STUDIES ON RARE MARINE ACTINOBACTERIA FOR THE INHIBITION OF BACTERIAL BIOFILMS AND ANTIBIOTIC RESISTANCE

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INDIAN INSTITUTE OF TECHNOLOGY DELHI

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by

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DEPARTMENT OF CHEMISTRY

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to the

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Dedicated to My Husband

CERTIFICATE

This is to certify that the thesis entitled "**Studies on rare marine actinobacteria for the inhibition of bacterial biofilms and antibiotic resistance**" being submitted by Ms. NIKKY GOEL to the Indian Institute of Technology Delhi for the award of the degree of Doctor of Philosophy in Chemistry is a record of bonafide research work carried out by her.

Ms. Nikky Goel has worked under my guidance and supervision and has fulfilled the requirements for the submission of the thesis which, to my knowledge, has reached the requisite standard.

The results contained in this dissertation 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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NIKKY GOEL

Abstract

Antimicrobial resistance (AMR), remains a significant worldwide public health issue, leading to 4.95 million deaths in 2019. If no action is taken, up to 10 million lives could be lost annually due to infections caused by drug-resistant microorganisms by the year 2050, resulting in an economic impact of approximately $100 trillion on the global economy. Biofilms, consisting of microbial cells embedded in self-produced exopolysaccharides, confer bacterial tolerance and resistance to antibiotics by obstructing penetration. As per the US National Institutes of Health, nearly eighty percent of microbial conditions in the body are associated with biofilms, a significant fraction of which exhibit resistance to conventional antimicrobial therapies. Actinobacterial species are recognised for their ability to produce secondary metabolites comprising antibiotic, antiviral, and anticancer compounds. With more than 7000 compounds produced by *Streptomyces* sp., most of them account for the secondary metabolites of potent antibiotics, making *Streptomycetes* the most exploited primary antibiotic-producing source.

The thesis aims to extensively investigate the isolation of rare marine actinobacteria for combating biofilm formation and drug resistance. Specifically, two strains of actinobacteria, namely, *Nocardiopsis lucentensis* EMB25 and *Streptomyces* sp. EMB24 were characterized and investigated for anti-biofilm and antibacterial potential. The highlight of the work is the isolation of rare actinobacteria inhibiting and eradicating the biofilm in *Pseudomonas aeruginosa*, reported for the first time.

The overall objectives of the thesis are as follows:

- Isolation of rare marine actinobacteria to study the effect of secondary metabolites on *P. aeruginosa* PseA biofilms
- Mechanism of secondary metabolites in inhibiting biofilm formation of *P. aeruginosa*

PseA

- Screening and isolation of actinobacteria for the antibacterial effect against drug-resistant bacteria
- Green synthesis of silver nanoparticles to study the antibacterial effect against drug-resistant bacteria.

The present study describes the whole genome analysis and biofilm-inhibiting and biofilm-eradicating potential of actinobacterial isolate identified as *N. lucentensis* EMB25. *N. lucentensis* EMB25 has a 6.5 Mbp genome with 71.62 % GC content. The genome analysis by Basic Local Alignment Search Tool (BLAST) Ring Image Generator (BRIG) revealed it to be closely related to *Nocardiopsis dassonvillei* NOCA502F. Moreover, the work intended to explore the antibiofilm potential of EMB25. Fermentation and extraction of the secondary metabolites was done employing the organic solvent extraction method. The organic extract at 1.56 µg/µL inhibited 50 % biofilm (BIC_{50}), and > 85 % biofilm inhibition was observed at 25 µg/µL. The gas chromatography-mass spectrometry (GC-MS/MS) technique identified the total compounds present. After identification, the ligands present in the extract were docked on the LasR and RhlR. Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET) analysis of the selected metabolites shows that these can be pursued as potential drug candidates. Finally, the mechanism was elucidated through expression analysis of genes regulated by LasR and RhlR in the presence and absence of organic extract. To the best of our knowledge, this is the first time the genus *Nocardiopsis* has been explored to inhibit and eradicate the biofilm of *P. aeruginosa.*

The current unprecedented rate of *Streptomyces* genome mining has led to the creation and usage of bioinformatics tools aimed explicitly at discovering Biosynthetic gene clusters (BGCs). Bacterial antiSMASH is a robust genome-mining tool that enables users to identify, annotate, and evaluate secondary metabolite BGCs easily. Based on the relevance of the

Streptomyces genus as a hub of novel antibiotics, we have isolated *Streptomyces* sp. strain EMB24 from Pen, Maharashtra, India. The *Streptomyces* sp. strain EMB24, with a GC content of 72.40 %, has a genome of 7,252,958 bp. The *Streptomyces* sp. strain EMB24 exhibited potent antibacterial activity against various drug-resistant bacteria growth, namely MRSA, tetracycline-resistant *N. gonorrhoeae*, and *N. gonorrhoea* WHO F evaluated using the agar-plug assay.

Subsequently, green synthesis of silver nanoparticles by *Streptomyces* extract resulted in a 10-30 nm size range confirmed by Field Emission Scanning Electron Microscopy (FESEM) and Transmission Electron Microscopy (TEM) images. The AgNPs exhibit significant antibacterial activity against drug-resistant bacteria, namely *P. aeruginosa*, tetracycline-resistant *N. gonorrhoeae*, *N. gonorrhoea* WHO (F) and MRSA at 5 µg/mL concentration. In addition, we have observed more than 80 % of biofilm inhibition in *P. aeruginosa* at 50 µg/mL concentration of AgNPs. This thesis makes a significant contribution by isolating and characterizing rare marine actinobacteria with promising potential for combating AMR and biofilm-related infections. The discovery of *N. lucentensis* EMB25 and *Streptomyces* sp. EMB24, along with their bioactive compounds, offers novel therapeutic strategies for addressing drug-resistant pathogens and biofilm-associated infections. The antimicrobial activity of green-synthesized AgNPs further highlights the potential of this research in developing alternative antimicrobial treatments.

सार

एंटीमाइक्रोबियल रेजिस्टेंस (एएमआर) एक गंभीर वैश्विक सार्वजनिक स्वास्थ्य समस्या बनी हुई है, जिसके कारण 2019 में लगभग 4.95 मिलियन मौतें हुईं। यदि इस पर ध्यान नहीं दिया गया तो 2050 तक ड्रग-रेजिस्टेंट सूक्ष्मजीवों के कारण होने वाले संक्रमणों से प्रति वर्ष लगभग 10 मिलियन लोगों की मौत हो सकती है, जिससे वैश्विक अर्थव्यवस्था पर लगभग $100 ट्रिलियन का आर्थिक प्रभाव पड़ सकता है। बायोफिल्म्स, जो कि स्वयं द्वारा निर्मित एक्सोपॉलीसैकेराइड्स में संलग्न माइक्रोबियल कोशिकाओं से बने होते हैं, एंटीबायोटिक्स के प्रवेश को रोककर बैक्टीरिया की सहिष्णुता और प्रतिरोध को बढ़ाते हैं। यूएस नेशनल इंस्टीट्यूट्स ऑफ हेल्थ के अनुसार, शरीर में लगभग अस्सी प्रतिशत माइक्रोबियल स्थितियाँ बायोफिल्म्स से जुड़ी होती हैं, जिनमें से अधिकांश परंपरागत एंटीमाइक्रोबियल उपचारों के प्रति प्रतिरोध प्रदर्शित करती हैं। एक्टिनोबैक्टीरियल प्रजातियों को उनके माध्यमिक मेटाबोलाइट्स, जिनमें एंटीबायोटिक, एंटीवायरल, और एंटी-कैंसर यौगिक शामिल हैं, का उत्पादन करने की क्षमता के लिए पहचाना जाता है। स्ट्रीप्टोमाइसीज प्रजातियों द्वारा उत्पादित 7000 से अधिक यौगिकों में से अधिकांश शक्तिशाली एंटीबायोटिक्स के माध्यमिक मेटाबोलाइट्स हैं, जिससे स्ट्रीप्टोमाइसीज एंटीबायोटिक उत्पादन का प्रमुख स्रोत बन गया है। इस शोध का उद्देश्य दुर्लभ समुद्री एक्टिनोबैक्टीरिया के पृथक्करण पर व्यापक अध्ययन करना है ताकि बायोफिल्म गठन और दवा प्रतिरोध का मुकाबला किया जा सके। विशेष रूप से, एक्टिनोबैक्टीरिया की दो प्रजातियों, नोकार्डियोप्सिस लुसेंटेंसिस EMB25 और स्ट्रीप्टोमाइसीज EMB24, का लक्षण निर्धारण और एंटी-बायोफिल्म तथा जीवाणुरोधी क्षमता के लिए परीक्षण किया गया। इस कार्य की विशेषता यह है कि इसमें पहली बार दुर्लभ एक्टिनोबैक्टीरिया को पृथक करके *Pseudomonas aeruginosa* में बायोफिल्म को रोकने और नष्ट करने की क्षमता का प्रदर्शन किया गया है।

इस शोध के समग्र उद्देश्य निम्नलिखित हैं:

- दुर्लभ समुद्री एक्टिनोबैक्टीरिया का पृथक्करण कर उनके माध्यमिक मेटाबोलाइट्स का *P. aeruginosa* PseA बायोफिल्म्स पर प्रभाव का अध्ययन।
- *P. aeruginosa* PseA के बायोफिल्म गठन को रोकने में माध्यमिक मेटाबोलाइट्स का तंत्र।
- दवा-प्रतिरोधी बैक्टीरिया के खिलाफ जीवाणुरोधी प्रभाव के लिए एक्टिनोबैक्टीरिया का स्क्रीनिंग और पृथक्करण।
- दवा-प्रतिरोधी बैक्टीरिया के खिलाफ जीवाणुरोधी प्रभाव का अध्ययन करने के लिए चांदी के नैनोकणों का हरित संश्लेषण।

वर्तमान अध्ययन में एक्टिनोबैक्टीरियल पृथक नोकार्डियोप्सिस लुसेंटेंसिस EMB25 का पूरे जीनोम विश्लेषण और बायोफिल्म-रोकने और नष्ट करने की क्षमता का वर्णन किया गया है। EMB25 में 6.5 Mbp का जीनोम और 71.62% GC सामग्री है। बेसिक लोकल अलाइनमेंट सर्च टूल (BLAST) रिंग इमेज जनरेटर (BRIG) द्वारा जीनोम विश्लेषण ने इसे *Nocardiopsis dassonvillei* NOCA502F से करीबी रूप से संबंधित पाया। इसके अतिरिक्त, EMB25 की एंटी-बायोफिल्म क्षमता की खोज का लक्ष्य रखा गया। माध्यमिक मेटाबोलाइट्स का किण्वन और निष्कर्षण जैविक सॉल्वेंट निष्कर्षण विधि का उपयोग करके किया गया। 1.56 µg/µL पर जैविक निष्कर्ष ने 50% बायोफिल्म (BIC50) को रोका और 25 µg/µL पर > 85% बायोफिल्म अवरोध देखा गया। गैस क्रोमैटोग्राफी-मास स्पेक्ट्रोमेट्री (GC-MS/MS) तकनीक ने सभी यौगिकों की पहचान की। पहचान के बाद, निष्कर्ष में मौजूद लिगैंड्स को LasR और RhlR पर डॉक किया गया। चुने गए मेटाबोलाइट्स का अवशोषण, वितरण, चयापचय, उत्सर्जन और विषाक्तता (ADMET) विश्लेषण से पता चला कि इन्हें संभावित दवा उम्मीदवारों के रूप में आगे बढ़ाया जा सकता है। अंततः, तंत्र को LasR और RhlR द्वारा नियंत्रित जीनों के अभिव्यक्ति विश्लेषण के माध्यम से स्पष्ट किया गया। हमारी जानकारी के अनुसार, यह पहली बार है जब नोकार्डियोप्सिस प्रजाति का उपयोग *P. aeruginosa* के बायोफिल्म को रोकने और नष्ट करने के लिए किया गया है।

वर्तमान में स्ट्रीप्टोमाइसीज जीनोम खनन की अभूतपूर्व दर ने जैवसिंथेटिक जीन क्लस्टर्स (BGCs) की खोज के लिए विशेष रूप से बायोइन्फॉर्मेटिक्स टूल्स का निर्माण और उपयोग संभव बना दिया है। बैक्टीरियल antiSMASH एक मजबूत जीनोम-खनन टूल है जो उपयोगकर्ताओं को माध्यमिक मेटाबोलाइट्स BGCs की पहचान, एनोटेशन और मूल्यांकन की सुविधा प्रदान करता है। स्ट्रीप्टोमाइसीज प्रजाति को नए एंटीबायोटिक्स के एक केंद्र के रूप में इसके महत्व को देखते हुए, हमने महाराष्ट्र, भारत के पेन से स्ट्रीप्टोमाइसीज प्रजाति EMB24 को पृथक किया है। EMB24 का जीनोम 7,252,958 bp का है और इसमें 72.40% GC सामग्री है। EMB24 ने विभिन्न दवा-प्रतिरोधी बैक्टीरिया, जैसे कि MRSA, टेट्रासाइक्लिन-प्रतिरोधी *N. gonorrhoeae*, और *N. gonorrhoeae* WHO F के खिलाफ एंटीबैक्टीरियल गतिविधि का प्रदर्शन किया है, जिसे एगर-प्लग विधि से आंका गया है।

इसके बाद, स्ट्रीप्टोमाइसीज निष्कर्ष द्वारा हरित संश्लेषित चांदी के नैनोकणों का आकार 10-30 nm पाया गया, जिसकी पुष्टि फील्ड एमिशन स्कैनिंग इलेक्ट्रॉन माइक्रोस्कोपी (FESEM) और ट्रांसमिशन इलेक्ट्रॉन माइक्रोस्कोपी (TEM) इमेजेस द्वारा की गई। 5 µg/mL पर इन AgNPs ने *P. aeruginosa*, टेट्रासाइक्लिन-प्रतिरोधी *N. gonorrhoeae*, *N. gonorrhoeae* WHO (F) और MRSA के खिलाफ महत्वपूर्ण जीवाणुरोधी गतिविधि का प्रदर्शन किया। इसके अतिरिक्त, 50 µg/mL AgNPs की सांद्रता पर *P. aeruginosa* में 80% से अधिक बायोफिल्म अवरोध देखा गया। यह शोध दुर्लभ समुद्री एक्टिनोबैक्टीरिया को पृथक कर, उनके जैव सक्रिय यौगिकों की खोज कर, एएमआर और बायोफिल्म-संबंधित संक्रमणों का मुकाबला करने के लिए एक महत्वपूर्ण योगदान देता है। EMB25 और *Streptomyces* EMB24 का पता लगाना और उनके जैव सक्रिय यौगिकों की पहचान नई चिकित्सीय रणनीतियों को प्रस्तुत करती है जो दवा-प्रतिरोधी रोगजनकों और बायोफिल्म-संबंधित संक्रमणों का समाधान प्रदान करती हैं। हरित संश्लेषित AgNPs की एंटीमाइक्रोबियल गतिविधि इस शोध की महत्वपूर्ण क्षमता को वैकल्पिक एंटीमाइक्रोबियल उपचार विकसित करने में रेखांकित करती है।

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confer no effect on preformed biofilm in the presence of AgNPs at 50 µg/mL concentration

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