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Rhamnolipid mediated enhanced degradation of chlorpyrifos by bacterial consortium in soil-water system

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ABSTRACT

The study was conducted with the aim to develop an environmentally compatible bio-based system which may rapidly detoxify soil and water polluted by inordinate use of organophosphate (OP) pesticides. Chlorpyrifos was used as model pesticide as it degrade slowly due to its low aqueous phase solubility (2 ppm) and formation of antibacterial intermediate 3,5,6, trichloropyridinol (TCP). Five potential bacteria used in this study belonging to genus *Pseudomonas*, *Klebsiella*, *Stenotrophomonas*, *Ochrobactrum* and *Bacillus* and their mixed culture system efficiently degraded chlorpyrifos and its toxic intermediates TCP and diethylthiophosphate (DETP) in aqueous medium. However, degradation rate in soil-water based slurry system was slow as it took 10 days to degrade 82% of added chlorpyrifos (50 mg/kg) by a potential mixed culture CS2 comprised of isolates F-3 and CH-y. This might be due to strong sorption affinity of chlorpyrifos to soil components which limits its bioavailability. Hence, a crude rhamnolipid biosurfactant produced by ChlD was used which improved the aqueous phase solubility of chlorpyrifos by 2–15 folds. This supported CS2 to attain 30% higher degradation within short period of 6 days as compared to biotic control without surfactant. Thus, this combination of mixed bacterial population with biosurfactant significantly improved the rate of chlorpyrifos degradation in soil without accumulation of toxic intermediates. This environmentally benign biosurfactant may be produced *"in situ"* and can replace commonly used toxic synthetic surfactants for bioremediation purposes.

1. Introduction

The organophosphate (OP) group of chemicals has multifarious applications ranging from commonly used insecticides, fire retardants/plasticizers to their use as chemical warfare agents (such as tabun, sarin, soman and cyclosarin) in Japan (Tokyo, 1995) and Syria in 2013 (UN mission report, 2013). Thus, excessive use and disposal of OP compounds is causing accumulation of high concentrations of these hydrophobic OPs and their toxic intermediates in the environment (Ogah and Coker, 2012; Estévez et al., 2008). Further, environmentally safe disposal of OP based expired stocks is another major challenge for many developing countries (Zhang et al., 2010). Eddleston et al. (2005) reported approximately 3,000,000 human poisoning across the world by OPs during 2002–2005. The OPs and nerve agents tend to disrupt the nervous system by inhibiting the enzyme acetylcholinesterase. Additionally, these could mimic hormones (estrogen and androgen) thus acting as endocrine disruptors leading to reproductive toxicity in humans and other non-target organisms (Choudhary et al., 2014). Moreover, detection of nerve agent degradation products (NADPs) in food products has further raised a serious issue related to human health (Zhang et al., 2010).

Chlorpyrifos [$C_9H_{11}C_{l3}NO_3PS$; O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate], used as model compound in this study is an extensively used chlorinated hydrophobic organophosphate insecticide. The transformation of chlorpyrifos leads to formation of 3,5,6, trichloropyridinol (TCP) and Diethylthiophosphate (DETP). TCP has been reported to be a potent toxin of bacterial metabolism which significantly restricts mineralization of chlorpyrifos leading to accumulation of both chlorpyrifos and TCP in the environment (Yang et al., 2011). Moreover, TCP has high binding affinity to functional groups in major and minor groves of DNA resulting in impairment of vital functions in animals and humans (Kashanian et al., 2012). Similarly, DETP has been reported to be a causative agent of DNA damage in hepatic cells (Vega et al., 2009). Thus, there is a need to isolate microbial populations which can efficiently degrade such toxic OPs and their hazardous intermediates.

The low aqueous phase solubility (2 ppm) of chlorpyrifos further limits its availability to potential microbial degraders which

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slows down the overall degradation process and become major cause of chlorpyrifos accumulation in soil. The slow and regular release of such accumulated OPs in water bodies causes acute poisoning to fishes which aggravates to the higher tropic levels (Singh, 2012, 2013). The reports are available regarding use of synthetic surfactants for improving bioavailability of pollutants to microbial degraders for their enhanced degradation (Mathurasa et al., 2012). However, synthetic surfactants, being of petrochemical origin are not easily biodegradable which may adversely affect the indigenous flora and fauna of polluted site. Bio-surfactants, the surface active molecules of microbial origin, with functional properties similar to those of synthetic surfactants are environmentally safe alternative to synthetic surfactants due to their biodegradability (Lima et al., 2011). Congiu et al. (2015) reported partitioning of pyrene by rhamnolipid based biosurfactant to improve its bioavailability and biodegradation. The bio-surfactants a molecule of 21st century can retain their emulsifying activities under extremes of temperature, pH and salinity which make them a promising asset for use in field scale bioremediation (Santos et al., 2016; Datta et al., 2011). However, high cost involved in extraction and purification of biosurfactant is the major hindrance in their field scale applications (Santa Anna et al., 2007). Thus, efficiency of crude biosurfactant preparations or "*in situ*" productions of biosurfactants at polluted sites by efficient isolates for their rapid bioremediation needs to be explored.

In light of this, efficiency of crude rhamnolipid biosurfactant of *Pseudomonas* sp. ChlD to partition soil bound chlorpyrifos from soil to the aqueous phase was evaluated. Further, the effect of enhanced bioavailability of chlorpyrifos on overall rate of degradation by a selected mixed bacterial culture system of potential isolates was studied. The pure indigenous bacterial isolates having ability to efficiently degrade chlorpyrifos and TCP were mixed to develop the consortia. Further, during the chlorpyrifos biodegradation process, formation and degradation of toxic intermediates TCP and DETP was evaluated.

2. Materials and methods

2.1. Media, chemicals and bacterial cultures

The mineral salt medium (MSM, pH 7.0) as described by Khehra et al. (2006) was used for isolation and screening of microbial isolates. The M-9 medium (pH 8.0) of following composition (g/l): $Na_2HPO_4 \cdot 7H_2O$ (12.8), NaH_2PO_4 (0.9), $MgSO_4$ (0.0096), NaCl (0.5), $CaCl_2 \cdot 2H_2O$ (0.00146) and $ZnSO_4$ (0.0287) was used for chlorpyrifos and TCP degradation studies. Analytical grade chlorpyrifos, 3,5,6-trichloropyridinol (99% purity, Supelco, Bellefonte, PA, USA) and diethylthiophosphate (98%, Sigma Aldrich, USA) were used throughout this study. The media components, chemicals and solvents used were of analytical/HPLC grade.

The soil samples collected from agricultural fields regularly sprayed with OP pesticides were incubated under shaking conditions in MSM supplemented with 100 mg/l of chlorpyrifos as sole carbon source for enrichment of potential microbes having ability to degrade chlorpyrifos. Five bacterial cultures designated as ChlD, F-3, CH-y, 13.9 and C-2 were isolated based upon their ability to efficiently transform chlorpyrifos. The bio-surfactant produced by isolate ChlD used in this study was identified as rhamnolipid as per protocol outlined in our recent report (Singh et al., 2009). The isolates were identified and submitted to Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh. The amplified 16S rDNA sequences of these isolates were also submitted to NCBI for their accession numbers.

2.2. Degradation of chlorpyrifos and TCP by pure isolates in M-9 medium

The isolates were activated in M-9 medium supplemented with 2% (w/v) glucose and 0.25% (w/v) yeast extract. The cells were harvested by centrifugation at 6000 g and washed twice with M-9 medium to remove medium components. The cells of respective isolates were inoculated separately to 50 ml M-9 medium, pH 8.0 (250 ml Erlenmeyer flasks) supplemented with 10 mg/l chlorpyrifos as sole carbon and energy source, at initial $O.D_{540} \approx 0.75$ ($1.4 \times 10^7 - 9.2 \times 10^7$ CFU/ml). The M-9 medium flask without inoculum was kept as abiotic control. The flasks were incubated at 30 °C in an orbital shaker at 150 rpm under shaking conditions. After 5 days incubation contents of the respective flasks were extracted twice with 50 ml of hexane to determine concentration of residual chlorpyrifos. However, to detect formation of TCP the medium in the flask was extracted twice with 50 ml of ethyl acetate. Further, the aqueous phase was saturated with sodium chloride and pH was lowered to 2.0. The contents of the flasks were then extracted twice with 50 ml of diethyl ether to recover DETP. The respective extracts were concentrated and analyzed by High performance liquid chromatography (HPLC).

Similarly, ability of respective isolates to degrade 10 mg/l TCP as a sole carbon source in M-9 was evaluated using similar set of physico-chemical conditions as described above. The contents of the flasks were extracted thrice with equal volume of ethyl acetate for further analysis.

2.3. Mixed culture study

The cells of five isolates ChlD, F-3, CH-y, 13.9 and C-2 were mixed as per combinations outlined in Table 1 to design seventeen (17) different consortia. The activated cells of different isolates with approximately same level of adjusted $O.D_{540}$ were mixed to achieve initial $O.D_{540} \approx 0.75$ for development of each consortium combination in 50 ml M-9 medium supplemented with 10 mg/l chlorpyrifos. The chlorpyrifos degradation potential of each mixed culture combination was evaluated, as per protocol described above. The flask without cells was used as abiotic control. The flasks were incubated at 30 °C in an orbital shaker at 150 rpm for 5 days. The content of the flasks was extracted thrice with equal volume of hexane to determine chlorpyrifos degradation by HPLC.

Table 1
Chlorpyrifos (10 mg/l) degradation potential of different mixed culture combinations in M-9 medium after 5 days of incubation.

Sr. no.	Combinations	[a]Degradation (%)
1	ChlD and 13.9	37.1 ± 0.93
2	ChlD and F-3	68.5 ± 1.25
3	ChlD and C-2	58.8 ± 0.73
4	ChlD and CH-y	68.2 ± 0.82
5	ChlD, 13.9 and F-3	70.7 ± 1.13
6	ChlD, 13.9 and C-2	78.6 ± 1.56
7	ChlD, 13.9 and CH-y	60.4 ± 0.55
8	ChlD, F-3 and C-2	78.2 ± 1.25
9	ChlD, F-3 and CH-y (CS3)	84.3 ± 0.91
10	ChlD, CH-y and C-2	57.4 ± 1.31
11	ChlD, 13.9, F-3 and CH-y	78.7 ± 1.15
12	ChlD, C-2, 13.9 and CH-y	60.5 ± 0.75
13	ChlD, 13.9, F-3 and C-2	57.1 ± 0.97
14	ChlD, CH-y, F-3 and C-2	76.4 ± 1.27
15	ChlD, CH-y, 13.9, F-3 and C-2 (CS5)	79.6 ± 0.81
16	CH-y, 13.9, F-3 and C-2 (CS4)	86.2 ± 1.32
17	F-3 and CH-y (CS2)	86.5 ± 1.56

[a] The experiment was repeated twice in duplicates to generate values with standard deviations.