

**BIOREMEDIATION OF XENOBIOTICS (RDX AND
CARBOFURAN) POLLUTED SOILS**

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INDIAN INSTITUTE OF TECHNOLOGY DELHI

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CARBOFURAN) POLLUTED SOILS**

by

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Submitted

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CERTIFICATE

This is to certify that the thesis entitled “**Bioremediation of xenobiotics (RDX and Carbofuran) polluted soils**”, being submitted by **Mr. Mohd Aamir Khan** to the **Indian Institute of Technology Delhi** for the award of “**Doctor of Philosophy**” is a record of bonafide research work carried out by him. He 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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*“...And if you would count the graces of Allah,
never could you be able to count them” (14:34)*

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Mohd Aamir Khan

ABSTRACT

Rapid industrialization and urbanization have led to the indiscriminate use of xenobiotics across the world. Xenobiotic compounds like nitramines and carbamates, due to their high recalcitrancy, reside in the environment for longer durations and thus contribute to the pollution of soil and water bodies. Thus, there is an imperative demand for feasible approaches for the removal of these xenobiotics from soil and water for sustainable rejuvenation of the surrounding ecosystem. Bioremediation is the most efficient approach for the clean-up of environmental bodies contaminated with hazardous xenobiotics. Several bacteria, fungi, and their respective metabolic enzymes are prime agents used in the bioremediation process.

Considering all these facts, the present work entitled “Bioremediation of xenobiotics (RDX and Carbofuran) polluted soils” focuses on the rejuvenation of explosives and pesticides contaminated soils using integrated bioaugmentation and biostimulation approach.

The study started with screening native bacterial strains isolated from explosives-contaminated sites for RDX degrading potential in nitrogen-limited minimal salt media. Four different bacterial isolates viz., *Bacillus oceanisediminis*, *Pelomonas aquatica*, *Kinneretiaasachharophila* and *Arthrobacter subterraneus* were initially isolated and identified by CSIR-Institute of Microbial Technology, IMTECH, Chandigarh, and kindly provided by Centre for Fire, Explosive and Environment Safety, Defence Research and Development Organisation (CFEES, DRDO) New Delhi. Of the four microbial isolates, *P. aquatica* and *K. asachharophila* were the most efficient RDX degraders with 82 and 74 % RDX (10 mg l^{-1}) degradation within 240 h of incubation with the highest microbial growth $8.7 \pm 0.52 \times 10^8$ and $3.1 \pm 0.07 \times 10^7 \text{ CFU ml}^{-1}$ respectively. Both *B. oceanisediminis* and *A. subterraneus* were poor RDX degraders with only 32 and 29 % RDX degradation after 240

h, thus left out for further steps. Alongside, six different carbofuran degrading bacterial strains were isolated from contaminated soils and screened for carbofuran degrading potential in minimal salt media. Bacterial isolates, *O. intermedium*, and *B. albus* were found to be the most effective carbofuran degraders, with 75.8 and 58.4 % carbofuran (300 mg l⁻¹) degradation within 120 h of incubation and thus selected for further steps.

Next, the interaction study was conducted between potent RDX (*P. aquatica* and *K. asachharophila*) and Carbofuran (*O. intermedium* and *B. albus*) degrading isolates to check compatibility for consortia development. However, no positive interaction was observed between RDX degraders and carbofuran degraders. Hence, no microbial consortium was developed, and monocultures of the potential RDX and carbofuran degrading strains were used in further studies.

After that, aqueous phase degradation studies were conducted to determine the RDX and carbofuran degradation kinetics and intermediate metabolites. *P. aquatica* and *K. asachharophila* isolated from explosives contaminated sites removed 80 and 75% RDX (30 mg l⁻¹) from MSM I within 240 h. The degradation of RDX was rapid and highly effective with RDX half-lives of 4.17 and 4.89 days with *P. aquatica* and *K. asachharophila* resp. The LCMS analysis of the aqueous extracts confirmed the presence of MNX, MEDINA, NDAB in case of *P. aquatica*, while only MEDINA and NDAB were detected as intermediates with *K. asachharophila*. In aqueous phase degradation of carbofuran, *O. intermedium* and *B. albus* degraded 92 and 68% carbofuran (100 mg l⁻¹) from aqueous media resp. *O. intermedium* showed rapid carbofuran removal with degradation rate constant 0.69 day⁻¹ and half-life of 1 day while a half-life of 29 days was observed in control treatments. GCMS analyses of the aqueous extract confirmed the formation of carbofuran-7-phenol (M.W. 164 Da) as major intermediate metabolite detected at RT of 11.4 min. Other intermediate 1,2-benzenecarboxylic acid, monoethyl ester (194 Da) was detected at RT of 15.2 min.

Followed by this, different potent bacterial strains were formulated into water-dispersible granules (WDG), talcum and charcoal-based dust and encapsulated beads formulation. The developed formulations were further checked for shelf life and contaminant (RDX and carbofuran) degrading potential over a storage period of six months at three different temperatures (4, 30 and 40 °C). The WDGs retained >80 % of initial viability, while a loss of >50 % in initial viable cell counts was recorded in talcum, charcoal and beads formulation after 6 months at 30 °C. However, at 4 °C all the developed formulations remained viably stable till 6 months of storage. The bacterial counts were greatly reduced within 2 months at 40 °C storage temperature.

WDGs of *P. aquatica* and *K. asachharophila* retained 95 and 96 % of the initial RDX degrading efficiency after storage period. While, WDGs of *O. intermedium* retained ~ 94 % carbofuran removing potential at the end of 6 months of storage at 30 °C. The remediating potential of powder and beads formulation was significantly decreased by >15 % within 3 months which further reduced to >50 % at the end of 6 months. Thus, the WDGs were finally selected for soil microcosm studies to study the impact of bioaugmentation and biostimulation on RDX and carbofuran degradation.

P. aquatica WDGs in sucrose amended microcosms showed a 77.57 % RDX removal while a 66.49 % RDX degradation was recorded in microcosms stimulated with wheat straw after one month of storage. The biostimulated microcosms recorded reduced RDX soil half-lives of 13.9 and 19.04 days resp. over control treatment with RDX half-life of 185.5 days. In case of carbofuran degradation in soil microcosms, the *O. intermedium* cells in WDGs stimulated by biogas slurry (BGS) recorded a 95 % carbofuran degradation within 30 days of treatment. The carbofuran half-life in control set was 78.3 days which was critically reduced to 8.04 days in microcosms treated with integrated bioaugmentation and biostimulation approach.

With successful remediation of RDX and carbofuran contaminated soils, the effect of our combinatorial approach was checked on soil and tomato plant health via in-planta assay with carbofuran as soil contaminant. The best treatment with co-application of WDGs and BGS showed significantly similar ($p < 0.5$) plant growth (shoot length, root length, fresh weight, chlorophyll) with control treatments inferring that our combinatorial approach does not produce any deleterious effect on plant growth and subsequently removes residual carbofuran from the soil.

The findings from present study presents an ecofriendly sustainable approach to remediate xenobiotics (RDX and carbofuran) contaminated soil. The synergistic effect of microbial formulation (WDG) and bio amendments (wheat straw, biogas slurry) not only enhances pollutant removal from soil but simultaneously provide a feasible option for agricultural waste management by utilizing them as nutritional source for potential autochthonous bacteria.

सारांश

तीव्र गति से बढ़ते हुए औद्योगीकरण और शहरीकरण के कारण दुनिया भर में ज़ेनोबायोटिक यौगिकों का अत्यधिक विवेकहीन उपयोग हुआ है। नाइट्रामाइन और कार्बामिट्स जैसे ज़ेनोबायोटिक यौगिकलंबे समय तक पर्यावरण में रहते हैं और इस प्रकार मिट्टी और जल निकायों के प्रदूषण में योगदान करते हैं। इस प्रकार, आसपास के पारिस्थितिकी तंत्र के स्थायी कायाकल्प के लिए मिट्टी और पानी से इन ज़ेनोबायोटिक्स को हटाने के लिए साध्य दृष्टिकोण की अनिवार्य मांग है। दूषित पर्यावरण निकायों को इन खतरनाक ज़ेनोबायोटिक्स से मुक्त करने हेतु, जैव उपचार सबसे कुशल उपाय है जिसे विश्व भर में व्यापक रूप से स्वीकार किया जा रहा है। विभिन्न प्रकार के जीवाणु, कवक और उनके संबंधित उपापचयी एंजाइम, जैव उपचार प्रक्रिया में उपयोग किए जाने वाले प्रमुख कारक हैं।

इन सभी तथ्यों को ध्यान में रखते हुए, "जेनोबायोटिक्स (आर. डी. एक्स तथा कार्बोफुरान) प्रदूषित मृदा का जैव उपचार" नामक वर्तमान शोधकार्य एकीकृत बायोएगमेंटेशन और बायोस्टिम्यूलेशन प्रविधि का उपयोग करके विस्फोटकों और कीटनाशकों से दूषित मिट्टी के उपचार तथा कायाकल्प पर केंद्रित है।

अध्ययन की शुरुआत नाइट्रोजन-सीमित न्यूनतम क्षार मीडिया में आर. डी. एक्स. क्षरणक्षमता के लिए विस्फोटक-दूषित साइटों से पृथक जीवाणुओं की जांच के साथ हुई। चार भिन्न प्रकार के जीवाणु, *बैसिलस ओशनिसिडमिनिस*, *पेलोमोनास एक्वाटिका*, *किनरेशिआ असक्रोफिला* और *आर्थ्रोबैक्टर सबट्रेनियस* को शुरू में सीएसआईआर-इंस्टीट्यूट ऑफ माइक्रोबियल टेक्नोलॉजी (आई. एम. टेक), चंडीगढ़ द्वारा पृथक किया गया, जिसे बाद में सेंटर फॉर फायर, एक्सप्लोसिव एंड एनवायरनमेंट सेफ्टी, रक्षा अनुसंधान एवं विकास संगठन (सी. एफ. ई. ई. एस., डी. आर. डी. ओ.) द्वारा हमें प्रदान किया गया। चारों सूक्ष्मजीवों में से, *पी. एक्वाटिका* और *के. असक्रोफिला* सबसे कुशल आर. डी. एक्स. डिग्रेडर पाए गए जिन्होंने $8.7 \pm 0.52 \times 10^8$ तथा $3.1 \pm 0.07 \times 10^7$ सी.एफ.यू./मि. ली., कोशिकीय वृद्धिके साथ 240 घंटे के भीतर, क्रमशः 82 और 74% आर. डी. एक्स. (10 मि.ग्रा./ली.) क्षरण किया। *बी. ओशनिसिडमिनिस* और *ए. सबट्रेनियस* दोनों ही निकृष्टतम आर. डी. एक्स. डिग्रेडर थे, जो कि 240 घंटे के बाद केवल 32 और 29% आर. डी. एक्स. गिरावट ही दर्ज कर पाए। साथ ही, छह अलग-अलग कार्बोफुरान क्षरण जीवाणुओं को दूषित मिट्टी से पृथक किया गया और न्यूनतम क्षार मीडिया में कार्बोफुरान क्षरण क्षमता के लिए जांच की गई। जीवाणु, *ओकरोबेक्ट्टम इंटरमीडियम*, और *बैसिलस एल्बस* को सबसे प्रभावी कार्बोफुरान डिग्रेडर पाया गया, जिसमें ऊष्मायन के 120 घंटे के भीतर 75.8 और 58.4% कार्बोफुरान (300 मि.ग्रा./ली.) का क्षरण हुआ और इस प्रकार आगे के चरणों के लिए चुना गया।

इसके बाद, संघ के विकास के लिए अनुकूलता की जांच करने के लिए शक्तिशाली आर. डी. एक्स. (*पी. एक्वाटिका* तथा *के. असक्रोफिला*) और कार्बोफुरान (*ओ. इंटरमीडियम* तथा *बी. एल्बस*) के बीच परस्पर क्रिया का अध्ययन किया गया। हालांकि, आर. डी. एक्स. तथा कार्बोफुरान डिग्रेडर्स के बीच कोई सकारात्मक परस्पर क्रियानहीं देखी गई। इसलिए, कोई माइक्रोबियल कंसोर्टियम विकसित नहीं किया गया था, और आगे के अध्ययनों में संभावित आर. डी. एक्स. और कार्बोफुरान क्षरण जीवाणुओं के मोनोकल्चर का उपयोग किया गया था।

उसके बाद, आर. डी. एक्स. और कार्बोफुरान डिग्रेडेशन कइनेटिक्स और इंटरमीडिएट मेटाबोलाइट्स को निर्धारित करने के लिए जलीय चरण क्षरण अध्ययन आयोजित किए गए थे। विस्फोटक दूषित स्थलों से पृथक किये गए *पी. एक्वाटिका* तथा *के. असक्रोफिला* ने 240 घंटे के भीतर 80 तथा 75% आर. डी. एक्स. (30 मि. ग्रा./ ली.) का क्षरण किया। आर. डी. एक्स. का क्षरण तेजी से और अत्यधिक प्रभावी था, जिसमें आर. डी. एक्स. की अर्ध-आयु 4.17 और 4.89 दिनों की पायी गयी। जलीय अर्क के LCMS विश्लेषण ने *पी. एक्वाटिका* के मामले में MNX, MEDINA, NDAB की उपस्थिति की पुष्टि की, जबकि *के. असक्रोफिला* के साथ मध्यवर्ती के रूप में केवल MEDINA और NDAB का पता चला। कार्बोफुरान के जलीय चरण क्षरणमें, *ओ. इंटरमीडियम* तथा *बी. एल्बसने* 92 तथा 68% कार्बोफुरान (100 मि. ग्रा./ ली.) क्षरण किया। *ओ. इंटरमीडियम* ने 0.69 / दिन की गिरावट दर के साथ तेजी से कार्बोफुरान क्षरणको 1 दिन की अर्ध-आयु के साथ दिखाया, जबकि नियंत्रण उपचार में 29 दिनों की अर्ध-आयु देखी गयी। जलीय अर्क के GCMS विश्लेषणों ने कार्बोफुरन-7-फिनोल (M.W. 164 Da) के गठन की पुष्टि की, जो 11.4 मिनट के RT पर प्रमुख मध्यवर्ती मेटाबोलाइट के रूप में पाया गया था। 15.2 मिनट के RT पर अन्य मध्यवर्ती 1,2-बेंजीनकार्बोक्सिलिक एसिड, मोनोएथिल एस्टर (M.W. 194 Da) का पता चला था।

इसके बाद, विभिन्न जीवाणुओंको डब्ल्यूडीजी, टैलकम तथा चारकोल आधारित पाउडर, और इनकैप्सुलेटेड बीड्सफार्मूलेशनके रूप में तैयार किया गया। तीन अलग-अलग तापमानों (4, 30 और 40°C) पर छह महीने की भंडारण अवधि में शेल्फ लाइफ और दूषित (आरडीएक्स और कार्बोफुरान) डिग्रेडिंग क्षमता के लिए विकसित की गयी फॉर्मूलेशन की जांच की गई। डब्ल्यूडीजीफार्मूलेशन ने 30°C तापमान पर 6 महीने के बाद, प्रारंभिक बैक्टीरिया की संख्या का >80% बरकरार रखा, जबकि टैलकम, चारकोल और बीड्स फॉर्मूलेशनमें >50% की हानि दर्ज की गई थी। हालांकि, 4 °C पर प्रारंभिक बैक्टीरिया की संख्या सभी विकसित की गयी फॉर्मूलेशन में 6 महीने के भंडारण तक स्थिर पायी गयी। 40 °C भंडारण तापमान पर 2 महीने के भीतर ही जीवाणुओं की संख्या बहुत कम हो गई थी।

पी. एक्वाटिका और *के. असक्रोफिला* के डब्ल्यूडीजी ने भंडारण अवधि के बाद प्रारंभिक आर. डी. एक्स अपघटन क्षमता का 95 और 96% बरकरार रखा। जबकि, *ओ. इंटरमीडियम* के डब्ल्यूडीजी ने 30 °C पर भंडारण के 6 महीने के अंत में ~ 94% कार्बोफुरान अपघटन क्षमता को बरकरार रखा। पाउडर और बीड्सफॉर्मूलेशन की उपचारात्मक क्षमता में 3 महीनों के भीतर >15% तक कमी पायी गई थी जो कि 6 महीने के अंत में और कम होकर >50% हो गई। इस प्रकार, आरडीएक्स और कार्बोफुरान क्षरण पर बायोएग्मेंटेशन और बायोस्टिम्यूलेशन के प्रभाव का अध्ययन करने के लिए डब्ल्यूडीजीफार्मूलेशन को अंततः चुना गया था।

सुक्रोज संशोधित मृदा में *पी. एक्वाटिका* डब्ल्यूडीजी ने 77.57% आर. डी. एक्स क्षरण किया, जबकि एक महीने के भंडारण के बाद गेहूं के भूसे से संशोधित मृदा में 66.49% आर. डी. एक्स क्षरण दर्ज किया गया। सुक्रोज तथा गेहूं के भूसे से संशोधित मृदा में आर. डी. एक्स की अर्ध-आयु में गिरावट पायी गयी (13.9 और 19.04 दिन क्रमशः), जबकि असंशोधित मृदा में आर. डी. एक्स की अर्ध-आयु 185.5 दिन पायी गयी।

इसी प्रकार, बायोगैस घोल (बीजीएस) संशोधित मृदा में *ओ. इंटरमीडियम* डब्ल्यूडीजी फार्मूलेशन ने 30 दिनों के भीतर 95% कार्बोफुरान क्षरण किया। असंशोधित मृदा में कार्बोफुरान अर्ध-आयु 78 दिन पायी गयी, जबकि एकिकृत बायोएग्मेंटेशन और बायोस्टिम्यूलेशन से उपचारित मृदा में कार्बोफुरान अर्ध-आयु गंभीर रूप से घटाकर 8.04 दिन पायी गयी।

आर. डी. एक्स और कार्बोफुरान दूषित मृदा के सफल उपचार के पश्चात्, मृदा और टमाटर के पौधों के स्वास्थ्य पर कार्बोफुरान के प्रभाव की जाँच की गई। डब्ल्यूडीजी और बीजीएस के सह-अनुप्रयोग के साथ सबसे अच्छा उपचारपाया गया जो कि नियंत्रण उपचार के साथ काफी समान ($p < 0.5$) पौधे की वृद्धि (शूट की लंबाई, जड़ की लंबाई, शुष्क भार, क्लोरोफिल) को दर्शाता है कि हमारा बायोएग्मेंटेशन और बायोस्टिम्यूलेशन संयोजन उपचार पौधे की वृद्धि पर कोई हानिकारक प्रभाव पैदा नहीं करता है और बाद में मृदा से अवशिष्ट कार्बोफुरान को हटा देता है।

वर्तमान अध्ययन के निष्कर्ष ज़ेनोबायोटिक्स (आर. डी. एक्स और कार्बोफुरान) दूषित मृदा के उपचार के लिए एक पर्यावरण के अनुकूल धारणीय दृष्टिकोण प्रस्तुत करते हैं। माइक्रोबियल फॉर्मूलेशन (डब्ल्यूडीजी) और जैव संशोधन (गेहूं का भूसा, बायोगैस घोल) का सहक्रियात्मक उपयोग न केवल मृदा से प्रदूषक अपघटन को बढ़ाता है बल्कि साथ ही संभावित मूल निवासी बैक्टीरिया के लिए पोषण स्रोत के रूप में उपयोग करके कृषि अपशिष्ट प्रबंधन के लिए एक संभव विकल्प प्रदान करता है।

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ABBREVIATIONS

USEPA	United States Environmental Protection Agency
RDX	Royal Demolition Explosive
TNT	Trinitrotoluene
HMX	High Melting Explosive
DPPQ&S	Directorate of Plant Protection Quarantine and Storage
EPA	Environment protection agency
MSM	Minimal salt medium
SSL	Soil screening level
HPLC	High performance Liquid Chromatography
LCMS	Liquid chromatography–mass spectrometry
GCMS	Gas chromatography mass spectrophotometer
FTIR	Fourier transform infrared spectroscopy
TLC	Thin layer chromatography
UV/VIS	Ultraviolet-Visible
MEDINA	Methylenedinitramine
ANOVA	Analysis of variance
PGPR	Plant growth promoting rhizobacteria
ACC	1-aminocyclopropane-1-carboxylate
IAA	Indole acetic acid
CFU	Colony forming unit
Fig.	Figure
MTCC	Microbial type culture collection
IMTECH	Institute of Microbial Technology
DMRT	Duncan's new multiple range test
EC	Electrical conductivity
MSM	Minimal salt medium
BGS	Biogas Slurry
Kg	Kilogram
mS/m	Milli siemens/ meter
M	Molar

w/v	Weight/volume
v/v	Volume/volume
%	Percent
mM	Millimolar
m/z	Mass/charge
d	Days
h	Hour
s	Seconds
g	Gram
mg	Milligram
cm	Centimeter
mm	Millimeter
rpm	Rotations per minute
mgL ⁻¹	Milligram per litre
gL ⁻¹	Gram per litre
mL	Milliliter
μl	Microlitre
°C	Degree centigrade