sample.

Soil sample	Viable count (cells/g)
Soil sample 1	1.5×10^6
Soil sample 2	1.3×10^6
Soil sample 3	1.2×10^6

CONCLUSION

The main focus of the present study was to isolate potential OPs degrading bacteria from agricultural

Table 3. Growth profile on BTB + MM + chlorpyrifos agar plate and TZC + MM + chlorpyrifos agar plate.

Name of the isolates	BTB dye assay (Yellow color formation)	Tetrazolium chloride assay (Red color formation)
MemCl1	Deep yellow (++)	Dee red (++)
MemCl4	Deep yellow (++)	Dee red (++)
MemCl New	Deep yellow (++)	Dee red (++)
MemCl6	Deep yellow (++)	Dee red (++)
N1A	Deep yellow (++)	Light red (+)
N3A	Deep yellow (++)	Dee red (++)
BUR4	Deep yellow (++)	Light red (+)
SanPs1	Deep yellow (++)	Dee red (++)
$N6A_1^1$	Deep yellow (++)	Dee red (++)
$N6A_1^2$	Deep yellow (++)	Dee red (++)
BURZ	Deep yellow (++)	Dee red (++)
BUR15	Deep yellow (++)	Light red (+)
N5B1	Light yellow (+)	Dee red (++)
BUR16B1	Deep yellow (++)	Dee red (++)
BUA	Light yellow (+)	Dee red (++)
BUR10	Light yellow (+)	Light red (+)
BUR11	Light yellow (+)	-
BUR16B22A	Deep yellow (++)	Dee red (++)
BUR16B22B	Deep yellow (++)	Dee red (++)

Abbreviations: ++ strong chlorpyrifos utilizer, + poor chlorpyrifos utilizer.

Table 4. Growth profile of isolates in different concentration of chlorpyrifos.

Name of isolates	Chlorpyrifos (200 ppm)	Chlorpyrifos (300 ppm)	Chlorpyrifos (500 ppm)	Chlorpyrifos (800 ppm)
N5B1	+++	+++	++	+
$N6A_1^1$	++	++	++	+
SanPS1	+++	+++	++	++
N3A	++	+++	++	+
$N6A_1^2$	++	+++	++	+
N1A	++	+++	++	+
BURZ	+++	+++	++	+
BUR15	++	+++	+	+
BUR10	++	+++	+	+
BUR11	+++	+++	+++	+
BUA	++	+++	+++	+
BUR4	++	++	+	+
BUR16B1	++	+++	++	+
MemCl4	+++	+++	++	+
MemCl	+++	+++	++	+
New				
Mem Cl1	+++	+++	++	+
MemCl6	+++	+++	+	+

Abbreviations: '+++' good growth, '++' moderate growth, '+' poor growth.

soil sample of Burdwan using enrichment culture technique, their identification and to study their growth profile in presence of different concentration chlorpyrifos and on other OP compound like parathion and their hydrolysis products (p-nitro phenol and 3,5,6 trichloro pyridinol). From the above mentioned

Table 5. Growth (at 37 ^{0}C after 24 hrs of incubation) profile of chlorpyrifos degrading isolates on other toxic compounds.

Name of isolates	Parathion	*p*- nitro phenol	3,5,6 tri chloro-2-pyridinol
N5B1	+++	+++	-
$N6A_1^1$	++	+++	-
SanPs1	+++	+++	++
N3A	+++	+++	-
$N6A_1^2$	++	+++	-
N1A	-	-	-
BURZ	+++	+++	-
BUR15	+++	+++	-
BUR11	+++	+++	++
BUA	++	+++	-
BUR4	+	+	-
BUR16B1	+	+++	-
MemCl4	+++	-	++
MemCl New	+++	-	++
Mem Cl 1	+++	-	++

Abbreviations: '+++' good growth, '++' moderate growth, '+' poor growth, '-' growth no found.

Table 6. Identification of OP degrading isolates.

Designation of isolates	Phylogenetic affiliation (Based on analyses carried out at RDP site)
BUR15	*Proteus* sp.
$N_6A_1^2$	*Staphylococcus s*p.
BUR10	*Proteus* sp.
BUR11	*Pseudomonas* sp.
N_3A	*Bacillus* sp.
BUA	*Staphylococcus* sp.
BUR16B1B	*Bacillus* sp.
BUR4	*Bacillus* sp.
BURZ	*Proteus* sp.
N6A1	*Bacillus* sp.
BUR16B22	*Staphylococcus* sp.
BUR16B1A	*Staphylococcus* sp.
BUR161A	*Staphylococcus* sp.
SanPS	*Bacillus* sp.
MemCl1	*Acinetobacter* sp.
MenCl4	*Acinetobacter* sp.
MemCl New	*Acinetobacter* sp.

research work, a diverse number of OP degrading bacterial strains were isolated based on culture dependent analyses of three soil samples. These strains are belonging to species of *Acinetobacter, Bacillus, Proteus, Pseudomonas* and *Staphylococcus.* Most of these strains can tolerate upto 800 ppm of chlorpyrifos, (the most common OP insecticide, used in Burdwan as well as in India) and can utilize parathion, and p-nitrophenol as sole source of carbon for growth. Four strains, belonging to *Acinetobacter* and one strain to *Pseudomonas* can also utilize 3,5,6-tri chloropyridinol (TCP) as sole source of carbon and energy for growth. Thus, all the strains, isolated from soil sample have the potential to be used for bioremediation of OP from their respective contaminated sites and these might play very important role in restoring the normal functional microbial community structure of Burdwan agricultural soil ecosystem. This will restore soil fertility, other parameters needed for better crop yield in pollution free eco-friendly habitats.

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