STUDIES ON β-LACTAMASE FROM *BACILLUS TROPICUS* EMB20 FOR ALLEVIATION OF ANTIBIOTIC POLLUTION AND ANTIMICROBIAL RESISTANCE

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by

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Dedicated to my family

CERTIFICATE

This is to certify that the thesis entitled "**Studies On β-Lactamase from *Bacillus tropicus* EMB20 For alleviation of Antibiotic Pollution and Antimicrobial Resistance**" being submitted by **Ms. HUMA FATIMA** 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. Huma Fatima has worked under my guidance and supervision and has fulfilled the requirements for the thesis submission, 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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Place: New Delhi

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Huma Fatima

Abstract

Antibiotics are extensively utilized across various domains including medicine, agriculture, aquaculture, and animal husbandry. In recent years, there has been a significant global increase in both prescribed and unprescribed antibiotic usage. However, due to the poor absorption of antibiotics in living organisms, substantial quantities of antibiotics and their metabolites end up in wastewater. Moreover, wastewater originating from healthcare and pharmaceutical industries further adds antibiotic load to the environment through their effluents. The presence of antibiotics in aquatic environments has a notable impact on both human and aquatic organisms, even at nano or microgram concentrations. The situation is exacerbated by the transfer of antibiotic-resistant genes (ARGs), leading to the emergence of multiple drug-resistant (MDR) bacteria. The escalating issue of antibiotic pollution has raised serious concerns regarding its remediation in recent years. The escalating threat of antibiotic pollution and antimicrobial resistance (AMR) necessitates innovative and sustainable remediation strategies. Based on the above background the present thesis investigates the potential of β-lactamase from *Bacillus tropicus* EMB20 in mitigating antibiotic contamination and vis-à-vis AMR.

B. tropicus EMB20 genome spans approximately 5.8 megabases (Mb) and contains 5,593 coding DNA sequences (CDSs). The CDSs encode a variety of proteins that contribute to the bacterium's metabolic capabilities and adaptability to different environments. One of the notable features of *B. tropicus* EMB20 is its ability to produce β-lactamase, an enzyme that confers resistance to β-lactam antibiotics. β-lactamase catalyzes the hydrolysis of the β-lactam ring, a core structural component of β-lactam antibiotics, rendering them ineffective. This enzymatic activity is important because it helps the bacterium survive in environments contaminated with β-lactam antibiotics and also has potential applications for the

bioremediation of antibiotics. The degradation of β-lactam antibiotics by *B. tropicus* EMB20 thus highlights its potential role in mitigating antibiotic pollution.

Building on this foundation, the study explores the application of β-lactamase from *Bacillus tropicus* EMB20 for *in-vitro* remediation of β-lactam antibiotics. The enzyme efficiently hydrolysed high concentrations of ampicillin, amoxicillin, and meropenem, demonstrating its capability to degrade different classes of β-lactam antibiotics, including carbapenems. The hydrolysed products were analyzed and found to be non-toxic, ensuring that the breakdown of these antibiotics does not produce harmful by-products.

To enhance the practical applicability of β-lactamase, the enzyme was immobilized onto magnetic nanoparticles (Fe_3O_4). This immobilization not only stabilized the enzyme but also facilitated its easy separation and reuse. The enzyme nanoconjugate demonstrated high removal efficiency for various antibiotics, including the simultaneous removal of multiple antibiotics from real aquaculture wastewater. Remarkably, the immobilized enzyme retained significant activity over multiple cycles of use, underscoring its practical viability for treatment processes. However, the enzyme immobilized onto magnetic nanoparticles was not suitable for scale-up due to the very small size of the nanoparticles and poor packing properties. Therefore, for further advancing the remediation techniques, a sustainable method was developed by entrapping β-lactamase within agarose discs. The enzyme entrapped in agarose efficiently hydrolyzed doripenem and other β-lactam antibiotics in both batch and continuous treatments. In a practical application, the fixed-bed column bioreactor setup, using these agarose discs, successfully treated synthetic hospital wastewater. This demonstrated the potential for large-scale applications, offering a feasible method for treating wastewater streams from healthcare facilities, which are significant sources of antibiotic contamination.

Complementing these practical approaches, the metallo-β-lactamase gene from *B. tropicus* EMB20 was cloned and expressed in *Escherichia coli* BL21(DE3) at various temperatures, resulting in the formation of inclusion bodies. These aggregates were solubilized and purified, then evaluated for their biological activity and structural characteristics. The recombinant enzyme displayed an enhanced activity over the native enzyme. Notably, β-lactamase activity within the inclusion bodies indicated proper protein folding. Lower expression temperatures correlated with reduced amyloid content and enhanced biological activity. The purified β-lactamase inclusion bodies effectively removed β-lactam antibiotics in vitro, highlighting their potential for environmental remediation.

Finally, the study addressed the broader issue of antimicrobial resistance (AMR) by exploring the development of meropenem-based nanoflowers (mNFs). These nanoflowers were synthesized by co-precipitating meropenem with metal ions, forming nanoflower structure with high surface area. The mNFs demonstrated significant antibacterial and anti-biofilm activities against *Pseudomonas aeruginosa* PA01, *Klebsiella pneumoniae*, and *Staphylococcus aureus.* The effective inhibition of biofilm formation and reduced bacterial viability, by mNFs, offers a promising therapeutic solution for biofilm-associated infections, which are notoriously difficult to treat and are often resistant to conventional antibiotics. This innovative approach provides a new avenue for combating AMR.

The present thesis collectively emphasizes the diverse applications of β-lactamase enzyme from *B. tropicus* EMB20 in addressing antibiotic pollution and AMR. Overall, the work paves the way for new bioremediation strategies and therapeutic interventions, showcasing the potential for these approaches to make a significant impact on public health and environmental sustainability.

सार

एंटीबायोटिक्स का उपयोग चिकित्सा, कृषि, जलीय कृषि और पशुपालन सहित विभिन्न क्षेत्रों में बड़े पैमाने पर किया जाता है। हाल के वर्षों में, उल्लेखित और गैर उल्लेखित दोनों तरह के एंटीबायोटिक के उपयोग में वैश्विक स्तर पर अत्यधिक वृद्धि हुई है। हालांकि, जीवों में एंटीबायोटिक के खराब अवशोषण के कारण, एंटीबायोटिक और उनके मेटाबोलाइट्स की पर्याप्त मात्रा अपशिष्ट जल में चली जाती है। इसके अलावा, स्वास्थ्य सेवा और दवा उद्योगों से निकलने वाला अपशिष्ट जल अपने अपशिष्टों के माध्यम से पर्यावरण में एंटीबायोटिक का प्रदूषण और बढ़ाता है। जलीय वातावरण में एंटीबायोटिक की मौजूदगी का मानव और जलीय जीवों दोनों पर अत्यधिक प्रभाव पड़ता है, यहां तक कि नैनो या माइक्रोग्राम स्तर तक सांद्रता पर भी। एंटीबायोटिक-प्रतिरोधी जीन के हस्तांतरण से स्थिति और खराब हो जाती है, जिससे कई दवा-प्रतिरोधी (एमडीआर) बैक्टीरिया उभर आते हैं। एंटीबायोटिक प्रदूषण के बढ़ते मुद्दे ने हाल के वर्षों में इनके उपचार के बारे में गंभीर चिंताएँ पैदा की हैं। एंटीबायोटिक प्रदूषण और रोगाणुरोधी प्रतिरोध (एएमआर) के बढ़ते खतरे के कारण अभिनव और टिकाऊ उपचार रणनीतियों की आवश्यकता है। उपरोक्त पृष्ठभूमि के आधार पर प्रस्तुत शोध प्रबंध न केवल एंटीबायोटिक संदूषण को कम करने बल्कि एएमआर के संबंध में बैसिलस ट्रोपिकस ईएमबी20 से प्राप्त β-लैक्टामेज की क्षमता की भी जांच करता है। बी. ट्रोपिकस ईएमबी20 जीनोम लगभग 5.8 मेगाबेस (एमबी) में फैला है और इसमें 5,593 कोडिंग डीएनए अनुक्रम (सीडीएस) हैं। सीडीएस विभिन्न प्रकार के प्रोटीन को एनकोड करते हैं जो जीवाणु की चयापचय क्षमताओं और विभिन्न वातावरणों के अनुकूल होने में योगदान करते हैं। बी. ट्रोपिकस ईएमबी20 की उल्लेखनीय विशेषताओं में से एक इसकी β-लैक्टामेज उत्पन्न करने की क्षमता है, जो एक ऐसा एंजाइम है जो β-लैक्टम एंटीबायोटिक दवाओं के लिए प्रतिरोध छमता प्रदान करता है। β-लैक्टामेज β-लैक्टम रिंग के हाइड्रोलिसिस को उत्प्रेरित करता है, यह एंजाइमेटिक गतिविधि महत्वपूर्ण है क्योंकि यह जीवाणु को β-लैक्टम एंटीबायोटिक दवाओं से दूषित वातावरण में जीवित रहने में मदद करती है और एंटीबायोटिक

दवाओं के बायोरेमेडिएशन के लिए भी इसके संभावित अनुप्रयोग हैं। इस प्रकार बी. ट्रोपिकस EMB20 द्वारा β-लैक्टम एंटीबायोटिक दवाओं का विघटन एंटीबायोटिक प्रदूषण को कम करने में इसकी संभावित भूमिका को उजागर करता है। इस आधार पर, अध्ययन β-लैक्टम एंटीबायोटिक दवाओं के इन-विट्रो उपचार के लिए बैसिलस ट्रोपिकस EMB20 से β-लैक्टामेज के अनुप्रयोग की खोज करता है। एंजाइम ने एम्पीसिलीन, एमोक्सिसिलिन और मेरोपेनम की उच्च सांद्रता को कुशलतापूर्वक हाइड्रोलाइज किया, जिससे कार्बापेनम सहित β-लैक्टम एंटीबायोटिक दवाओं के विभिन्न वर्गों को विघटित करने की इसकी क्षमता का प्रदर्शन हुआ। हाइड्रोलाइज्ड उत्पादों का विश्लेषण किया गया और पाया गया कि वे गैर विषैले हैं, जिससे यह सुनिश्चित होता है कि इन एंटीबायोटिक दवाओं के टूटने से हानिकारक उप-उत्पाद उत्पन्न नहीं होते हैं। β-लैक्टामेज की व्यावहारिक प्रयोज्यता को बढ़ाने के लिए, एंजाइम को चुंबकीय नैनोकणों (Fe_3O_4) पर स्थिर किया गया था। इस स्थिरीकरण ने न केवल एंजाइम को स्थिर किया, बल्कि इसके आसान पृथक्करण और उसके पुनः उपयोग को भी सुगम बनाया। एंजाइम नैनोकंजुगेट ने विभिन्न एंटीबायोटिक दवाओं के लिए उच्च निष्कासन दक्षता का प्रदर्शन किया, जिसमें वास्तविक जलीय कृषि अपशिष्ट जल से कई एंटीबायोटिक दवाओं को एक साथ हटाना शामिल है। उल्लेखनीय रूप से, स्थिर एंजाइम ने उपयोग के कई चक्रों में महत्वपूर्ण गतिविधि बनाए रखी, जो उपचार प्रक्रियाओं के लिए इसकी व्यावहारिक व्यवहार्यता को रेखांकित करता है। हालाँकि, चुंबकीय नैनोकणों पर स्थिर किया गया एंजाइम नैनोकणों के बहुत छोटे आकार और खराब पैकिंग गुणों के कारण स्केल-अप के लिए उपयुक्त नहीं था। इसलिए, उपचार तकनीकों को आगे बढ़ाने के लिए, एगरोज़ डिस्क के भीतर β-लैक्टामेज़ को फंसाकर एक स्थायी विधि विकसित की गई। एगरोज़ में फंसा एंजाइम बैच और निरंतर उपचार दोनों में डोरिपेनम और अन्य β-लैक्टम एंटीबायोटिक दवाओं को कुशलतापूर्वक हाइड्रोलाइज़ करता है। एक व्यावहारिक अनुप्रयोग में, इन एगरोज़ डिस्क का उपयोग करके फिक्स्ड-बेड कॉलम बायोरिएक्टर सेटअप ने सिंथेटिक अस्पताल अपशिष्ट जल का सफलतापूर्वक उपचार किया। इसने बड़े पैमाने पर अनुप्रयोगों क्षमता को प्रदर्शित किया, जो स्वास्थ्य देखभाल सुविधाओं से अपशिष्ट जल धाराओं के उपचार के लिए एक सरल

व्यावहारिक विधि प्रदान करता है, जो एंटीबायोटिक संदूषण के महत्वपूर्ण स्रोत हैं। इन व्यावहारिक दृष्टिकोणों को पूरक करते हुए, बी। ट्रोपिकस ईएमबी 20 से मेटालो-β-लैक्टामेज जीन को क्लोन किया गया और विभिन्न तापमानों पर एस्चेरिचिया कोली बीएल 21 (डीई 3) में व्यक्त किया गया, जिसके परिणामस्वरूप समावेशन निकायों का निर्माण हुआ। इन समुच्चयों को घुलनशील और शुद्ध किया गया, फिर उनकी जैविक गतिविधि और संरचनात्मक विशेषताओं के लिए मूल्यांकन किया गया। पुनः संयोजक एंजाइम ने मूल एंजाइम की तुलना में एक बढ़ी हुई गतिविधि प्रदर्शित की। विशेष रूप से, समावेशन निकायों के भीतर β-लैक्टामेज गतिविधि ने उचित प्रोटीन तह का संकेत दिया। कम अभिव्यक्ति तापमान कम एमिलॉयड सामग्री और बढ़ी हुई जैविक गतिविधि के साथ सहसंबंधित है।

अंत में, अध्ययन ने मेरोपेनम-आधारित नैनोफ्लॉवर (mNFs) के विकास की खोज करके रोगाणुरोधी प्रतिरोध (AMR) के व्यापक मुद्दे को संबोधित किया। इन नैनोफ्लॉवर को मेरोपेनम को धातु आयनों के साथ सह-अवक्षेपित करके संश्लेषित किया गया था, जिससे उच्च सतह क्षेत्र के साथ नैनोफ्लॉवर संरचना का निर्माण हुआ। mNFs ने स्यूडोमोनस एरुगिनोसा PA01, क्लेबसिएला न्यूमोनिया और स्टैफिलोकोकस ऑरियस के खिलाफ महत्वपूर्ण जीवाणुरोधी और एंटी-बायोफिल्म गतिविधियों का प्रदर्शन किया। बायोफिल्म निर्माण का प्रभावी अवरोध और बैक्टीरिया की संख्या में कमी, mNFs द्वारा बायोफिल्म से जुड़े संक्रमणों के लिए एक आशाजनक चिकित्सीय समाधान प्रदान करता है, जिसका इलाज करना बेहद मुश्किल है और जो अक्सर पारंपरिक एंटीबायोटिक दवाओं के लिए प्रतिरोधी होते हैं। यह अभिनव दृष्टिकोण AMR का मुकाबला करने के लिए एक नया रास्ता प्रदान करता है।

वर्तमान थीसिस सामूहिक रूप से एंटीबायोटिक प्रदूषण और AMR को संबोधित करने में B. ट्रोपिकस EMB20 से β-लैक्टामेज एंजाइम के विविध अनुप्रयोगों पर जोर देती है। कुल मिलाकर, यह शोध कार्य नई जैव-उपचार रणनीतियों और उपचारात्मक हस्तक्षेपों का मार्ग प्रशस्त करता है, तथा इन तरीकों से

सार्वजनिक स्वास्थ्य और पर्यावरणीय स्थिरता पर महत्वपूर्ण प्रभाव पड़ने की संभावना को भी प्रदर्शित करता है।

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LIST OF ABBREVIATIONS

µg/L	micrograms per liter
µl	Microliter
µg	Microgram
ACN	Acetonitrile
Amox	Amoxicillin
Amp	Ampicillin
AMR	Antimicrobial resistance
ANI	Average nucleotide identity
APTES	3-aminopropyltriethoxisilane
AQW	Aquaculture wastewater
ARGs	Antimicrobial resistance genes
CDSs	Coding DNA sequences
CFS	Cell-free supernatant
CFU	Colony Forming Units
CFX	Cefuroxime (CFX)
CGIA	Colloidal gold immunoassay
CLIA	Chemiluminescence immunoassay
CLSI	Clinical and Laboratory Standards Institute
Conc.	Concentration
CPR	Cefpirome (CPR)
CTX	Cefotaxime (CTX)

CV	Crystal violet
dDDH	Digital DNA-DNA hybridization
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ELISA	Enzyme-Linked Immunosorbent Assay
EPS	Extracellular polymeric substances
FBCR	Fixed-bed column bioreactor
FCC	Face centered cubic
FDA	Food and Drug Administration
FE-SEM EDS	Field emission scanning electron microscope Energy Dispersive Spectroscopy
FIA	Fluorescence immunoassay
FT-IR	Fourier transform infrared spectroscopy
HGT	Horizontal gene transfer (HGT)
HPLC	High performance liquid chromatography
HR-MS	High-resolution mass spectrometry
HWW	Hospital wastewater
IMP	Imipenemase
IPTG	Isopropyl β-d-1-thiogalactopyranoside
*is*DDH	*in-silico* DNA-DNA hybridization
JCPDS	Joint Committee on Powder Diffraction Standards
KPC	*Klebsiella pneumoniae* carbapenemase

LB	Luria Bertani
LCMS	Liquid Chromatography-Mass Spectrometry (LC-MS),
LIBs	Lactamase Inclusion Bodies
LOD	Limit of detection
MBLs	Metallo-β-lactamases
MDR	Multidrug-resistant
Mer	Meropenem
mg	Milligram
MICs	Minimum inhibitory concentrations MICs
mm	Millimetre
mM	Milli molar
mNFs	Meropenem nanoflowers
MNPs	Magnetic nanoparticles
MTCC	Microbial type culture collection
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MWCO	Molecular weight cut-off
NB	Nutrient Broth
NCBI	National Center for Biotechnology Information
NDM	New Delhi metallo-β-lactamase
ng/L	Nanograms per liter
nm	Nanometre
PAGE	Polyacrylamide gel electrophoresis

PCR	Polymerase chain reaction
PGAP	Prokaryotic Genomes Annotation Pipeline
PMSF	Phenylmethylsulfonyl fluoride
QS	Quorum sensing
RAST	Rapid Annotation using the Subsystem Technology
RIA	Radioimmunoassay
SBLs	Serine-β-lactamases
SDGs	Sustainable Development Goals
SDS	Sodium Dodecyl Sulphate
SEM	Scanning electron microscopy
SHV	Sulf-hydryl variable active site
TDS	Total dissolved solids
TEM	Transmission electron microscope
ThT	Thioflavin T
TSB	Tryptic soya broth
TSS	Total suspended solids
TYGS	Type (Strain) Genome Server
UV	Ultraviolet
VIM	Verone integron-encoded metallo-beta-lactamase
WWTPs	Wastewater treatment plants
XRD	X-ray diffraction