STUDIES ON PRODUCTION AND OPTIMIZATION OF BIOACTIVE COMPOUNDS FROM *STREPTOMYCES FRAGILIS* AND *STREPTOMYCES CLAVULIGERUS*

THESIS SUBMITTED IN PARTIAL FULFILLMENT
FOR THE AWARD OF DEGREE

DOCTOR OF PHILOSOPHY

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This Thesis is Dedicated To My Beloved Parents

MEERA DEVI

ROHTASH SAINI

My Wife

SONALI KUMARI

My Grand Parents

SANTU RAM

SUKHDEVI

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ACKNOWLEDGEMENT

Through this page, I offer my respectful salutation to the founder of this esteemed seat of learning, Bharat Ratna Mahamana Pt. Madan Mohan Malviya Ji.

It is indeed my proud privilege to express my deep sense of gratitude and indebtedness to my supervisor, Prof. Abha Mishra, Professor, School of Biochemical Engineering, Indian Institute of Technology (BHU), Varanasi, for her invaluable guidance, constant encouragement, and unwavering support throughout the course of this research. Her parental care and sustained interest have been instrumental in the successful completion of this work.

I am sincerely thankful to Dr. Pranjal Chandra, coordinator, and Prof. Vikas Kumar Dubey, former coordinator, School of Biochemical Engineering, IIT (BHU), for providing essential facilities and constant motivation during my research journey.

I would also like to extend my gratitude to the RPEC members, Prof. Siva Hemalatha, Department of Pharmaceutical Engineering and Technology, and Dr. Sanjay Kumar, School of Biochemical Engineering, IIT (BHU), for their valuable suggestions and kind cooperation.

I am grateful to all the faculty members of the School of Biochemical Engineering, including Prof. Pradeep Srivastava, Dr. Vishal Mishra, Dr. Abhishek Suresh Dhoble, Dr. Prodyut Dhar, Dr. Sumit Kumar Singh, Dr. Aditya Kumar Padhi, Dr. Sharon Mano Pappu J and Dr. Rajendra Prasad, for their support and encouragement.

I also wish to acknowledge the former faculty members Prof. Subir Kundu, Prof. S. K. Srivastava, Prof. R. M. Banik, and Prof. Meera Debnath for their past guidance and inspiration, which have played a significant role in shaping my academic foundation.

I express my special thanks to Prof. Amit Singh, Professor, Institute of Medical Sciences, BHU, and Dr. Subhash Mohan Agarwal, Scientist F, National Institute of Cancer Prevention and Research (NICPR–ICMR), for their unconditional support, timely insights, and consistent encouragement throughout my research work.

I am also deeply thankful to all the non-teaching staff of the School of Biochemical Engineering, IIT (BHU), whose technical assistance was crucial to the smooth progress of my research.

I gratefully acknowledge the Ministry of Human Resource Development (MHRD), New Delhi, India, for financial support in the form of a Teaching Assistantship (TA), which enabled me to carry out this research.

My heartfelt appreciation goes to my dear lab mates Mr. Aprit, Mr. Abhay, Ms. Aditi, Ms. Shruti, Dr. Alok, and Dr. Deepankar for their camaraderie, encouragement, and support, which made this journey both fruitful and joyful.

I would like to express my sincere gratitude to Agneesh Pratim Das (Technical Officer, NICPR), Dr. Ankit Verma (MMMUT), Dr. Vikash Kumar Vasistha, Dharmendra Kumar (Thapar University) and Prof. Julian Davies (University of British Columbia) for their unwavering support and generous provision of resources, which were instrumental in the continuation of my thesis work.

Above all, I owe everything to the unwavering love, patience, and blessings of my family- my mother (Meera Devi), father (Rohtash Saini), brother (Rahul Saini), and wife (Sonali Kumari). Their sacrifices, prayers, and steadfast belief in me have been the cornerstone of this achievement.

Lastly, I offer my deepest gratitude to the Almighty God for giving me the strength and perseverance to complete this work.

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List of Abbreviations

ADME - Absorption, Distribution, Metabolism, and Excretion

ALA - Alanine

ANN - Artificial Neural Networks

ASP - Aspartic Acid

ATP - Adenosine Triphosphate

BBD - Box-Behnken Design

BCL2: B-Cell Lymphoma 2

BCR-ABL: Breakpoint Cluster Region-Abelson

BFGS: Broyden-Fletcher-Goldfarb-Shanno

BGCs - Biosynthesis Gene Clusters

BLAST: Basic Local Alignment Search Tool

BLIP - Beta-Lactamase Inhibitory Protein

CCD - Central Composite Design

CDK4: Cyclin-Dependent Kinase 4

cDNA: Complementary DNA

DEGs: Differentially Expressed Genes

DGF - Di-Glycine Motif

DMEM-HG: Dulbecco's Modified Eagle Medium-High Glucose

DMSO: Dimethyl Sulfoxide

DNA - Deoxyribonucleic Acid

DPGA - Diphosphoglyceric Acid

DPPH: 2,2-Diphenyl-1-Picrylhydrazyl

EGFR - Epidermal Growth Factor Receptor

ER - Endoplasmic Reticulum

FDA - Food and Drug Administration

FGFR - Fibroblast Growth Factor Receptor

FtsZ - Filamenting Temperature-Sensitive Mutant Z

g/L: Grams per Liter

GC - Guanine-Cytosine

GLN - Glutamine

GO: Gene Ontology

GRNN: General Regression Neural Network

HERG - Human Ether-à-go-go-Related Gene

HGFR - Hepatocyte Growth Factor Receptor

HR-LCMS: High-Resolution Liquid Chromatography-Mass Spectrometry

IC50: Half Maximal Inhibitory Concentration

IGFR - Insulin Growth Factor Receptor

JAK2: Janus Kinase 2

KEGG: Kyoto Encyclopedia of Genes and Genomes

LEU - Leucine

LM: Levenberg-Marquardt

LUSC: Lung Squamous Cell Carcinoma

m/z: Mass-to-Charge Ratio

MAE - Maestro File Format

Mb - Megabase

MD - Molecular Dynamics

MET - Methionine

ML: Machine Learning

MM/GBSA - Molecular Mechanics/Generalized Born Surface Area

MRSA - Methicillin-Resistant Staphylococcus Aureus

NCBI: National Centre for Biotechnology Information

NEB: New England Biolabs (likely referring to NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina)

NPT - Number of particles, Pressure, and Temperature

ns - Nanoseconds

NSC - Non-Small Cell

NSCLC - Non-Small Cell Lung Cancer

NVT - Number of particles, Volume, and Temperature

OPLS - Optimized Potentials for Liquid Simulations

PAINS - Pan Assay Interference Compounds

PDGFR - Platelet-Derived Growth Factor Receptor

PHE - Phenylalanine

PKC - Protein Kinase C

PKI: Protein Kinase Inhibitor

PKS - Polyketide Synthases

PPI: Protein-Protein Interaction

QbD - Quality by Design

R^2: Coefficient of Determination

RCSB-PDB - Research Collaboratory for Structural Bioinformatics Protein Data Bank

Rg - Radius of Gyration

rGyr: Radius of Gyration

RMSD - Root Mean Square Deviation

RMSF - Root Mean Square Fluctuation

RNA - Ribonucleic Acid

RSM - Response Surface Methodology

RTK - Receptor Tyrosine Kinase

SASA - Solvent Accessible Surface Area

SCG: Scaled Conjugate Gradient

SCLC: Small-cell lung cancer

SDF - Structure Data File

SER - Serine

SVM: Support Vector Machine

TIP3P - Transferable Intermolecular Potential with 3 Points

TSS: Trace Salt Solution

UD: Uniform Design

VAL - Valine

VEGFR - Vascular Endothelial Growth Factor Receptor

List of Symbols

- °C - Degrees Celsius
- α - Alpha
- μM - Micromolar
- Å - Angstrom
- Δ - Delta
- ΔGbind - Change in binding free energy
- ΔGcomp - Change in complex free energy
- ΔGprotein - Change in protein free energy
- ΔGlig - Change in ligand free energy
- ± - Plus-Minus
- γ - Gamma
- λ - Lambda
- Σ - Sigma
- π - Pi
- β - Beta
- μ - Mu
- σ - Sigma
- ρ - Rho
- τ - Tau
- Ω - Omega

- κ - Kappa
- θ - Theta
- φ - Phi
- ψ - Psi
- χ - Chi
- ∞ - Infinity
- ≠ - Not equal to
- ≈ - Approximately equal to
- ≤ - Less than or equal to
- ≥ - Greater than or equal to
- → - Right arrow
- ↔ - Double-headed arrow
- + - Plus
- − - Minus
- × - Multiplication
- ÷ - Division
- √ - Square root
- ∑ - Summation
- ∫ - Integral
- µg/mL - Micrograms per milliliter
- 2θ - Two theta

PREFACE

The genus *Streptomyces*, a member of the phylum Actinomycetota, is notable for its high GC content (70%) in its genetic material. This filamentous, aerobic, gram-positive, saprophytic, soil-dwelling bacterium has the ability to synthesize a wide range of secondary metabolites, including antibiotics. *Streptomyces* is a significant source of antibiotics, responsible for synthesizing about 80% of the antibiotics used to combat various diseases. Synthetic drugs often have numerous side effects, so there is a pressing need to discover alternative drugs based on natural sources with fewer side effects and lower costs. *Streptomyces*, with its bioavailability, abundant secondary metabolites, biotic friendliness, low toxicity, and high effectiveness, can serve as a perfect alternative for producing bioactive compounds.

Furthermore, *Streptomyces* possesses both eukaryotic signaling systems and prokaryotic two-component regulatory systems, making it a promising candidate for developing an assay for screening kinase inhibitors in eukaryotic signaling pathways. *Streptomyces 85E* strain has been identified as a suitable strain for testing kinase inhibitors using agar diffusion methods due to its growth and development characteristics on solid media and its ability to reveal the cytotoxicity of tested inhibitors. With known eukaryotic kinase inhibitors, such as tyrphostin and genistein, inhibition of hyphae formation has been observed, leading to the formation of "bald" colonies. The *Streptomyces 85E* assay exhibits three distinct phenotypes that can be utilized to deduce the persistence of eukaryotic protein kinase inhibitors (PKIs). These phenotypes encompass the absence of a zone, indicating the drug's inactivity; a transparent zone, signifying the cytotoxicity toward the cell, inhibiting both growth and sporulation; and a turbid zone, characterized by the creation of "bald" colonies that inhibit sporulation and aerial hyphae formation. *Streptomyces clavuligerus* and *Streptomyces fragilis* are gram-positive, filamentous

actinobacteria that comprise aerial mycelium with branched hyphae and spores. *Streptomyces clavuligerus* is known for its production of β-lactam antibiotics, such as clavulanic acid, cephamycin C, penicillin N, O-carbamoyl derivative deacetylcephalosporin C, and deacetoxycephalosporin C. Clavulanic acid is a novel β-lactamase inhibitor with broad-spectrum antibiotic properties effective against both gram-positive and gram-negative bacteria. Apart from its antibiotic activity, *Streptomyces clavuligerus* is also capable of producing anticancer metabolites, including bleomycin, Clavulactones, and Tunicamycin. Furthermore, this species produces kinase and phosphatase inhibitors, showing potential as lead molecules in cancer treatment targeting specific cellular processes. *Streptomyces fragilis*, although less studied within the *Streptomyces* genus, is also notable for its production of Azaserine, an antibiotic with tumor-inhibiting properties effective against a range of organisms, including gram-positive and gram-negative bacteria, protozoa, and fungi. Further exploration of *Streptomyces fragilis* could uncover novel bioactive compounds, potentially including new anticancer agents and other valuable biochemical products, making it an exciting prospect for future scientific and industrial applications.

The research aimed to evaluate the antioxidant, protein kinase inhibitory (PKIs) potential and cytotoxicity activity of the extracts from *Streptomyces clavuligerus* and *Streptomyces fragilis*. The bioactive compounds were obtained through submerged fermentation of the strains at 30 degrees Celsius, 200 rpm, for 3 days. After the 3rd day, the broth was sonicated, centrifuged, and subjected to a two-phase separation technique to obtain glycosidic anthracycline. The purified extract obtained from four different media was tested for the presence of S. 85E kinase inhibitor in the fermented broth. The media showing the highest bald zone in the kinase inhibitory test was chosen for further studies. The DPPH assay demonstrated that the organic extract had a strong free radical

scavenging capacity, with an IC_{50} value of 28.90 ± 0.24 µg/mL for *Streptomyces clavuligerus* and 38.76 ± 0.24 µg/mL for *Streptomyces fragilis*. In addition, the PKIs test indicated that *S. clavuligerus* and *S. fragilis* extracts produced a white bald zone, suggesting the existence of PKIs. The MTT assay was used to evaluate the cytotoxic activity of the organic extract on MCF-7, Hop-62, SiHa, and PC-3 cell lines. It showed the lowest IC_{50} value against the MCF-7 cell line (128.93±3.1 µg/mL), followed by the SiHa cell line for *S. clavuligerus*. Similarly, in the case of *S. fragilis* organic extract, SiHa cell lines (58.46±2.0 µg/mL) showed the lowest $IC_{50,}$ followed by the MCF-7 cell line (61.14 ± 2.5 µg/mL). This indicates potent growth inhibitory potential against human breast cancer and human cervical cancer cell lines. Multiple secondary metabolites from the organic and aqueous extracts of S. clavuligerus and S. fragilis were identified through HR-LCMS analysis after incubation at 30°C under 200 rpm for 3 days. The maximization of anthracycline compound production from *S. fragilis* was assessed through fermentation condition optimization. This included different carbon sources (dextrose, sucrose, fructose, corn flour, tapioca, lactose, and maltose) and nitrogen sources (beef extract, yeast extract, casein peptone, and ammonium sulfate) as well as varied ranges of temperatures (26°C-34°C), pH (6.5-7.5), C: N ratio (0.53-1.6) and agitation rates (150-250 rpm). The maximum production of anthracycline metabolites was achieved with molasses and yeast extract at a C: N ratio (1.15) at 29.7 °C temperature, pH 7.09, and a 203-rpm agitation rate using batch fermentation. The RSM model predicted a maximum metabolite yield of 4.65 g/L, which closely matched the experimental yield of 4.59 g/L. In addition to RSM, machine learning (ML) models (LM, SVM, GRNN, BFGS, and SCG) were utilized to predict metabolite production, and the LM algorithm emerged as the most effective, demonstrating exceptional predictive accuracy. The RSM and ML

models predicted metabolite productions of 4.65 g/L and 4.58 g/L, respectively, with the experimental yield being 4.59 g/L.

Computational studies revealed that epirubicin demonstrated superior inhibitory activity against key kinase proteins such as CDK4, EGFR, PDGFR, and PI3K when compared to the FDA-approved drug doxorubicin. Additionally, the downstream process of the secondary metabolites and the isolation and structural characterization of epirubicin (anthracycline) were conducted using various analytical techniques such as HPLC, FTIR, and NMR. The thermal characteristics of epirubicin were elucidated by XRD, DSC, and TGA analysis. The DPPH assay revealed the potent free radical inhibitory capacity of epirubicin (IC_{50} 12.56 ± 0.22 µg/mL) compared to ascorbic acid (IC_{50} 14.38 ± 0.14 µg/mL). In MTT assays, epirubicin demonstrated effectiveness against human lung cancer cells (A549), with an IC_{50} of 5.435 µg/mL and a 37% reduction in cancer cell viability at an 8 µg/mL concentration. Subsequent transcriptomics analysis of A549 cells, using RNAseq analysis with a phred score of 35, revealed that 428 genes were significantly altered, with the highest number of downregulated genes primarily involved in cell cycle regulation. KEGG analysis indicated upregulation of the p53 pathway, with all other pathways containing genes that directly or indirectly enhance p53 pathway activation, leading to apoptosis and cell death. This research highlights the potential of compounds derived from *Streptomyces fragilis* and *Streptomyces Clavuligerus* in cancer treatment. It also demonstrates the feasibility of optimizing fermentation processes for large-scale production of metabolites using agricultural waste molasses and yeast extract.