IMPACT OF ZINC OXIDE NANOPARTICLES ON CELLULOSE DEGRADING ACTINOMYCETES

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MAY 2025

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by

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Submitted

in partial fulfillment of the requirements for the degree of Doctor of Philosophy

to the

Indian Institute of Technology Delhi

May 2025

Certificate

This is to certify that the thesis entitled **"Impact of Zinc Oxide Nanoparticles on Cellulose degrading Actinomycetes"**, submitted by **Ms. Zoya Javed** to the Indian Institute of Technology, Delhi, for the award of the degree of **Doctor of Philosophy,** is a record of original, bonafide research work carried out by her under my supervision and guidance. The thesis has reached the standard fulfilling the requirements of the regulations related to the award of the degree.

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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ACKNOWLEDGEMENTS

I sincerely express the Almighty God my heartfelt thanks for giving me the vitality and divine intervention to complete this work.

First and foremost, I want to express my gratitude to my supervisor, Prof. Kavya Dashora, for her motivational talks, compassion, enthusiastic guidance, instruction, and mentoring. Throughout my PhD, she had an immense stake in my overall welfare, much like my family, and has been an excellent advisor outside of the academic realm.

I am grateful to all the faculty members of the Centre for Rural Development and Technology, IIT Delhi, for their help and encouragement while I finished this thesis, I want to express my gratitude to my SRC committee members, Prof. Anushree Malik (Chairperson, CRDT), Prof. Satyawati Sharma (Internal Expert, CRDT) and Prof. Arun Kumar (External Expert, Department of Civil Engg. IIT Delhi) for bringing me the outstanding support they offered, always prepared with insightful suggestions, constructive feedback, and sincere, valuable advice to enhance the quality of my research endeavours. I extend my heartfelt thanks to the laboratory personnel and administrative staff at CRDT, IIT Delhi, for their assistance and support concerning research and academic services and resources.

I would like to profoundly appreciate my colleagues in the lab and seniors, Dr. Mansi Mishra, Dr. Vinayak Fasake, Dr. Ayushi Srivastava, Ajay Yadav, Gyan Datta Tripathi, Meghana Gattupalli, Mohona Munshi, Onkar Sarma, Keerti Reddy and Mr. Chetan Kumar for assisting me across this journey, constantly motivating me to keep a positive attitude and accomplish my objectives.

My deepest gratitude to my good friends Mr. Salman Khan, Ms. Nidhi Maurya, Ms. Sadaf Khan, Mr. Anand Datta Tripathi, Ms. Kiran Rana, Dr. Manali Somani, and all my hostel friends for their unwavering support and tenacious presence during my thesis journey

Above all, I cannot overstate the tremendous influence and contribution of my beloved parents Mr. Javed Ahmad Siddiqui and Mrs. Khursheed Fatima. Their frequent epistolary contact and unwavering encouragement got me through the hardest of times. Without their boundless love and motivation, this accomplishment would have remained an unrealised dream, as this thesis attests. siblings Dr. Summaiya Javed and Dr. Tabish Javed deserve special recognition for their intense support, encouragement, and prayers, which helped me feel emotionally connected even though we were miles apart. I am thankful to have you all on this road with me since you have all given me so much in so many ways. Numerous more people have offered me support and assistance in different ways. I want to express my gratitude to each one of them and ask for their pardon for not naming them here.

Zoya Javed

ABSTRACT

This study addresses the effect of zinc oxide nanoparticles (ZnO NPs) on cellulose-degrading actinomycetes isolated from soil. The actinomycetes are widely researched and reported as the diverse micro-machinery with incredible capabilities to synthesized and secrete the enzymes like cellulase, chitinase, lipases and many other bioactive compounds which are involved in soil reclamation and the fertility of soil. The metabolic traits associated with actinomycetes present significant opportunities for industrial uses in the paper, pharmaceutical, food, textile and leather industries. Their degradation of cellulose and lignin rich agri-biomass other polysaccharides present in soil is well documented. As saprophytes, they may decompose complex polysaccharides like lignin and cellulose to produce soil organic matter. Cellulases produced from the actinomycetes have additional advantages over other sources such as bacteria due to their thermal and pH stability, as well as availability in diverse environmental conditions. However, exploring more suitable strains for cellulase production due to their slow growth, spore formation, and other challenges, is still an area of interest for scientists.

The enhanced use of nanoparticles has increased concern about risk for the environment and soil because it affects microbial processes and may damage soil fertility. Microbial ecotoxicity of the NPs has gained huge attention from the researchers across the globe; however, unavailability of consolidated data and detailed analysis, it would be difficult to reach the conclusion about the ZnO NPs mediated toxicity in soil. Presently, ZnO NPs found multiple applications in various industries and agriculture, however their interaction with soil microbial communities is still not well understood. The present thesis investigates the interaction between ZnO nanoparticles and actinomycetes to find out the possible mechanism of toxicity on cellular and functional level (cellulose degradation potential).

In the study, actinomycetes with cellulose degradation potential were isolated from the soil of different locations because of the availability and adaptation of the wide range of novel species distribution in diverse soil conditions. These isolates were screened for their cellulose-hydrolysing potential, and out of 45 isolates, 3 isolates formed clear zones on CMC agar, indicating the degradation of cellulose. The 16S genomic studies showed that the cellulolytic actinomycetes belonged to the *Streptomyces* genus. Further, these isolates were screened on the basis of pH (5-11), temperature (30-90 ^{0}C) and salinity (2-10% NaCl) stability, results showed that species *Streptomyces calvus* was more stable in these conditions.

As the next step, ZnO NPs were synthesized using Cow dung extract and characterized with the UV-VIS spectra, FTIR, XRD, SEM, TEM microscopy and EDX spectroscopy. The Antimicrobial potential of these ZnO NPs synthesized from Cow dung extract were tested against *Escherichia coli*, *Staphylococcus aureus* as well as isolates *Streptomyces* sp. Compared with control (water), the impact of biologically synthesized ZnO NPs on seed-germination, root and shoot lengths were also explored. Results suggested that ZnO NPs could significantly induce the germination of mung bean and does not disturb microbial flora and fauna due to minimal *invitro* toxicity on test microorganisms. The lower cellular leakage and reactive oxygen species (ROS) in comparison with commercial ZnO NPs (1mg/ml), made Cow dung extract mediated NPs more suitable for finding the possibilities in agricultural and industrial applications.

In the next step, the catalytic potential of ZnO NPs on cellulose degradation was also investigated with the help of solid-state fermentation (SSF) of rice straw and crude cellulase was also recovered as another important outcome of the study. The ZnO NPs (1 mg/gm) were supplemented in a solid-state fermentation medium with the isolated actinobacterial species *Streptomycetes calvus* for cellulase production at 30 ^{0}C for 15 days. It was noticed that the addition of the NPs catalyzes the biodegradation of rice straw complex structure and facilitated cellulase production. It was also found that cellulase activity increased by 22.5% (29.9 IU/gds with ZnO NPs vs. 24.4 IU/gds in control) due to application of NPs. Further, SEM and FTIR clearly showed the degradation of lignocellulose in the form of morphological changes in rice straw structure due to microbial action during fermentation process. Additionally, 36.6 % higher saccharification rate (sugar yield) with respect to non-treated control indicated that nanoparticles-mediated cellulases might be helpful in releasing sugar more efficiently.

The pure culture-based studies were also performed to evaluate the toxicity of cow dung based and commercial ZnO NPs on the isolated *Streptomyces calvus*. The result showed various concentration (0.05-50 mg/ml) concentration of NPs causes toxic effect (zone of inhibition) on gram positive *S. calvus.* On comparing both the NPs the level of ROS is gradually increased in commercially purchase ZnO NPs. The outcomes revealed that the toxicity of biologically synthesized ZnO NPs was lower (MIC-2 mg/ml) than the commercial ZnO (MIC-1 mg/ml) and mainly attributed due to destruction of membranes and entrance of Zn^{+2} through membranes. Further, BacLight™ live-dead analysis highlighted the toxicity of ZnO NPs on *S. calvus*, due to both contact mode (damaged membrane) and concentration of the NPs.

The microcosm study was also performed to observe the ecotoxicological potentials of ZnO NPs on bacterial and actinobacterial diversity. The effect of 1000 mg/kg of biologically synthesized and commercial ZnO NPs was investigated using culture plate study (CFU) as well as the amplicon-based sequencing of 16S rRNA genes present in the soil extracted DNA from microcosms. It was observed that microbial diversity was found to be reduced by 34% in the soils treated with ZnO NPs (ZnO B: 1.5×10^7; ZnO C: 1.01×10^7). Further, 16S based genomic sequencing of the soil revealed that the relative abundances ecologically significant and agriculturally important phyla specifically *Acidobacteria*, *Actinobacteria*, *Proteobacteria* and *Firmicutes* were positively or negatively affected in the presence of ZnO NPs ranges from 1 to 4% with respect to non-spiked soil. To the best of my knowledge, this is the first study to evaluate the impact of biologically synthesized ZnO NPs on cellulase production and soil microbial diversity using a combination of solid-state fermentation and next-generation sequencing. The overall outcome of the study suggests that biologically synthesized ZnO NPs could be a safer alternative to commercial ZnO NPs for agricultural applications, minimizing toxicity to soil microorganisms while enhancing cellulase production.

सार

वर्तमान अध्ययन में मिट्टी के विभिन्न नमूनों से पृथक किये गए सेल्यूलोज डिग्रेडिंग एक्टिनोमाइसिटीज पर जिंक ऑक्साइड नैनोकणों के प्रभाव पर प्रकाश डाला गया है। एक्टिनोमाइसिटीज को बहुत बहुमुखी सूक्ष्म मशीनरी के रूप में जाना जाता है जिसमें एमाइलेज, लाइपेस, और सेल्युलेज़ और एंटीबायोटिक्स जैसे एंजाइमों का उत्पादन करने की क्षमता होती है जो मिट्टी के सुधार और टिकाऊ मिट्टी के स्वास्थ्य में सहायक होते हैं। वे लंबे समय से मिट्टी में मौजूद जटिल बायोमास, सेल्यूलोज, लिग्नोसेल्यूलोज, जाइलीन, पेक्टिन और अन्य जटिल पॉलीसेकेराइड के विघटित करने के लिए जाने जाते हैं। ज्यादातर सैप्रोफाइट्स के रूप में माना जाता है, यह उन्हें मिट्टी के कार्बनिक पदार्थों और खाद के उत्पादन के लिए लिग्निन और सेलूलोज़ सामग्री जैसे जटिल पॉलीसेकेराइड को विघटित करने के लिए एक उपयुक्त उम्मीदवार बनाता है। एक्टिनोमाइसेट्स से उत्पादित सेल्यूलस में उनके थर्मल और पीएच स्थिरता और विविध पर्यावरणीय परिस्थितियों में उपलब्धता के कारण बैक्टीरिया जैसे स्रोतों पर अतिरिक्त फायदे हैं। हालांकि, उनकी धीमी वृद्धि, बीजाणु गठन और अन्य चुनौतियों के कारण सेल्यूलेज़ उत्पादन के लिए अधिक उपयुक्त उपभेदों की खोज करना, अभी भी वैज्ञानिकों के लिए रुचि का क्षेत्र है।

नैनोकणों के बढ़ते उपयोग ने पर्यावरण और मिट्टी के लिए जोखिम के बारे में चिंता बढ़ा दी है क्योंकि यह सूक्ष्मजीव उत्पादकता को प्रभावित करता है और मिट्टी के स्वास्थ्य को नुकसान पहुंचाता है। यहां, यह पहली बार है जब हमने मिट्टी के एक्टिनोमाइसिटीज पर ZnO नैनोकणों के प्रभाव की जांच की है। सूक्ष्मजीवों पर नैनोकणों (एनपी) के विषाक्त प्रभावों ने ध्यान आकर्षित किया है; हालांकि, इस पर कुछ रिपोर्टें हैं कि क्या एनपी सूक्ष्मजीवों के विकास को प्रभावित कर सकते हैं। वर्तमान में, जिंक ऑक्साइड नैनोकणों ने अपने विभिन्न कृषि और जैविक अनुप्रयोगों के कारण बहुत ध्यान आकर्षित किया, हालांकि मिट्टी के सूक्ष्मजीव के साथ जेडएनओ एनपी की बातचीत अभी भी अच्छी तरह से समझ में नहीं आई है।

वर्तमान अध्ययन के लिए, सेल्युलेज़-उत्पादक एक्टिनोमाइसेट्स को विभिन्न स्थानों की मिट्टी से अलग किया गया था। इन आइसोलेट्स को उनकी सेलूलोज़-हाइड्रोलाइज़िंग क्षमता के लिए जांचा गया था, और 45 आइसोलेट्स में से 3 आइसोलेट्स ने सीएमसी अगर पर स्पष्ट ज़ोन का गठन किया, जो सेलूलोज़ के क्षरण का संकेत देता है। 16S जीनोमिक अध्ययनों से पता चला है कि सेल्यूलोलाइटिक एक्टिनोमाइसेट्स *स्ट्रेप्टोमाइसेस* जीनस से संबंधित थे। इसके अलावा, इन आइसोलेट्स को पीएच (5-11) और तापमान (30-90°C) और लवणता (2-10% NaCl) स्थिरता पर जांच की गई और परिणामों से पता चला कि प्रजाति *स्ट्रेप्टोमाइसेस कैल्वस* इन स्थितियों पर अधिक स्थिर थी।

निम्नलिखित चरण के रूप में, ZnO नैनोकणों को गाय के गोबर के अर्क का उपयोग करके संश्लेषित किया गया था और यूवी-वीआईएस स्पेक्ट्रम, फूरियर ट्रांसफॉर्म इन्फ्रारेड स्पेक्ट्रोस्कोपी (एफटीआईआर), एक्स-रे विवर्तन (एक्सआरडी), स्कैनिंग इलेक्ट्रॉन माइक्रोस्कोपी (एसईएम), और ऊर्जा-फैलाने वाले एक्स-रे स्पेक्ट्रोस्कोपी (ईडीएक्स) का उपयोग करके विशेषता थी। उनकी रोगाणुरोधी क्षमता पर अध्ययन *एस्चेरिचिया कोलाई, स्टैफिलोकोकस ऑरियस* और *फ्यूजेरियम ऑक्सीस्पोरम* के खिलाफ आयोजित किए गए थे। नियंत्रण की तुलना में, अधिकतम बीज अंकुरण, जड़ की लंबाई और शूट लंबाई पर जैविक रूप से संश्लेषित जेडएनओ एनपी का प्रभाव देखा गया। इससे पता चलता है कि ZnO बीज के अंकुरण में काफी वृद्धि कर सकता है और इन विट्रो स्थितियों में मिट्टी, माइक्रोबियल वनस्पतियों और जीवों को परेशान नहीं कर सकता है। वाणिज्यिक नैनोकणों (1 मिलीग्राम / एमएल) की तुलना में कम सेलुलर रिसाव और प्रतिक्रियाशील ऑक्सीजन प्रजातियों (आरओएस) उत्पादन ने गाय के गोबर निकालने की मध्यस्थता नैनोकणों को कृषि और औद्योगिक अनुप्रयोगों के लिए उपयुक्त बना दिया।

इसके अतिरिक्त, इस अध्ययन का उद्देश्य ठोस-अवस्था किण्वन (SSF) सेल्यूलेज़ उत्पादन के दौरान चावल के भूसे के क्षरण में ZnO नैनोकणों के प्रभाव को निर्धारित करना है। जिंक ऑक्साइड (ZnO) नैनोकणों 1

मिलीग्राम/ग्राम को 15 दिनों के लिए 30°C पर सेल्यूलेज़ उत्पादन के लिए पृथक एक्टिनोबैक्टीरियल प्रजातियों स्ट्रेप्टोमाइसेट्स कैल्वस के साथ एक ठोस-अवस्था किण्वन माध्यम में पुरा किया गया था। यह देखा गया कि नैनोकणों के अलावा चावल के भूसे की जटिल संरचना के बायोडिग्रेडेशन को उत्प्रेरित करता है और सेल्युलेज़ उत्पादन की सुविधा प्रदान करता है। तो, सेल्युलेज़ गतिविधि में 22.5% की वृद्धि हुई (नियंत्रण में ZnO NPs बनाम 24.4 IU/gds के साथ 29.9 IU/gds। इसके अलावा, एसईएम और एफटीआईआर स्पष्ट रूप से किण्वन के दौरान चावल के भूसे की संरचना में लिग्नोसेल्यूलोज और रूपात्मक परिवर्तनों के क्षरण को दर्शाते हैं। इसके अतिरिक्त, गैर-उपचारित नियंत्रण के संबंध में 36.6% उच्च सैकरीफिकेशन दर (चीनी उपज) ने संकेत दिया कि नैनोकणों-मध्यस्थता वाले सेल्युलेज़ चीनी को अधिक कुशलता से जारी कर सकते हैं।

स्ट्रेप्टोमाइसिटीज पर जैविक और वाणिज्यिक जेडएनओ एनपी के विषाक्त प्रभाव का अध्ययन करने के लिए संस्कृति-आधारित अध्ययन भी किया गया था, जिसके बाद परिणाम से पता चला कि एनपी की विभिन्न सांद्रता (0.05-50 मिलीग्राम / एमएल) एकाग्रता ग्राम पॉजिटिव एस कैल्वस पर विषाक्त प्रभाव (निषेध का क्षेत्र) का कारण बनती *है*। दोनों एनपी की तुलना करने पर वाणिज्यिक रूप से खरीदे जाने वाले जेडएनओ एनपी में आरओएस के स्तर में धीरे-धीरे वृद्धि होती है। जैविक रूप से संश्लेषित ZnO NPs की विषाक्तता वाणिज्यिक ZnO (MIC-2 mg/ml) की तुलना में कम (MIC-1 mg/ml) थी - और मुख्य रूप से Zn^{2+} की रिहाई के लिए जिम्मेदार ठहराया गया, कॉन्फोकल लेजर स्कैनिंग माइक्रोस्कोप (CLSM) द्वारा विश्लेषण से पता चला कि मायसेलियम का आकार नहीं बदला हालांकि कुछ व्यक्तिगत बैक्टीरिया की मृत्यु हो गई। यह संभवतः झिल्ली के माध्यम से कोशिकाओं में प्रवेश करने वाले एनपी से Zn^{2+} जारी होने के कारण था, इस शोध से संकेत मिलता है कि जिंक ऑक्साइड नैनोकणों से *S. calvus* की विषाक्तता, नैनोकणों के संपर्क मोड और एकाग्रता दोनों से संबंधित है।

बैक्टीरियल और एक्टिनोबैक्टीरियल विविधता पर जिंक ऑक्साइड नैनोकणों के विषाक्त प्रभावों का मूल्यांकन करने के लिए सूक्ष्म जगत अध्ययन किया गया था। 1000 मिलीग्राम/किग्रा जैविक संश्लेषित और वाणिज्यिक ZnO नैनोकणों के प्रभाव की जांच संस्कृति-आधारित अध्ययन के साथ-साथ 16S rRNA जीन के इलुमिना MiSeq अनुक्रमण का उपयोग करके की गई थी। इस अध्ययन में,ZnO NPs (ZnO B: 1.5×10^7; ZnO B: 1.510^7; जेडएनओ सी: 1.01×10^7)। उसके बाद मिट्टी के 16S आधारित जीनोमिक अनुक्रमण ने प्रमुख और कृषि रूप से महत्वपूर्ण फ़ाइला के उन सापेक्ष बहुतायत पर प्रकाश डाला, अर्थात्, प्रोटियोबैक्टीरिया, एक्टिनोबैक्टीरिया, गैर-नुकीली मिट्टी के संबंध जिंक ऑक्साइड 1 से 4% की सीमा जिंक ऑक्साइड ZnO नैनोकणों की उपस्थिति में बदल गए थे।

मेरे ज्ञान में, यह ठोस तथ्य और अगली पीढ़ी के अनुक्रमण के संयोजन का उपयोग करके सेल्यूलेज़ उत्पादन और मिट्टी माइक्रोबियल विविधता पर जैविक रूप से संश्लेषित जिंक ऑक्साइड नैनोकणों के प्रभाव का मूल्यांकन करने वाला पहला अध्ययन है। निष्कर्ष बताते हैं कि जैविक रूप से संश्लेषित जिंक ऑक्साइड नैनोकण कृषि अनुप्रयोगों के लिए वाणिज्यिक जेडएनओ एनपी का एक सुरक्षित विकल्प हो सकता है, जो सेल्यूलेज़ उत्पादन को बढ़ाते हुए मिट्टी के सूक्ष्मजीवों के लिए विषाक्तता को कम कर सकता है।

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