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Bioremediation of Chlorpyrifos-contaminated Soil by Exopolysaccharide, Surfactant and Biofilm Synthesising Plant Growth Promoting Rhizobacteria

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This research paper aims to investigate the ability of plant growth promoting rhizobacteria, Bacillus tropicus to degrade chlorpyrifos in soil. Plant growth promoting rhizobacteria (PGPR) have the ability to degrade various xenobiotic compounds, including pesticides and enhance plant growth. The bacterial isolate DK5 identified as Bacillus tropicus, showed biofilm production, exopolysaccharide synthesis and surfactant analysis under abiotic stress. Chlorpyrifos degradation by DK5 was examined using liquid phase extraction followed by HPLC. In HPLC analysis, DK5 degraded 96.1% of chlorpyrifos within 30 days under laboratory conditions. DK5 can be used for remediation of chlorpyrifos form pesticide contaminated soil. The inoculation of DK5 in pesticide contaminated soil can be a promising bioremediation technique for chlorpyrifos removal.

Keywords: Abiotic-stress; Biofilm; Chlorpyrifos; HPLC; Exopolysaccharide; PGPR; Remediation; Surfactant.

Chlorpyrifos is an organophosphorus pesticide used as a broad-spectrum insecticide in various agricultural and non-agricultural conditions[1]. Chlorpyrifos is a persistent pesticide that contaminates soil and water, harms non-target organisms, disrupts soil biodiversity, and bioaccumulates through the food chain. Its residues can damage soil health and lead to pest resistance, posing long-term environmental risks. Extensive use of chlorpyrifos adversely affects the environment and human health, inhibiting the activity of acetylcholinesterase enzyme in nervous system, leading to the accumulation of acetylcholine, overstimulation of nerve cells, paralysis and death[2]. Now a days, researchers have been exploring eco-friendly solutions to mitigate the adverse effects of pesticides. Plant growth-promoting rhizobacteria along with plant growth promotion have the ability to degrade a wide range of xenobiotic compounds[3]. Interaction of PGPR and plants enhances the degradation of chlorpyrifos in soil. The production of organophosphorus hydrolase enzyme by PGPR converts chlorpyrifos into its metabolites, 3, 5, 6-trichloro-2-pyridinol (TCP) and diethylthiophosphate (DETP)[4]. These metabolites are further metabolized by other enzymes, such as monooxygenases, dehydrogenases and dehalogenases into non-toxic compounds that can be utilized by other microorganisms present in the soil[5].

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Bacillus tropicus and related PGPR strains play a dual role in remediating chlorpyrifos-contaminated soils and supporting plant growth. Their enzymatic breakdown of chlorpyrifos, combined with nutrient cycling, stress tolerance, and growth promotion, makes them valuable allies for sustainable agriculture and soil health restoration. Exopolysaccharides produced by PGPR enhance plant tolerance towards pesticides by acting as chelating agents, sequestering and detoxifying harmful chemicals[6]. Additionally, surfactants secreted by PGPR promote the degradation of pesticides and reduce their toxic effects on plants. Furthermore, biofilm synthesis by PGPR offer a protective shield against pesticide-induced stress, preventing their entry into plant tissues. These findings highlight the potential of PGPR synthesizing exopolysaccharide, surfactant and biofilm as promising biocontrol agents for sustainable agriculture in pesticide-contaminated environments[7]. Maize inoculation with PGPR resulted in plant growth promotion with a decreased chlorpyrifos residue in plant tissues[8]. In this study PGPR, DK5 was examined for synthesizing exopolysaccharide, surfactant, biofilm and ability to degrade chlorpyrifos in spiked soil.

MATERIALS AND METHODS

The bacterial isolate DK5 identified as *Bacillus tropicus*, was isolated from pesticide contaminated soil by enrichment method. Commercial-grade chlorpyrifos was obtained from a local market in Kurukshetra. The reference material was procured from Sigma-Aldrich, India. HPLC grade solvents and water were purchased from HiMedia.

Biofilm production

The overnight grown DK5 bacteria isolate was transferred to a 96-well plate containing different concentrations of pesticides (P1 (20 µg/ml), P2 (40 µg/ml) and P3 (100 µg/ml))and incubated for 24 hr at 30 !. After incubation, the wells were washed with sterile phosphate-buffered saline to remove the unattached bacteria. Bacterial isolates adhering to the wall were stained with 0.1% crystal violet solution and incubated for 10 minutes. After incubation 200 µL of 95% ethanol was added to each well to dissolve the crystal violet bound to the biofilm. The biofilm synthesis was estimated by calculating the optical density of crystal violet-stained biofilm at 490 nm by using a microplate reader[9].

Exopolysaccharide synthesis

The overnight grown bacterial isolate DK5 in presence of different concentrations of pesticides (P1 (20 µg/ml), P2 (40 µg/ml) and P3 (100 µg/ml)) was centrifuged at 8000 rpm. The cell-free supernatant of DK5 was mixed with equal volume of ice-cold ethanol and incubated for 24 hr at 4 °C. The mixture was centrifuged for 15 minutes, followed by the addition of an equal volume of 5 % phenol and concentrated sulfuric acid. The solution was kept at room temperature for 30 minutes. The mixture was centrifuged and pellet was washed with ethanol before drying at 60 °C. Pellet was suspended in ethanol and absorbance of mixture was taken at 490 nm by using a spectrophotometer[10].

Surfactant analysis

The overnight grown bacterial isolate DK5 in presence of different concentrations of pesticides (P1 (20 µg/ml), P2 (40 µg/ml) and P3 (100 µg/ml)) was treated with dichloromethane for extraction of surfactant. The extracted surfactant was dried with anhydrous sodium sulphate and concentrated using a rotary evaporator. The extract was dissolved in small amount of methanol and spotted on TLC plate coated with silica gel[11]. The absorbance of dissolved extract was taken at 540 nm by using a spectrophotometer.

Ex-situ chlorpyrifos degradation

The 120g soil was airdried and autoclaved; soil sample was then spiked with chlorpyrifos (35 ppm g^{-1}) and inoculated with bacterial isolate DK5 (10^8 CFU g^{-1}). The soil samples were incubated at 30 ! with 45 % water-holding capacity in dark conditions. Then 20 g soil samples were drawn at an interval of 0, 10, 20 and 30 days. The soil samples were treated with 0.25 ml of 25 % ammonia solution, 0.5 g activated charcoal and 0.5 g florisil and packed in the column. The solvent (1:1 ethyl acetate: acetonitrile) was passed through the column. The eluted solvent was dried and reconstituted in 20 µl of solvent for HPLC analysis. HPLC for detection of chlorpyrifos a mobile phase of Acetonitrile–ultrapure water(50:50 v/v) at a flow rate of 1.2 mL/min, 20 ìL of the sample was injected at 30°C and detected at 254nm. The pesticide degradation was estimated by considering the area

under the peak specified for chlorpyrifos. Removal efficiency of pesticides by was calculated by using formula. Where, (R %, removal percentage), ($A_{0,}$ peak area of under pesticide residue) and (A, peak area of 100 µg/ml concentration of pesticide)[12].

$$R\% = A - A_0 / A \times 100$$

RESULTS AND DISCUSSION

Biofilm, exopolysaccharide and biosurfactant production by bacterial isolate DK5

The biofilm, exopolysaccharide and biosurfactant production by DK5 is shown in figure 1. Biofilm formation is a complex and adaptive mechanism used by many bacteria to protect themselves in challenging environments[13]. Biofilm synthesis by bacterial isolate DK5 exponentially increase with rise in the concentration of chlorpyrifos. The maximum synthesis of biofilm by DK5 was found at 100 µg/ml concentration of chlorpyrifos. This suggests the resilience and adaptation to of DK5 towards environmental stress caused by the pesticide. The biofilm produced by PGPR helps to colonize plant roots, which lead to improved nutrient uptake, increased plant growth, and enhanced resistance to diseases and pests[14].

The maximum synthesis of EPS by DK5 was 213.47 µg/ml at 100 µg/ml concentration of chlorpyrifos. EPS provides structural integrity to bacterial communities, serves as a protective barrier against adverse environmental conditions. The increased EPS production was found in the presence of pollutants[15]. PGPR can promote plant growth, enhance plant tolerance to biotic and abiotic stresses and improve soil structure with water-holding capacity[16].

Qualitative analysis of biosurfactant was analysed by spot inoculating all the samples on TLC labelled as c, p1, p2 and p3. The surfactant

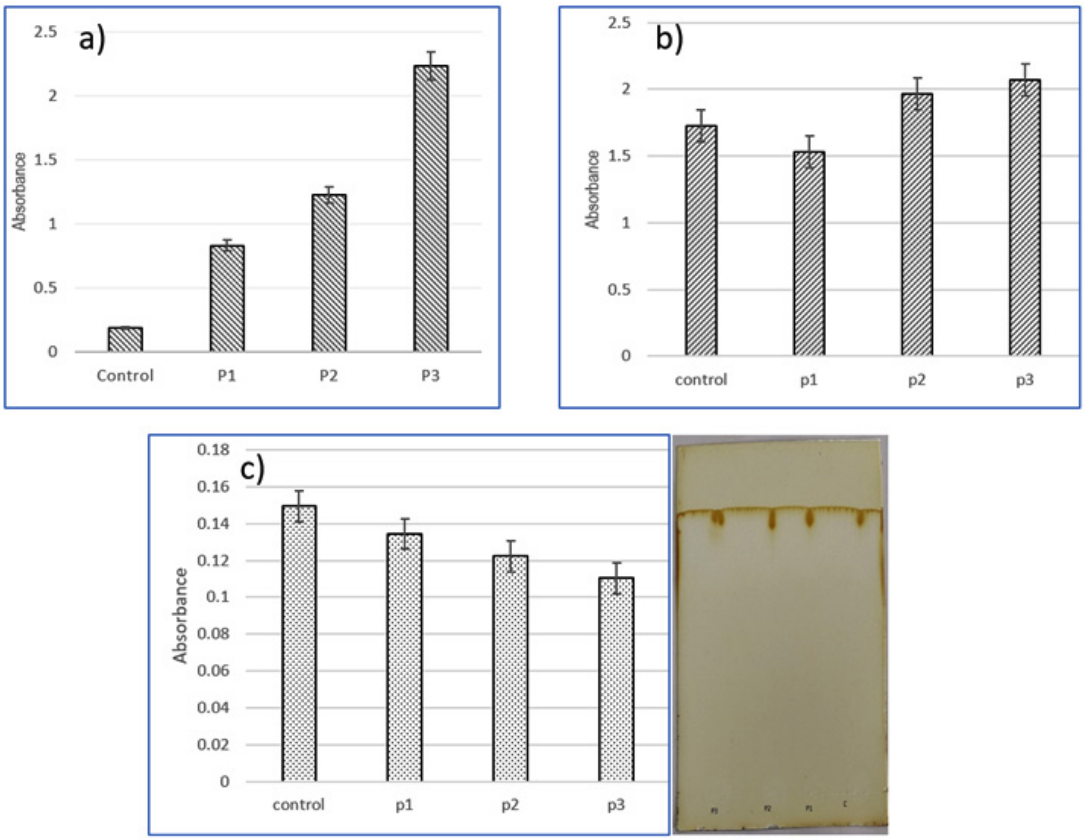

Fig. 1. a) Biofilm, b) EPS and c) biosurfactant production by DK5 at P1 (20 µg/ml), P2 (40 µg/ml) and P3 (100 µg/ml) concentration of chlorpyrifos

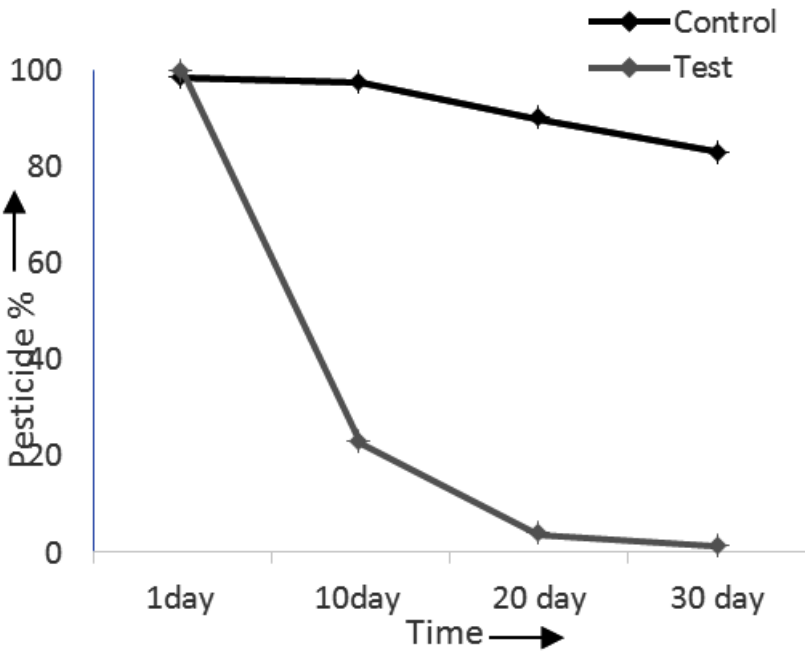

Fig. 2. Chlorpyrifos degradation in soil by DK5

synthesis by DK5 was slightly decline with the increase concentration of chlorpyrifos. The biosurfactants produced by *Pseudomonas aeruginosa* increased the solubility of chlorpyrifos in water and facilitated its breakdown, leading to a reduction in its persistence in the environment[17]. Biosurfactants also improves the plant growth and reduces the toxicity of pesticides towards plants by forming micelles[18].

In-situ chlorpyrifos degradation

In laboratory experiment, DK5 was showing higher rate of chlorphrifos degradation in soil. In HPLC analysis, after 30 days of incubation 96.1% chlorpyrifos was degraded by DK5 as shown in figure 2. In control only 16.33 % chlorpyrifos degradation was observed. In previous study a *Bacillus* strain was able to remove 68.14% of 30 mg L-1 chlorpyrifos in 96 h[23]. These results suggest that the inoculation of PGPR, DK5 into soil enhance the degradation of chlorpyrifos. Inoculation of PGPR reduce the harmful effects of pesticides on the environment[19]. *Bacillus* species helps in the degradation of various organic pollutants, including chlorpyrifos[20].

The unintentional adverse effects of chlorpyrifos breakdown by *Bacillus tropicus* include 3,5,6-Trichloro-2-pyridinol (TCP), an environmentally stable and moderately toxic chemical that has a negative impact on terrestrial and marine ecosystems. Other metabolites that appear are Diethyl Thiophosphate (DETP) and Diethyl Phosphate (DEP); though less lethal, they may bioaccumulate if not completely eliminated[21]. The inoculation of PGPR into soil significantly enhanced the degradation of chlorpyrifos and alter the soil microbial community composition[22].

The ability of DK5 to synthesize biofilm, EPS, and biosurfactants in the presence of chlorpyrifos indicates its potential to enhance plant growth, improve soil health, and reduce pesticide toxicity. These findings highlight the promising role of DK5 as a potential biocontrol agent for mitigating the adverse impacts of chlorpyrifos in agriculture. Further field studies are necessary to explore the practical application of DK5 as a PGPR and its long-term effects on soil quality, crop productivity and environmental sustainability.

CONCLUSION

In conclusion, this study demonstrates the potential of the Bacillus tropicus strain

DK5 as a promising biocontrol agent for mitigating the environmental and agricultural impacts of chlorpyrifos. Biosurfactants and exopolysaccharides synthesized by DK5 help in the growth of plants and concurrently aid in the degradation of chlorpyrifos in the soil. The result demonstrates that 96.1% of chlorpyrifos could be degraded after incubation for 30 days. Therefore, it has been proved that DK5 reduces pesticide residues and enhances the health of soils. Results show a crucial role for DK5 in sustainable agriculture. Soil improvements of microbial communities together with reduced pesticide toxicity will promote the use of such a microbial synergist. However, more field studies must be performed under real agricultural conditions to validate its long-term efficacy as well as potential adverse environmental impacts.

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This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration

This research does not involve any clinical trials.

Author Contributions

Deepak Kumar Malik: Conceptualization, Methodology, Writing – Original Draft; Vivek Singh: Data Collection, Analysis, Writing – Review & Editing; Rajesh Agnihotri: Visualization; Meenu Rathi: Resources and Supervision

REFERENCES

1. Afzal, M., Khan, Q. M., and Sessitsch, A, Endophytic bacteria: prospects and applications for the phytoremediation of organic pollutants. *Chemosphere*, 2014; 117, 232-242.
2. Polla, W. M., Regaldo, L., Reno, U., Popielarz, A., Gervasio, S., Fernández, V., and Gagneten, A. M., Effect of a glyphosate formulation on freshwater plankton: A community combined metric approach. *Journal of Environmental Biology*, 2022; 43, 35-42.
3. Sehrawat, A., Phour, M., Kumar, R., and Sindhu, S. S., Bioremediation of pesticides: an eco-friendly approach for environment sustainability. *Microbial Rejuvenation of Polluted Environment:* 2021; 1, 23-84.
4. Egamberdieva, D., Wirth, S. J., Behrendt, U., and Ahmad, P, Interaction between phosphate-solubilizing bacteria and Rhizobium strains for improving growth and nutrient uptake of chickpea (*Cicer arietinum* L.). *Journal of plant growth regulation*, 2017; 36, 608-617.
5. Bose, S., Kumar, P. S., and Vo, D. V. N., A review on the microbial degradation of chlorpyrifos and its metabolite TCP. *Chemosphere*, 2021; 283, 131447.
6. Ajijah, N., Fiodor, A., Pandey, A. K., Rana, A., and Pranaw, K., Plant Growth-Promoting Bacteria (PGPB) with Biofilm-Forming Ability: A Multifaceted Agent for Sustainable Agriculture. *Diversity*, 2023; 15(1), 112.
7. Singh, A., and Chauhan, P. S., Ecological significance of soil associated plant growth–promoting biofilm forming microbes for stress management. *Biofilms in plant and soil health*, 2017; 291-326.
8. Saeed, Q., Xiukang, W., Haider, F. U., Kuèerik, J., Mumtaz, M. Z., Holatko, J., and Mustafa, A., Rhizosphere bacteria in plant growth promotion, biocontrol, and bioremediation of contaminated sites: A comprehensive review of effects and mechanisms. *International Journal of Molecular Sciences*, 2021; 22, 10529.
9. Waters, E. M., McCarthy, H., Hogan, S., Zapotoczna, M., O'Neill, E., and O'Gara, J. P., Rapid quantitative and qualitative analysis of biofilm production by *Staphylococcus epidermidis* under static growth conditions. *Staphylococcus Epidermidis: Methods and Protocols*, 2014; 157-166.

10. Gani, G., Asif, M., Wani, P. A., Malik, M. A., Dar, Z. M., Masood, A., and Shafi, S., Chlorpyrifos degradation, biocontrol potential and antioxidant defence activation under pesticide stress by rhizosphere bacteria isolated from rhizosphere of peach (*Prunus persica*) plants. *Chemistry and Ecology*, 2021; 37, 866-881.
11. Parthipan, P., Preetham, E., Machuca, L. L., Rahman, P. K., Murugan, K., and Rajasekar, A., Biosurfactant and degradative enzymes mediated crude oil degradation by bacterium*Bacillus subtilis* A1. *Frontiers in microbiology*, 2017; 8, 193.
12. Nasiri, A., Amirahmadi, M., Mousavi, Z., Shoeibi, S., Khajeamiri, A., and Kobarfard, F., A multi residue GC-MS method for determination of 12 pesticides in cucumber. *Iranian Journal of Pharmaceutical Research*: IJPR, 2016; 15, 809.
13. Ozma, M. A., Khodadadi, E., Rezaee, M. A., Kamounah, F. S., Asgharzadeh, M., Ganbarov, K., and Kafil, H. S., Induction of proteome changes involved in biofilm formation of *Enterococcus faecalis* in response to gentamicin. *Microbial Pathogenesis*, 2021; 157, 105003.
14. Santoyo, G., Urtis-Flores, C. A., Loeza-Lara, P. D., Orozco-Mosqueda, M. D. C., and Glick, B. R., Rhizosphere colonization determinants by plant growth-promoting rhizobacteria (PGPR). *Biology*, 2021; 10, 475.
15. Pompilio, A., Scribano, D., Sarshar, M., Di Bonaventura, G., Palamara, A. T., and Ambrosi, C., Gram-negative bacteria holding together in a biofilm: the *Acinetobacter baumannii* way. *Microorganisms*, 2021; 9(7), 1353.
16. Morcillo, R. J., and Manzanera, M., The effects of plant-associated bacterial exopolysaccharides on plant abiotic stress tolerance. *Metabolites*, 2021; 11, 337.
17. Gilani, R. A., Rafique, M., Rehman, A., Munis, M. F. H., Rehman, S. U., and Chaudhary, H. J., Biodegradation of chlorpyrifos by bacterial genus *Pseudomonas*. *Journal of basic microbiology*, 2016; 56, 105-119.
18. Karamchandani, B. M., Pawar, A. A., Pawar, S. S., Syed, S., Mone, N. S., Dalvi, S. G., and Satpute, S. K., Biosurfactants' multifarious functional potential for sustainable agricultural practices. *Frontiers in bioengineering and biotechnology*, 2022; 10, 1-20.
19. Li, Q., Wang, P., Zou, C., Ge, F., Li, F., Liu, Y., and Tian, J., Dynamics of dominant rhizospheric microbial communities responsible for trichlorfon absorption and translocation in maize seedlings. *Journal of Hazardous Materials*, 2023; 451, 131096.
20. Bhandari, S., Poudel, D. K., Marahatha, R., Dawadi, S., Khadayat, K., Phuyal, S., and Parajuli, N., Microbial enzymes used in bioremediation. *Journal of Chemistry*, 2021, 1-17.
21. Bose, S., Kumar, P., & Vo, D. (2021). A review on the microbial degradation of chlorpyrifos and its metabolite TCP.. *Chemosphere*, 283, 13(1),447 .
22. Jaiswal, D. K., Verma, J. P., Krishna, R., Gaurav, A. K., and Yadav, J., Molecular characterization of monocrotophos and chlorpyrifos tolerant bacterial strain for enhancing seed germination of vegetable crops. *Chemosphere*, 2019; 223, 636-650.
23. Suryono, C. A., Subagiyo, & Sabdono, A. (2021). Chlorpyrifos pesticide-degrading bacteria isolated from bivalves of Surabaya coastal waters, East Java, Indonesia.