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AK Sharma
Department of Biotechnology, P P Savani University, Kosamba, Surat, Gujarat, India

J Pandit
Department of Environment Sciences, Dr. Y S Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India

V Gautam
Department of Molecular Biology & Biotechnology, Dr. Y S Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India

P Shirkot
Department of Molecular Biology & Biotechnology, Dr. Y S Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India

Correspondence
AK Sharma
Department of Biotechnology, P P Savani University, Kosamba, Surat, Gujarat, India

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Optimization of culture conditions for maximizing Extracellular Organophosphorus hydrolase activity in pseudomonas strains with Chlorpyrifos degradation potential

AK Sharma, J Pandit, V Gautam and P Shirkot

Abstract
In present study, two bacterial strains from apple orchard soils of Himachal Pradesh were isolated, characterized via biochemical tests followed by molecular characterization using 16S *rrna* gene technology. Bacterial isolates were identified as *Pseudomonas indoloxydans* $ASK_{3.2}$ and *Pseudomonas resinovorans* strain AST2.2. Both *Pseudomonas* strains exhibits higher extracellular organophosporus hydrolase (OPH) activities ranging between 0.012-0.165 U/ml and showed resistance to grow at higher concentration of chlorpyrifos. Effect of modification in culture conditions on extracellular OPH activities was studied. In both strains highest OPH activity was observed at pH 8.0, temperature 37 °C, after 48 hrs in presence of glucose as carbon source. Activity of extracellular OPH enzyme in both the strains was found to be enhanced in presence of Co^{+2} metal ions, while Cu^{+2}, Mg^{+2} and Fe^{+2} ions had lesser effect on OPH activity. SDS was found to be a potent inhibitor for extracellular OPH activity in both strains, while OPH activity was found to be enhanced in presence of DTT. Both strains have chlorpyrifos degrading potential which may be attributed to presence of OPH activities and further purification of this enzyme and its use for bioremediation, biosensor for organophosphorus detection can be tried.

Keywords: *Pseudomonas sp.*, 16S *r*RNA *technology, Organophosphorus hydrolase, Chlorpyrifos*

Introduction
Organophosphorus compounds consist of diverse group of chemicals which are being used extensively for both domestic as well as in industrial settings. Chlorpyrifos is one of the common organophosphorus compound used as pesticide in agriculture and its indiscriminate usage in apple orchards has contaminated soil as well as underground water. Numerous reports are available in literature where exposure of chlorpyrifos to humans has caused death by respiratory failure or cardiac arrest, mild exposure may lead to headache, dizziness (Environment Research Laboratory, 1984) [1].
Biological methods for detoxification especially microbial degradation of organophosphorus compounds have attracted growing interest in recent years and many researchers have reported isolation, characterization of various micro-organisms like bacteria, fungi, actinomycetes etc. with degradation potential (Efremenko & Sergeeva, 2001, Sharma *et al.* 2016) [2, 3].
Microbes possessing potential of organophosphorus degradation must have degrading genes, which encodes enzymes having catabolic or co-metabolic modes of degradation. Micro-organisms with degradation abilities may use chlorpyrifos as source of carbon or phosphate. Researchers have reported few organophosphorus degrading genes like *opd*, *opd* A, *opa*, *mpd* etc.and further these gene translates into degrading enzymes such as organophosphorus hydrolase, organophosphorus acid anhydrolase, methyl parathion hydrolase. Organophosporus hydrolase (OPH) enzyme exhibits ability to degrade numerous organophosphorus pesticides by hydrolysing phosphodiester bonds. OPH enzyme was initially reported to be present in bacteria *Pseudomonas diminuta* and *Flavobacterium* species, which were isolated from soil contaminated by Organophosphorus pesticides (Munnecke *et al.* 1976; Munnecke *et al.* 1989;

De Frank & Cheng, 1991; Dave *et al*. 1993; Sharma *et al*. 2016) [4, 5, 6, 7, 3]. Degradation of chlorpyrifos by microbes is mainly attributed to presence of organophosphorus hydrolase activity. In present study, two bacterial strains were isolated employing enrichment technique, from apple orchard soils contaminated due to regular and extensive use of chlorpyrifos. Bacterial strains were screened for the chlorpyrifos degrading activities, characterized, identified and further culture conditions were optimized for maximal extracellular OPH activities.

Material & Methods

Isolation and characterization of bacterial isolates

Bacterial strains were to be isolated from apple orchard soils of Himachal Pradesh employing enrichment technique, and only those isolates exhibiting high tolerance to chlorpyrifos concentration were further selected and screened. Biochemical characterized of bacterial isolates were done by biochemical tests *viz*. Methyl-red & Voges-Proskauser, Catalase, Citrate Utilization, Oxidase, Urease, Casein Hydrolase, fermentation of sugars employing standard protocols (Aneja, 2003) [8]. Molecular characterization was done by genomic DNA extraction from bacterial isolates and further amplification of using 16S *r*RNA gene by PCR utilizing universal primers 27f(5'-AGAGTTTGATCCTGGCTCAG-3'forward) and 1492r (5'-GGTTACCTTGTTACGACTT-3' reverse). After DNA sequencing of amplified product, using *in- silico* analysis (EzTaxon server & BLASTn), bacterial isolates were identified and provided accession number by submitting sequence to NCBI.

Assay for Organophosphorus hydrolase activity in bacterial strains

Protocol standardized by Ningfeng *et al*., (2004) [9] was employed for the detection of organophosphorus activity in bacterial strains. Overnight grown broth culture of bacterial strains were used to obtain culture supernatant at 8,000 X g, 4 °C for 10 minutes and this was used as crude extracellular enzyme. 100µl of crude extracellular enzyme preparations was added to 900 µl of 50 mmol/l Tris-Cl (pH 9.0) assay buffer combined with 5.0 µl of 10 mg/ml methyl-parathion, (which act as a substrate) and incubated at 37 °C for 10 minutes. Reaction termination was completed by addition of 1.0 ml of 10 % CCl_3COOH (Trichloroacetic acid) followed by addition of 1.0 ml of 10 % Na_2CO_3 for color development. The optical density was recorded at 410 nm wavelength by means of spectrophotometer (Perkin Elmer Lambda 25). Enzyme activity can be calculated from the amount of p-nitrophenol (PNP) liberated which is the product of hydrolysis. One unit of OPH activity is defined as the amount of enzyme liberating 1.0 µmol of p-nitrophenol per minute at 37 °C.

Effect of culture conditions for maximizing OPH activity

Effects of various factors such as incubation time (duration range 24-120hrs), incubation temperature (24 °C-60 °C), pH (4.0-9.0), carbon source (1.0% w/v of glucose, sucrose, maltose and mannose), different metal ions (1mM concentration of $MgSO_4$, $FeSO_4$, $CoSO_4$, $CaCl_2$ and $CuSO_4$ in minimal salt medium) and chemical compounds like sodium dodecyl sulphate (SDS), Ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT) etc. on extracellular organophosphate hydrolase activity in both bacterial strains of *Pseudomonas* were investigated.

Results and Discussion:

Bacteria were isolated from soils samples by employing enrichment technique (Sharma *et al*. 2016) [3], where 10g of soil samples were added to mineral salt medium which was fortified with 50mg/l chlorpyrifos. After 3 weeks of incubation at 37 °C in MSM with 50mg/l final concentration of chlorpyrifos and the detectable growth (colony forming unit) of bacterial population was recorded from all the soil samples. Seventy two bacterial isolates were isolated and further exposed to high concentration of chlorpyrifos (1600 mg/l) and it was observed that two bacterial isolates (AST2.2 & ASK3.2) were tolerant to very high concentration of chlorpyrifos (1600mg/l). Bhagobaty and Malik (2008[10]) have also screened chlorpyrifos tolerant bacteria which utilize chlorpyrifos as carbon source. Two bacterial isolates were further studied for various morphological characteristics including colony descriptors and microscopic characters (Table1). Both isolates exhibited almost similar morphological characters except that ASK3.2 isolate colony colour was light yellow while AST2.2 colony was creamish. Farhan *et al*. (2012) [11] has characterized bacteria as *Pseudomonas* sp. on the basis of morphological, biochemical and physiological characteristics.

Table 1: Morphological characterization of chlorpyrifos tolerant bacterial isolates

Colony Morphology					Microscopic characters				
Isolate code	**Colony color**	**Colony shape**	**Colony texture**	**Margin**	**Gram Reaction**	**Shape**	**Spore formation**	**Motility**	**Pellicle formation**
$ASK_{3.2}$	Light yellow	Circular	Smooth	Entire	Gram -ve	Rod	Non-sporous	Motile	Yes
$AST_{2.2}$	Creamish	Irregular	Smooth	Entire	Gram -ve	Rod	Non-sporous	Motile	Yes

Chlorpyrifos tolerant bacterial isolates were then examined for biochemical tests *viz*., catalase, citrate, urease, triple sugar iron agar, indole, methyl red, Voges-Proskauer, oxidase, casein hydrolase, gelatin and fermentation of sugars i.e. lactose, glucose and sucrose. Isolate ASK3.2 gave positive test for indole while isolate AST2.2 was found to hydrolyze casein. On the basis of various morphological and biochemical characteristics, the bacterial isolate ASK3.2 & AST2.2 were identified as member of the genus *Pseudomonas* by standard protocol set in Bergey's Manual of Determinative Bacteriology (Holt *et al*. 1994) [12].

Table 2: Biochemical characterization of selected chlorpyrifos tolerant bacterial isolates

Biochemical Tests													
Isolate code	**Catalase**	**Citrate utilization**	**Urease**	**Triple sugar test**	**Indole**	**MR**	**VP**	**Oxidase**	**Casein hydrolase**	**Gelatin**	**Lactose**	**Glucose**	**Sucrose**
$ASK_{3.2}$	+	+	+	-	+	-	-	+	-	-	-	+	-
$AST_{2.2}$	+	+	+	-	-	-	-	+	+	-	-	+	-

Total genomic DNA of each bacterial isolate was extracted successfully using Genomic DNA extraction Mini kit (Real Genomics) and presence of DNA and its quality was checked using 1.0% agarose gel. Researchers commonly use 16S *rrna* gene technology for molecular characterization of bacteria with chlorpyrifos degradation abilities (Singh *et al*. 2004; Yang *et al*. 2006; Rani *et al*. 2008; Latifi *et al*. 2012; Sharma *et al*., 2016) [13, 14, 15, 16, 3]. After 35 cycles of PCR amplification, universal primers for 16S *rrna* gene were able to successfully amplify 16S *rrna* gene of bacterial isolates and followed by sequencing. BLAST algorithm as well as EzTaxon is employed frequently by researchers for exact identification of bacterial strains on the basis of homology (Kim *et al*. 2012; Chun *et al*. 2007) [17, 18]. *In-Silico* analysis of 16S *rrna* gene amplicon comprised of BLASTn as well as EzTaxon analysis, confirmed nucleotide test sequence of strain ASK3.2 showed 98.48% homology with *Pseudomonas indoloxydans* IPL-1(T) while nucleotide test sequence of strain AST2.2 showed 97.80% homology with *Pseudomonas resinovorans* strain LMG 2274(T). Chlorpyrifos degrading bacterial isolates 16S *rrna* gene sequences were submitted to GeneBank-NCBI database and have specific accession number (KP322757 & KP322753 respectively). Both bacterial strains have shown considerable chlorpyrifos degradation under *in–vitro* conditions (Sharma *et al*. 2016 & Sharma *et al*. 2017) [6, 19] and probable reason for chlorpyrifos degradation may be attributed to presence of certain enzyme system in both bacterial strains.

Organophosphate compounds having P-O, P-F, P-CN, P-S bonds are reported to be hydrolyzed commonly by organphosphorous hydrolase enzymes (Lai *et al*. 1995; Dumas *et al*. 1989) [20, 22] and detoxification is resulted by attack of bridging water molecule on phosphate centre (Efremenko & Sergeeva 2001; Benning *et al*. 2001; Dong *et al*. 2005) [2, 22, 23]. OPH activity have been reported by researchers in wide range of *Pseudomonas* species like *P. stutzeri*, *P. diminuta*, *P. pseudoalcaligenes*, as well in other bacterial species like *Flavobacterium* sp., *Agrobacterium radiobacter*, *Burkholderia* sp. etc. (Mulbry *et al*. 1987; Mulbry *et al*. 1989; Horne *et al*. 2002, Singh, 2009) [24, 25, 26, 27] Organophosphate hydrolase activity was determined by protocol (Ningfeng *et al*. 2004) [9] for the two bacterial strains and it was observed that extracellular activity is significantly higher in both bacterial strains as compared to the respective intracellular activity (Sharma *at al*. 2017) [19]. This result is consistent with the previous report of *Ningfeng et al*. (2004) [9] who reported extracellular OPHC2 activity 0.208 U/ml and 0.198 U/ml was confined to cell membrane in *Pseudomonas pseudoalcaligenes*. To improve OPH activity levels in our bacterial strains, we optimized various culture conditions (Verma *et al.* 2014) [28]. In both strains highest OPH activity was observed at pH 8.0, temperature 37 °C, after 48 hrs in presence of glucose as carbon source. Singh *et al*. (2003) [29] also reported that rate of chlorpyrifos degradation by microbes is enhanced under alkaline pH condition and our result fall in line as high OPH activity was observed at pH 8.0. Similarly other authors had reported maximum OPH activity at 35 °C and 37 °C in *Pseudomonas stutzeri* S7B4 and *Pseudomonas aeruginosa* NL01 (Bayoumi *et al*. 2009; Najavand *et al*. 2012) [30, 31]. Many researchers reported alkaline pH to be the optimum for maximum OPH activity (Najavand *et al*. 2012; DeFrank and Cheng, 1991; Chu *et al*., 2006) [31, 6, 32].

Activity of extracellular OPH enzyme in both the strains was found to be enhanced in presence of Co^{+2} metal ions, while Cu^{+2}, Mg^{+2} and Fe^{+2} ions had lesser effect on OPH activity. SDS was found to be a potent inhibitor for extracellular OPH activity in both strains, while OPH activity was found to be enhanced slightly in presence of DTT (Table 3). Our results correspond with those of Chu *et al*. (2006) [32] who reported that OPHC2 activity was enhanced by 14.2 % in presence of Co^{2+} in *Pichia pastoris* and Najavand *et al*. (2012) [31] who found OPH enzyme activity enhancement by 1.28 fold in the presence of $CoCl_2$. SDS inhibited strongly the activity of the OPHC2 in *Pseudomonas pseudoalcaligenes* and *Pichia pastoris* (Ningfeng *et al*. 2004; Chu *et al*. 2006) [9, 32]. The OPH enzyme activity was inhibited competitively by dithiothreitol (DTT) in *Pseudomonas diminuta* (Dumas *et al*. 1989) [21] while in our study DTT slightly enhanced OPH activity in *Pseudomonas indoloxydans* strain $ASK_{3.2}$ and *Pseudomonas resinovorans* strain $AST_{2.2}$.

Table 3: Optimization of culture conditions for enhancing OPH activity in bacterial strains

Optimization of culture conditions	Variation in Parameter	Bacterial Strains exhibiting OPH activity (U/ml)	
		P. indoloxydans ASK3.2	*P. resinovorans AST2.2*
Incubation Time (hrs)	24	0.034	0.032
	48	0.165	0.158
	72	0.163	0.151
	96	0.157	0.153
	120	0.150	0.147
Incubation Temperature (°C)	24	0.128	0.114
	30	0.150	0.131
	37	0.204	0.195
	42	0.155	0.148
	50	0.143	0.129
	60	0.103	0.095
pH	4.0	0.064	0.050
	5.0	0.086	0.072
	6.0	0.111	0.099
	7.0	0.163	0.150
	8.0	0.210	0.199
	9.0	0.178	0.170
Carbon Source (1%)	Sucrose	0.180	0.177
	Glucose	0.217	0.204
	Lactose	0.182	0.178

	Mannose	0.177	0.173
Metal Ions (1mM)	$CuSO_4$	0.212	0.203
	$CoSO_4$	0.254	0.241
	$MgSO_4$	0.214	0.204
	$FeSO_4$	0.211	0.202
	$CaCl_2$	0.037	0.028
Chemical compounds	SDS	0.000	0.000
	EDTA	0.210	0.204
	DTT	0.223	0.209

Conclusion

OPH enzymes are powerful tool in the bioremediation of organ phosphorus compounds. In our study both *Pseudomonas* strains exhibits presence of high extracellular OPH activities which are probable reason for the bacterial strains showing high chlorpyrifos degradation potential. Further, this organophosphorus hydrolase enzyme can be purified and catalytic activity as well as efficiency may be enhanced using site directed mutagenesis tool of gene and protein engineering. Bio-nanotechnology interventions like nanostructures/nanoparticles can be used to immobilize OPH enzyme and these enzyme carriers can be tested for potential application in bioremediation, biosensor development for organophosphorus detection, treating organ phosphorous poisoning.

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