

**DEVELOPMENT OF MICROBIAL FORMULATIONS FOR THE  
BIOREMEDIATION OF SOIL CONTAMINATED WITH  
EXPLOSIVES AND CHEMICAL PESTICIDES**

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*by*

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## CERTIFICATE

This is to certify that the thesis entitled “**Development of microbial formulations for the bioremediation of soil contaminated with explosives and chemical pesticides**”, being submitted by **Ms. Sonal Yadav** to the **Indian Institute of Technology Delhi** for the award of “**Doctor of Philosophy**” is a record of bonafide research work carried out by her. She has worked under our guidance and supervision and has fulfilled the requirements for the submission of this thesis. To the best of our knowledge, the results contained in this thesis have not been submitted in part or full to any other university or institute for the award of any degree or diploma.



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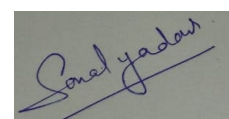
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Sonal Yadav

## ABSTRACT

In this industrialized world, soil contamination is the major concern to environment. Xenobiotic compounds like nitroaromatics, organohosphates are the main source of polluting soil ecosystem as their residues accumulate in soil for prolonged period. Therefore, it is necessary to remediate such contaminants from natural environment to restore the ecosystem. Bioremediation is the process to decontaminate and mineralize the contaminants using biological agents like bacteria, fungi or their enzymes to obtain sustainable environment. Present study is an attempt to develop a suitable strategy for bioremediation of soil contaminated by explosives and chemical pesticides.

This study focuses on the use of indigenous bacterial isolates (obtained from explosive contaminated sites) immobilized to develop novel bioformulations, for achieving the remediation goals. Seven bacterial isolates, namely, *Bacillus oceanisediminis*, *Dydobacter jiangsuensis*, *Dienococcus misasensis*, *Arthrobacter subterraneus*, *Janibacter cremeus*, *Pseudomonas entomophila* and *Microbacterium esteraromaticum* were kindly provided by Centre for Fire, Explosive and Environment Safety, Defence Research and Development Organisation (CFEES, DRDO) New Delhi (identified by Institute of Microbial Technology, IMTECH, Chandigarh). Initially the interaction study among all bacterial isolates was carried out to check their compatibility among themselves. The results obtained indicated that there was no positive interaction among isolates indicating no compatibility with each other hence all studies were carried out using monoculture system instead of their consortium.

After that these isolates were subjected for examining their bioremedial potential by growing them in presence of Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and *Microbacterium esteraromaticum* MTCC 12849, gram-positive, aerobic bacterium was found as an efficient degrader. The strain detoxified 72.6% of 30ppm RDX in minimal nutrient

medium in 240 h and in soil, 63.93% of 30 mg RDX/kg of soil degradation was exhibited with viable cell count of  $3.2 \times 10^4$  cfu/g within 30 days of incubation.

Subsequently, the different potent strains were formulated in water-dispersible granules (WDG), talcum/charcoal based powder and alginate beads formulations. Developed WDG with the 90% of inert material with the active ingredient in ratio of 1:2, 2% acacia gum as good binder and 8% alginic acid as an efficient dispersing agent gave best results. The tested properties of WDG fulfilled the guidelines of Collaborative International Pesticides Analytical Council (CIPAC). Shelf life of WDG formulation was found to be 120 days when stored at 30°C, however at 45°C the bacterial growth was not observed. At low temperature (4°C) storage condition, the viability of the bacterial isolates in different formulation was found to be quite stable with the loss of only one log unit at the end of 180 days.

Among all developed microbial formulations, WDG showed best degrading efficiency, and formulated *M. esteraromaticum* exhibited highest degradation. However, a mesocosm study of RDX in soil biocolumn reactor indicated 9.88% increment in RDX degradation through developed *M. esteraromaticum* WDG i.e. 73.2%. with the subsequent formation of intermediates N-methyl-N, N'-dinitromethanediamine, and methylenedintramine detected through LCMS analysis, in soil during the RDX degradation. Interestingly, no significant difference was observed in the rate of RDX degradation due to the formulation process. The first-order kinetics was seen in RDX degradation with a degradation coefficient of 0.04 and  $0.0339 \text{ day}^{-1}$  by formulated and unformulated strain, respectively. The current investigation implies *M. esteraromaticum* as a potential microbe for RDX degradation and opens up the possibility of exploiting it in its effective WDG form for the remediation of explosive contaminated sites.

Similar studies were conducted for another contaminant i.e. Chlorpyrifos (O, O-diethyl O-3, 5, 6-trichloropyridin-2-yl phosphorothioate), a toxic and chlorinated organic contaminant in soil across the globe. The qualitative testing of all bacterial isolates against CP degradative ability on selective media showed that majorly three bacteria, *D. jiangsuensis*, *M. esteraromaticum* and *D. misasensis* exhibited change in colour indicating the capability of strains to degrade CP. The effect of chlorpyrifos on bacterial growth was checked and among all three selected strains, the proliferation of *D. jiangsuensis* was highest signifying the ability of this strain to utilize CP as a sole carbon source and also confirmed the utilization of 3,5,6-trichloro-2-pyridinyl (TCP) through silver nitrate assay.

*D. jiangsuensis* degraded 80.36 and 76.93% chlorpyrifos (CP) in aqueous medium and soil environment, respectively. The trend observed in organophosphorus hydrolase (OPH) activity exposed its localization extracellularly, with highest activity noted in *D. jiangsuensis*, which were in concurrent with the degradation results. The *D. jiangsuensis* WDG achieved 21.13% enhanced CP degradation in soil under microcosm condition as compared to the unformulated one on 15th day of the treatment. The intermediate metabolites namely 3,5,6-trichloro-2-pyridinol (TCP), tetrahydropyridine, thiophosphate and phenol, 1, 3-bis (1,1-dimethylethyl) were detected during the CP degradation.

The current investigation reveals *D. jiangsuensis* as a potential microbe for CP degradation and opens up the possibility of exploiting its formulations to remediate the CP polluted soils. In this study, *D. jiangsuensis* proved as an efficient degrader of chlorpyrifos in soil environment. In *in planta* study, it was notable that WDG of *D. jiangsuensis* was most efficient in enhancing the growth parameters of tomato plants. In pot study, *D. jiangsuensis* WDG showed 25.7% increment in chl a and 59.3 % increase in chl b (total chlorophyll content

by 35.84%) in comparison to the control. Also, there was increase of 17.33 and 41.28% in height and fresh weight respectively of tomato plant over control.

The present finding identified a sustainable and environmental friendly technology package for bioremediation of soil contaminated by chemical pesticides and explosives. The use of microbes in formulated form aids in degradation efficacy and enhanced the plant growth.

## सारांश

इस औद्योगिकृत दुनिया में, मिट्टी का प्रदूषण पर्यावरण के लिए प्रमुख चिंता का विषय है। नाइट्रोएरोमैटिक्स, ऑर्गनोफॉस्फेट जैसे ज़ेनोबायोटिक यौगिक मिट्टी के पारिस्थितिकी तंत्र को प्रदूषित करने का मुख्य स्रोत हैं क्योंकि उनके अवशेष मिट्टी में लंबे समय तक जमा रहते हैं। इसलिए, पारिस्थितिकी तंत्र को बहाल करने के लिए ऐसे दूषित पदार्थों को प्राकृतिक पर्यावरण से हटाना आवश्यक है। बायोरेमेडिएशन, सतत स्वच्छ वातावरण प्राप्त करने के लिए बैक्टीरिया, कवक या उनके एंजाइम जैसे जैविक एजेंटों का उपयोग करके दूषित पदार्थों को विसंदूषित एक करने की प्रक्रिया है। वर्तमान अध्ययन विस्फोटकों और रासायनिक कीटनाशकों से दूषित मिट्टी के जैव उपचार के लिए एक उपयुक्त रणनीति विकसित करने का एक प्रयास है।

यह अध्ययन उपचारात्मक लक्ष्यों को प्राप्त करने के लिए, नए बायोफॉर्म्यूलेशन विकसित करने के लिए स्थिर स्वदेशी बैक्टीरियल आइसोलेट्स (विस्फोटक दूषित साइटों से प्राप्त) के उपयोग पर केंद्रित है। DRDO-CFEES, नई दिल्ली (IMTECH, चंडीगढ़ द्वारा पहचाने गए) द्वारा सात बैक्टीरियल आइसोलेट्स, जैसे की, बैसिलस ओशनाइज़िडमिनिस, डाइडोबैक्टर जियांगसुएन्सिस, डायनोकोकस मिसेन्सिस, आर्थ्रोबैक्टर सबट्रेनियस, जेनिबैक्टर क्रीमियस, स्यूडोमोनास एंटोमोफिला और माइक्रोबैक्टीरियम एस्टरोमैटिकम प्रदान किए गए थे। प्रारंभ में सभी बैक्टीरियल आइसोलेट्स के बीच परस्पर क्रिया का अध्ययन किया गया ताकि आपस में उनकी अनुकूलता की जांच की जा सके। प्राप्त परिणामों ने संकेत दिया कि आइसोलेट्स के बीच कोई सकारात्मक परस्परक्रिया नहीं थी जो एक दूसरे के साथ कोई अनुकूलता नहीं दर्शाती थी इसलिए सभी अध्ययन उनके संघ के बजाय मोनोकल्चर प्रणाली का उपयोग करके किए गए थे।

इसके अलावा, हमने इन आइसोलेट्स की हेक्साहाइड्रो-1,3,5-ट्राइनाइट्रो-1,3,5-ट्राएज़िन (आरडीएक्स) की उपस्थिति में उनके दूषित मिट्टी के उपचार क्षमता की जांच की और जांच के दौरान, माइक्रोबैक्टीरियम एस्टरोमैटिकम एमटीसीसी 12849, ग्राम-पॉजिटिव, एरोबिक जीवाणु को एक कुशल डिग्रेडर के रूप में पाया। इस स्ट्रेन ने 240 घंटे में न्यूनतम पोषक माध्यम में 30 ppm आरडीएक्स का 72.6% और मिट्टी में, 30 मिलीग्राम आरडीएक्स/किलोग्राम मिट्टी के 63.93% क्षरण को ऊष्मायन के 30 दिनों के भीतर प्रदर्शित किया।

उसके बाद विभिन्न बैक्टीरिया फॉर्मूलेशन जैसे की डब्ल्यूडीजी, टैल्कम और चारकोल आधारित पाउडर फॉर्मूलेशन और एल्लिगेट मोतियों के रूप में तैयार किए गए। 1:2 के अनुपात में सक्रिय संघटक के साथ 90% अक्रिय सामग्री, अच्छे बाइंडर के रूप में 2% बबूल का गोंद और एक कुशल फैलाव एजेंट के रूप में 8% एल्लिगनिक एसिड से विकसित डब्ल्यूडीजी ने सर्वोत्तम परिणाम दिए। डब्ल्यूडीजी के परीक्षण किए गए गुणों ने सहयोगात्मक अंतर्राष्ट्रीय कीटनाशक विश्लेषणात्मक परिषद (CIPAC) के दिशानिर्देशों को पूरा किया। डब्ल्यूडीजी फॉर्मूलेशन जब 30°C पर संग्रहीत किया गया तब उसका शेल्फ

जीवन 120 दिनों का पाया गया, हालांकि 45°C पर बैक्टीरिया की वृद्धि नहीं देखी गई। कम तापमान (4°C) भंडारण की स्थिति में, अलग-अलग फॉर्मूलेशन में बैक्टीरियल आइसोलेट्स की व्यवहार्यता 180 दिनों के अंत में केवल एक लॉग यूनिट के नुकसान के साथ स्थिर पाई गई।

सभी विकसित माइक्रोबियल फॉर्मूलेशन के बीच, डब्ल्यूडीजी ने सबसे अच्छी अपघटन क्षमता दिखाई, हालांकि डब्ल्यूडीजी के मामले में, तैयार किए गए एम.एस्टेरोमैटिकम ने अनफॉर्मलेटेड के विपरीत 7.19% तक उच्चतम अपघटन का प्रदर्शन किया। जबकि मृदा बायोकोलम रिएक्टर में आरडीएक्स के मेसोकोस्म अध्ययन ने विकसित एम. एस्टेरोमैटिकम डब्ल्यूडीजी के माध्यम से आरडीएक्स क्षरण में 9.88% वृद्धि का संकेत दिया यानी 73.2%। मिट्टी में आरडीएक्स क्षरण के दौरान एलसीएमएस विश्लेषण के माध्यम से मध्यवर्ती मेटाबोलाइट्स, एन-मिथाइल-एन, एन'-डिनिट्रोमेथेनेडियम, और मेथिलेंडिनिट्रामाइन को पाया गया। दिलचस्प बात यह है कि निर्माण प्रक्रिया के कारण आरडीएक्स के क्षरण की दर में कोई महत्वपूर्ण अंतर नहीं देखा गया। पहले क्रम के काइनेटिक्स को आरडीएक्स अवक्रमण में क्रमशः 0.04 और 0.0339 हर दिन के अवक्रमण गुणांक के साथ सूत्रबद्ध और असंगठित तनाव द्वारा देखा गया था। वर्तमान जांच में आरडीएक्स क्षरण के लिए संभावित सूक्ष्म जीव के रूप में एम. एस्टेरोमैटिकम सक्षम है और विस्फोटक दूषित साइटों के लिए इसके प्रभावी डब्ल्यूडीजी रूप में इसके उपयोग की संभावना को खोलता है।

इसी तरह के अध्ययन एक अन्य संदूषक यानी क्लोरपाइरीफोस (ओ, ओ-डायथाइल ओ-3, 5, 6-ट्राइक्लोरोपाइरिडिन-2-वाइएल फॉस्फोरोथियोएट) के लिए किए गए थे, जो दुनिया भर में मिट्टी में एक विषाक्त और क्लोरीनयुक्त कार्बनिक संदूषक है। चयनात्मक मीडिया पर क्लोरपाइरीफोस (सीपी) अपघटन क्षमता के लिए सभी बैक्टीरिया के गुणात्मक परीक्षण से पता चला है कि प्रमुख रूप से तीन बैक्टीरिया, डी. जियांगसुएन्सिस, एम. एस्टेरोमैटिकम और डी. मिसासैंसिस ने रंग में परिवर्तन का प्रदर्शन किया, जो सीपी का अपघटन करने की उपभेदों की क्षमता को दर्शाता है। बैक्टीरिया के विकास पर क्लोरपाइरीफोस के प्रभाव की जांच की गई और सभी तीन चयनित उपभेदों में, डी. जियांगसुएन्सिस का प्रसार सीपी को एकमात्र कार्बन स्रोत के रूप में उपयोग करने की क्षमता को दर्शाता है और सिल्वर नाइट्रेट परख के माध्यम से 3,5,6-ट्राइक्लोरो-2-पाइरिडिनिल (टीसीपी) के उपयोग की भी पुष्टि करता है

डी. जियांगसुएन्सिस ने जलीय माध्यम और मिट्टी के वातावरण में क्रमशः 80.36% और 76.93% क्लोरपाइरीफोस (सीपी) का क्षरण किया। ऑर्गनोफॉस्फोरस हाइड्रॉलेज़ (ओपीएच) गतिविधि में देखी गई प्रवृत्ति ने इसके स्थानीयकरण को अतिरिक्त रूप से उजागर किया, जिसमें डी. जियांगसुएन्सिस में उच्चतम गतिविधि का उल्लेख किया गया था, जो गिरावट के परिणामों के साथ समवर्ती थे। उपचार के 15वें दिन अनफॉर्मलेटेड की तुलना में डी. जियांगसुएन्सिस डब्ल्यूडीजी ने सूक्ष्म जगत की परिस्थितियों में मिट्टी में 21.13% बढ़ा हुआ सीपी क्षरण हासिल किया। मध्यवर्ती मेटाबोलाइट्स अर्थात् 3,5,6-ट्राइक्लोरो-2-पाइरिडिनोल (टीसीपी), टेट्राहाइड्रोपाइरीडीन,

थियोफॉस्फेट और फिनोल, 1, 3-बीआईएस (1,1-डाइमिथाइलथाइल) सीपी क्षरण के दौरान पाए गए थे। वर्तमान जांच से पता चलता है कि डी. जियांगसुएंसिस सीपी क्षरण के लिए एक संभावित सूक्ष्म जीव के रूप में है और सीपी प्रदूषित मिट्टी को ठीक करने के लिए इसके योगों के दोहन की संभावना को खोलता है। इस अध्ययन में, डी. जियांगसुएंसिस मिट्टी के वातावरण में क्लोरपाइरीफोस के एक कुशल डिग्रेडर के रूप में साबित हुआ। प्लांट अध्ययन में, यह उल्लेखनीय था कि डी. जियांगसुएंसिस के अधिकांश डब्ल्यूडीजी टमाटर के पौधों के विकास मानकों को बढ़ाने में सबसे अधिक कुशल थे। पॉट अध्ययन में, डी. जियांगसुएंसिस डब्ल्यूडीजी ने नियंत्रण कंट्रोल की तुलना में क्लोरोफिल ए में 25.7% वृद्धि और क्लोरोफिल बी में 59.3% की वृद्धि (कुल क्लोरोफिल सामग्री 35.84%) दिखाई। साथ ही, ऊंचाई में 17.33% और 41.28% की वृद्धि हुई और पौधे का ताजा वजन नियंत्रण से अधिक था।

वर्तमान खोज ने रासायनिक कीटनाशकों और विस्फोटकों से दूषित मिट्टी के जैव उपचार के लिए एक स्थायी और पर्यावरण के अनुकूल प्रौद्योगिकी पैकेज की पहचान की। फॉर्मूलेशन के रूप में बैक्टीरिया का उपयोग मिट्टी एवं पाली पानी के वातावरण को शुद्ध करता है एवं पौधे की वृद्धि को भी बढ़ाता है।

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## ABBREVIATIONS

RDX	Royal Demolition Explosive
TNT	Trinitrotoluene
CP	Chlorpyrifos
LCMS	Liquid chromatography–mass spectrometry
US EPA	United States environment protection agency
EPA	Environment protection agency
SSL	Soil screening level
MEDINA	Methylenedinitramine
TCP	3,5,6- trichloro-2-pyridinyl
UXO	Unexploded ordnance
HMX	High Melting Explsoive
HPLC	High performance Liquid Chromatography
NED	N-(1-naphthyl)ethylenediamine
FTIR	Fourier transform infrared spectroscopy
GCMS	Gas chromatography mass spectrophometer
ANNOVA	Analysis of variance
ACC	1-aminocyclopropane-1-carboxylate
CFU	Colony forming unit
Fig.	Figure
MTCC	Microbial type culture collection
DMRT	Duncan’s new multiple range test
EC	Electrical conductivity
PGPR	Plant growth promoting rhizobacteria
MSM	Minimal salt medium
mS/m	Milli siemens/ meter
TLC	Thin layer chromatography
UV/VIS	Ultraviolet-Visible
w/v	Weight/volume
v/v	Volume/volume
mm	Millimeter
mg	Milligram

Kg	Kilogram
mM	Millimolar
M	Molar
IAA	Indole acetic acid
m/z	Mass/charge
d	Days
h	Hour
g	Gram
cm	Centimeter
nm	Nanometer
rpm	Rotations per minute
ppm	Parts per million
mg/L	Milligram per litre
g/L	Gram per litre
mL	Milliliter
°C	Degree centigrade
μl	Microlitre
s	Seconds