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Chlorpyrifos Degradation Using Bacterial Consortium Obtained From Soil

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Abstract—Widespread and indiscriminate use of Chlorpyrifos (CP) - an organophosphate pesticide, leads to severe environmental problems. It poses a great threat to different trophic levels of the ecosystem from soil microorganisms to human beings. As the risk of their off-site migration pose health risk to non-target organisms, it is essential to remove this pesticide from their point source of contamination. The current research paper attempts to develop a bacterial consortium capable of remediating CP effectively. The degradatory efficiency of thirteen morphologically different soil bacterial isolates obtained by selective enrichment on mineral medium containing CP (25ppm) or Trichloro2-pyridinol (TCP- antimicrobial byproduct of CP degradation) (25 ppm) as sole source of carbon and nitrogen was investigated for five days. The isolates were equally capable of degrading TCP compared to CP. Using these isolates different bacterial consortia were developed and the consortium C2 (S5, S6, S12) was efficient and showed 95.38±0.02 % of degradation of CP (25 ppm) in three days. The efficiency of the consortium to mineralize CP was investigated under different culture conditions. The study revealed that the consortium could transform CP to non-toxic products. Immobilisation of developed consortium was done and rate of CP degradation using immobilized system in presence of inorganic fertilizer (N: P: K) and coir pith was investigated. The obtained results demonstrated that the immobilized consortium could be recommended for usage in biobeds as a viable alternative to prevent CP dissipation in the environment.

Keywords—Chlorpyrifos, biodegradation, consortium, organophsphate pesticide

I. Introduction

Large-scale manufacture and handling of the organophosphate insecticide Chlorpyrifos (CP) have led to the contamination of soil, air, surface and groundwater in many parts of the world. If pesticides are not degraded or detoxified rapidly enough, besides causing environmental contamination the risk of their off-site migration may pose a health risk not only to variety of beneficial arthropods, fishes at concentrations as low as a few parts per trillion, birds and plants but also to humans also. Increasing awareness of the potential adverse effects of CP has resulted in greater public concern to assess, monitor and minimize its off-site impacts.

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Due to the environmental concerns associated with the accumulation of CP in food products and water supplies, efforts are currently underway to develop safe, convenient and economically feasible environmental friendly methods for detoxification. Current methods to detoxify organophosphate pesticides mainly rely on chemical treatment, incineration and landfills [1] Soil micro flora is another potential candidate for detoxification of CP pesticides. Microbiological remediation is usually the major route of mineralization of pesticides. Microbial degradation of pesticides such as parathion, methyl parathion, malathion, monocrotophos, dimethoate, some organosulphates etc. has been reported in soil, flooded soil and water by pure culture and consortia. Several attempts to isolate a CP-degrading microbial system by repeated treatments or enrichment of soils and other media with CP have not been successful [2]. The resistance of CP to enhanced degradation in soil was attributed for this failure. However, CP has been shown to be degraded co-metabolically in liquid media by bacteria. With this point in view the current research paper attempted to develop a bacterial consortium capable of remediating CP effectively in an environmental friendly manner.

II. Materials and methods

A. *Isolation and screening of CP degrading bacteria*

Soil samples were collected from crop fields across Kerala State where CP was sprayed extensively. Soil enrichment was carried out in mineral salt medium (pH 7.6) containing (gL^{-1}) K_2HPO_4, 1.5; KH_2PO_4, 0.5; NaCl, 0.5; $MgSO_4$, 0.2; $CaCl_2$, 0.05; $FeSO_4$, 0.02. Enrichment was done in higher concentrations of CP ranging from 50 ppm to 400ppm. Morphologically distinct strains with the tolerance to the highest CP concentration were selected, inoculated in mineral salt medium (MSM) containing CP (25ppm) and incubated at 30°C for 5 days. Residual CP in the medium was extracted with dichloromethane and dissolved in HPLC grade acetonitrile. The biodegradation of CP was detected and confirmed by reverse phase liquid chromatography (RP-HPLC) (Shimadzu), with C-18 column equipped with SPD 20 A UV detector (220 nm) at 290nm. The mobile phase used was acetonitrile: water: glacial acetic acid (82:17.5:0.5) at a flow rate of 1ml/min. Data acquisition and processing was done by using LC solution system software (Shimadzu). The efficient CP degrading microorganisms were selected for developing a consortium.

B. Development of a consortium for CP degradation

CP degrading bacterial consortium was developed using different combinations of the screened potential isolates. One OD log phase culture isolates were mixed in equal proportion to get final I mL suspension and inoculated in sterilized MSM containing CP and further incubated to develop the consortium. The biodegradation was estimated by HPLC analysis and expressed as the percentage depletion of the CP.

C. Identification of selected isolates

Identification of the isolates in the consortium was done by Gram stain, shape, and motility under the microscope and tested for their all biochemical reactions. Strains were characterized by analysis of their 16S rRNA gene also. The determined sequence was compared with those available in the GenBank database using the NCBI BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

D. Optimization of pH and temperature

The optimum pH and temperature for degradation were determined by observing the degradation of CP at various pHs, i.e. 5, 6, 7, 8, 9 and various temperatures, i.e., 25, 30, 35 and 40 °C.

E. Analysis of metabolic byproducts

The biodegradation of CP to different metabolites was confirmed using analytical techniques FTIR (Shimadzu –IR Prestige 21 FTIR - ATR attached) and GC-MS (Shimadzu) Column (30 m x 0.25 mm x 0.25 µm) temperature was isothermal at 60°C, while the injector and detector temperatures were 250°C and 300°C, respectively. Carrier gas used was helium.

F. Biodegradation enhancement studies

Biodegrdation enhancement studies were assessed by immobilization mehods (alginate beed based or coir pith based) and NPK supplementation analysis. 1 OD (600nm) consortium inoculum and alginate solution (4%) mixed in equal proportion was dropped into a solution of 0.2M $CaCl_2$ to get bead and these seeds were used to inoculate MSM with 25 ppm CP and incubated. At the same time a suspension of washed bacterial cells in sodium-phosphate buffer (pH 7.0) at a concentration corresponding to 1 OD density value (OD 600nm) was used for immobilization of bacteria on the coir pith block. The incubation was carried out in an orbital shaker (120 rpm) at 37°C for 4 days. Degradation was calculated against control using HPLC.

To check the effect of N:P:K in biodegradation N:P:K (20:10:10, 1%) was added to mineral salt medium which was seeded with 1ml of freshly made consortium and also the coir pith block with adhered microorganisms as seed in separate flasks and incubated at 37°C at 120rpm for 4 days. Extracted samples were analyzed in HPLC.

III. Results

Thirteen colonies (S1-S13) showing different morphological characters were selected by soil enrichment for screening. From these isolates 6 were selected and using these isolates different bacterial consortia were developed Among these, the consortium C2 (S5, S6, S12) was efficient and showed 95.38±0.02 % of degradation of CP (25 ppm) in three days (Fig. 1). The members of the consortium were identified by both biochemical and molecular methods as two different strains of *Pseudomonas aeruginosa* (S5 and S12) and *Klebsiella* sp. There are reports on degradation of chlorpyrifos by a *Pseudomonas* [3] and *Klebsiella* [4] strains. A consortium consist of *Klebsiella* sp., *Pseudomonas aeruginosa*, *Pseudomonas stutzeri*, *Pseudomonas putida* was isolated from chlorpyrifos contaminated agricultural soil [5]. Vidhya Lakshmi et al., [6] have developed three aerobic consortia consisting of *Pseudomonas aeruginosa*, *Bacillus cereus*, *Klebsiella* sp., and *Serratia marscecens*. Nancy and Gustavo [7] could obtain a consortium of *Pseudomonas putida*, *Bacillus* sp, *Pseudomonas aeruginosa*, *Citrobacter freundii*, *Stenotrophomonas* sp, *Flavobacterium* sp, *Proteus vulgaris*, *Pseudomonas* sp, *Acinetobacter* sp, *Klebsiella* sp and *Proteus* sp. In culture medium enriched with each of the pesticides, the consortium was able to degrade 150 mgL^{-1} of methyl parathion and chlorpyrifos in 120 h.

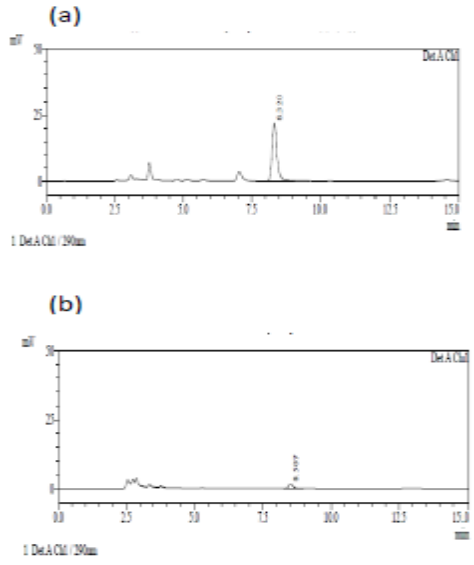

Fig. 1 HPLC chromatogram of (a) Control (b) C2 treated sample after 3 days of incubation

Maximum degradation rate occurred at 30°C (97.34±0.10) and followed was the degradation rate at 35°C (73.66±0.05), while the degradation capability of the consortium in liquid at 25 and 40°C was relatively weak (Fig 2). The degradation of CP was evaluated after 3 days incubation at 30°C and 120 rpm. As noted in fig.1 the optimum pH was 7 (96.76±0.09), and the degradation of CP by consortium was still

considerable at pH 8 (86.12±0.19), but became significantly inhibited at pH below 6 and above 8. Thus the optimum temperature and pH was found to be 30°C and pH 7. Zhiyuan Liu et al., [8] have reported the optimum temperature and pH, of CP degradation were 30°C, 7.0. Singh et al., [13] have suggested that only soils with a pH of ≥6.7 were able to maintain this degrading ability 90 days after inoculation.

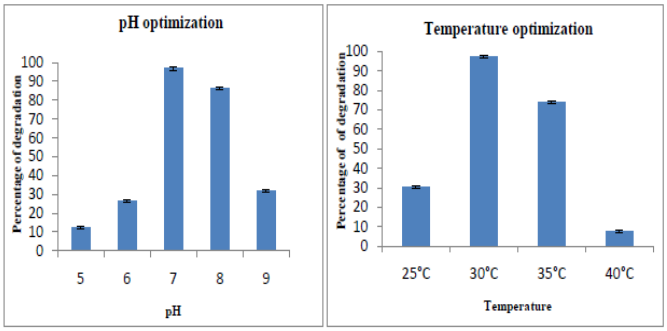

Fig. 2 Optimization of pH and temperature: optimum pH was pH 7 and temperature was 30°C

The FTIR spectra gives a picture of structural changes occurred in CP after treatment with bacterial consortium (Fig. 3). The IR spectrum of CP showed band at The absorption bands of CP were located between 1549 to 968 cm^{-1}due to C=N stretching (1660 cm^{-1}), pyridine stretching, ring vibration, ring breathing, C-Cl stretching (850-500 cm^{-1}), trigonal ring breathing and P=S stretching (750-535 cm^{-1}) [9]. During the biodegradation of CP, the IR peaks of C-Cl and P=S stretching were dropped and from that it could be concluded that the cleavage of C-Cl and P=S bonds occurred and chlorine was further converted. And also the IR spectrum of CP, such as 675, 744, 831, 1168 and 1548cm^{-1}bands, disappeared in the chlorpyrifos degraded sample.

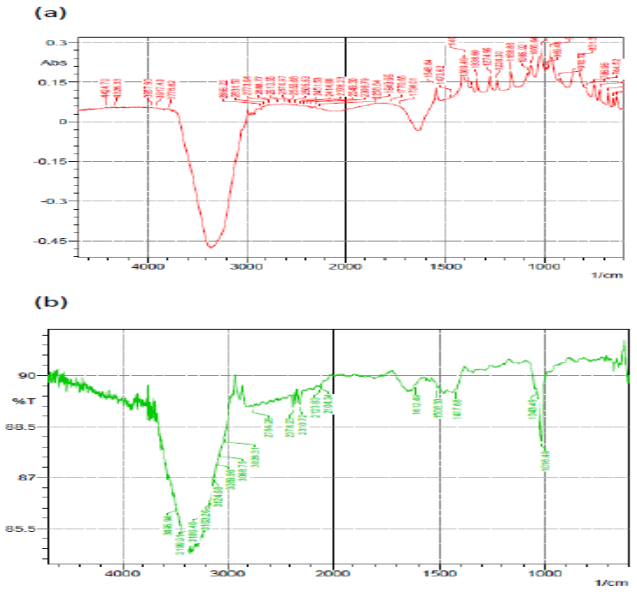

Fig. 3 FTIR spectra of (a) Standard CP and (b) treated CP pesticide

Based on the GC-MS analysis, the parent chlorpyrifos (m/z=351) was metabolized to produce TCP (m/z=197) a major byproduct of CP degradation. Previous researchers have reported that the removal of CP resulted in the formation of metabolites like CP-oxon, 3,5,6-trichloro-2-methoxypyridine, 2-chloro-6-hydroxypyridine [10, 11] and TCP, which was the major degradation product accumulated in pure cultures, water and soils [12, 13]. Peng Lu et al., [14] has reported that CP was degraded to TCP and the TCP degradation results in 2-pyridinol.

In the enhancement study of CP degradation, the consortium as free cells in the medium was able to degrade CP equally with and without N: P: K amendment. The consortium adhered to coir pith showed reduced rate of degradation (62%), but when amended with N: P: K the rate of degradation was found to be increased (81.53%). Similarly immobilized beads amended with N: P: K could give higher degradation rate than beads without N: P: K. When the immobilized consortium was amended with N: P: K, the percentage of degradation was calculated as 97.81%. Tortella et al., [15] have demonstrated that the biostimulation of the biomix with low NPK concentration (0.1% and 0.5%) has a significant effect during the first 10 days of incubation on the CP degradation. Thus it could be concluded that N: P: K fertilizer application on consortium increases the rate of degradation.

Sodium alginate immobilized consortium was found to enhance degradation on HPLC analysis. A possible reason for this could be that the alginate beads allow optimal diffusion of contaminants and as because the support will be acting as a protective barrier against the detrimental changes in the medium, also enhancing the degradation ability of the cells [16, 17]. For instance, a *Pseudomonas* strain immobilized in calcium alginate mineralized a 50% more of phenol than free cells under the same conditions [18]. Also, Yanez-Ocampo et al., [19] studied the removal of two organophosphate pesticides by a bacterial consortium, and they obtained a percentage of methyl parathion removed 31% higher when the consortium was immobilized in alginate beads, compared with a suspension culture.

IV. Conclusion

Our results reveal that the consortium C2, of two different strains of *Pseudomonas aeruginosa* (S5 and S12) and *Klebsiella* sp, could be used in mixed cultures and in immobilized systems as a potential tool for remediation of environments contaminated with CP pesticide. In conclusion, the obtained results demonstrated that the biomix prepared with immobilized cells and biostimulated with NPK nutrient can be recommended in biobeds as a viable alternative of chlorpyrifos dissipation avoiding soil and water contamination probability.

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