STUDIES ON SIDEROPHORE PRODUCING BACTERIA ISOLATED FROM MANGROVES OF GOA, INDIA: DOCKING STUDIES WITH ANTIBIOTIC

Dissertation Report

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by

AKASH RAJWAR

Roll no – 22P0500001

P.R. No: 202208790

Under the supervision of

DR. SANIKA SAMANT

School Of Biological Science and Biotechnology

M.sc Marine Biotechnology

Goa University

April 2024

Seal of the school:

Examined by:

DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "Studies on siderophore producing bacteria isolated from mangroves of Goa, India: Characterization and Application" is based on the results of investigations carried out by me in the M.Sc. in Marine Biotechnology at the, Goa University SBSB under the Supervision of Dr. Sanika Samant and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observations/experimental or other findings given the dissertation.

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This is to certify that the dissertation report '**STUDIES ON SIDEROPHORE PRODUCING BACTERIA ISOLATED FROM MANGROVES OF GOA, INDIA: DOCKING STUDIES WITH ANTIBIOTIC**' is a bonafide work carried out by **MR. AKASH RAJWAR** under my supervision in partial fulfilment of the requirements for the award of the degree of **MASTERS of SCIENCE** in the Discipline **MARINE BIOTECHNOLOGY** at the **SCHOOL OF BIOLOGICAL SCIENCES AND BIOTECHNOLOGY**, Goa University, Goa.

2/5/24

Signature and Name of Supervising Teacher: Dr. Sanika Samant

Name of Discipline: Marine Biotechnology

Prof. B. F. Rodrigues

Dean School of Biological Sciences & Biotechnology

Dean of School of Biological Sciences & Biotechnology
Goa University, Goa-403206

School of Biological Sciences and Biotechnology

Date: 8/4/24

Place: Goa University

SBSB: Biotechnology
Taleigao Plateau, Goa 403206
GOA UNIVERSITY

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PREFACE

My fascination with the microbial diversity of the mangroves of Goa led me to investigate siderophore-producing bacteria. Siderophores, with their remarkable iron-chelating abilities, represent a compelling area of research due to their potential applications in various fields. This dissertation focuses on the isolation and characterization of siderophore-producing bacteria from these unique ecosystems, with a particular emphasis on their siderophore type.

The primary objective of this research was to elucidate the potential of these naturally occurring siderophores in drug delivery systems. By strategically conjugating them with antibiotics, we envisioned a novel approach for targeted therapy. Herein, I present a comprehensive investigation into the characteristics of these bacterial siderophores and their subsequent evaluation as potential carriers for antibiotic delivery.

This dissertation serves as a scientific record of my research journey. It details the isolation techniques employed to obtain siderophore-producing bacteria from the mangroves of Goa. Furthermore, it presents the thorough characterization of the isolated siderophores. Finally, the dissertation explores the feasibility of utilizing these siderophores for antibiotic conjugation, paving the way for potential future applications in drug delivery.

The research contained within these pages contributes to our understanding of siderophore diversity and their potential as drug delivery vehicles. It is my hope that this work will stimulate further research in this exciting field, ultimately leading to the development of novel and effective therapeutic strategies.

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In closing, I would like to express my deepest appreciation to all those who have contributed to this thesis, directly or indirectly. Your support has been invaluable, and I am forever grateful for the opportunity to undertake this scholarly endeavour.

List of abbreviations

Abs	Absorbance
BLAST	Basic local alignment search tool
bp	Base pair
CAS	Chrome Azurol Sulphonate
EDTA	Ethylene diamine tetra acetic acid
Fig	Figure
g	Gram
L	Liter
h	Hour (s)
ZMA	Zobell Marine Agar
ZMB	Zobell Marine Broth
rpm	Revolutions per minute
RNA	Ribonucleic Acid
DNA	Deoxyribonucleic Acid
SDS	Sodium dodecyl sulphate
TE	Tris EDTA
TAE	Tris acetate EDTA
PCR	Polymerase chain reaction
UV	Ultra violet
Vis	Visible

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Introduction

Iron is a crucial component for the growth, metabolism and replication of bacteria (Schaible & Kaufmann, 2004). Iron is often needed at micromolar concentrations for the growth and reproduction of marine bacteria, while only 0.1–2 nM of iron are present in ocean surface water (Paul & Dubey, n.d.). In environments where iron is limited, bacteria produce siderophores to sequester iron and sustain their development. (Granger & Price, 1999). Low molecular weight ligands specific to iron are synthesized and called siderophores. Insufficient iron levels stimulates the production of Fe^{3+} (Neilands, 1995). To overcome the extreme iron scarcity, several marine bacteria have developed siderophore-mediated iron transport mechanisms. Siderophores are low-molecular-weight ligands that scavenge iron [Fe(III)] from the environment and transport it into cells via iron-regulated outer membrane proteins (IROMP). Microbial siderophores are classified as hydroxamates, catecholates, carboxylates, or mixed kinds (b-hydroxyl aspartate/catecholate) depending on the chemical makeup of their coordination sites (Baakza et al., 2004). Siderophore production gets influenced by iron bioavailability and growth conditions (temperature, nutrition, carbon supply, and so on) (McNeil & Matear, 2006). Siderophores have a strong affinity for ferric iron, but they may also form complexes with metals other than Fe3+, although with a lesser affinity (Braud et al., 2009). When studying siderophores by genera, the most frequently researched iron chelators are Gram-negative bacteria siderophores, namely the catechol and hydroxamate groups found in pseudomonas and enterobacteria. Siderophores from agrobacteria, Yersinia, and vibrions have also been investigated. Hydroxamates have been largely researched from Gram-positive organisms, although catechols were discovered in a very modest number of members of this group (Temirov et al., 2008).

During this study, the bacteria producing siderophores were isolated from a mangrove of Goa. Mangroves are extremely productive ecosystems that support a diverse spectrum of coastal and offshore marine creatures. Mangroves offer a distinct ecological setting for different bacterial

populations (Ramanathan & Carmichael, 2008). Mangroves are extremely productive ecosystems that support a diverse spectrum of coastal and offshore marine creatures. Mangroves offer a distinct ecological setting for different bacterial populations (Shrivastav et al., 2011). Microorganisms thrive in environments characterized by diverse conditions such as temperature, pH, salinity, and nutrient availability. Previous studies have revealed the presence of a vast array of microorganisms within these ecosystems (Fusconi & Godinho, 2002). This study was thus focused to isolating siderophore-producing bacteria from sediment samples and coastal ecosystems, as well as studying their kind.

The outer membrane permeability barrier stands as a significant determinant of resistance in bacterial pathogens. When coupled with mechanisms such as drug inactivating enzymes, target modification, and efflux, this barrier can profoundly augment resistance levels. An effective strategy to combat this form of resistance involves exploiting bacterial transport systems, particularly those implicated in iron uptake. These systems play indispensable roles in bacterial virulence and survival, especially in the context of iron-depleted host environments, which serve as innate defence mechanisms against bacterial invasion. To address this challenge, we have engineered biomimetic siderophores to serve as molecular shuttles for facilitating the active transport of antibiotics across bacterial membranes. Through systematic structure-activity relationship investigations, we have developed siderophore-aminopenicillin conjugates that demonstrate potent activity against Gram-negative pathogens (Möllmann et al., 2009).

Antibiotics are super important for fighting bacterial infections. But if we use them too much, it can mess up the balance of bacteria in our bodies, making the antibiotics less effective over time (Murray et al., 2022). One challenge in creating new antibiotics is making sure they can

get inside bacteria efficiently. Recently, scientists have been focusing more on developing precise methods to transport antibiotics effectively into bacteria. This area of research has become increasingly important in the development of new antibiotic drugs (Richter et al., 2017). Cefiderocol (S-649266) is a new combination of a catechol-type siderophore and a cephalosporin antibiotic. It utilizes the siderophore-iron complex pathway to enter the outer membrane of Gram-negative bacteria, alongside the typical passive diffusion through membrane porins. (Zhanel et al., 2019).

Chapter 1

Literature survey

1.1 Need for iron and impacts of iron deficiency.

Iron is required for the growth and development of nearly all living things. It serves as a catalyst for some of the most essential enzymatic activities, including as oxygen metabolism, electron transport, and DNA/RNA synthesis. Despite its abundance on Earth and the necessary micromolar amounts for microbial development, iron is often absent in most habitats (Lankford & Byers, 1973).

Iron deficiency can affect the activity of some enzymes. Iron-limitation affects enzyme activity and polypeptide chain composition. Iron shortage can significantly increase NADase activity and reduce cytochrome, peroxidase, and catalase concentrations. NADH dehydrogenase, a membrane-bound enzyme containing iron-sulfur centers, oxidizes NADH in the terminal electron transport systems of aerobes. Iron deficiency leads to a reduction in microbial cell biomass.

1.2 Siderophore

Siderophores are low molecular weight ligands (20–2000 Da) generated by bacteria, fungi, and plants that solubilize and take up iron (Chu et al., 2010). Siderophores bind iron with an affinity constant of almost 1030 M^{-1}. Over 500 distinct siderophores, largely from bacteria, have been identified. Iron ligation groups are classified into three types: hydroxamate, catecholate, and hydroxycarboxylates **(Fig. 1.1)**. Other siderophore structures, such as oxazoline, thiazoline, hydroxypyridinone, α- and β-hydroxy acids, and α-keto acid components, have also been identified. Siderophores' denticity (the number of iron coordinating atoms per molecule) varies from bidentate to hexadentate (Dertz & Raymond, 2004). The peptide backbone of siderophores is frequently constructed of numerous non-protein amino acid analogs comprising

both modified and D-amino. Bacteria may manufacture numerous forms of siderophores, allowing them to flourish in diverse settings.

Siderophores are of three types-

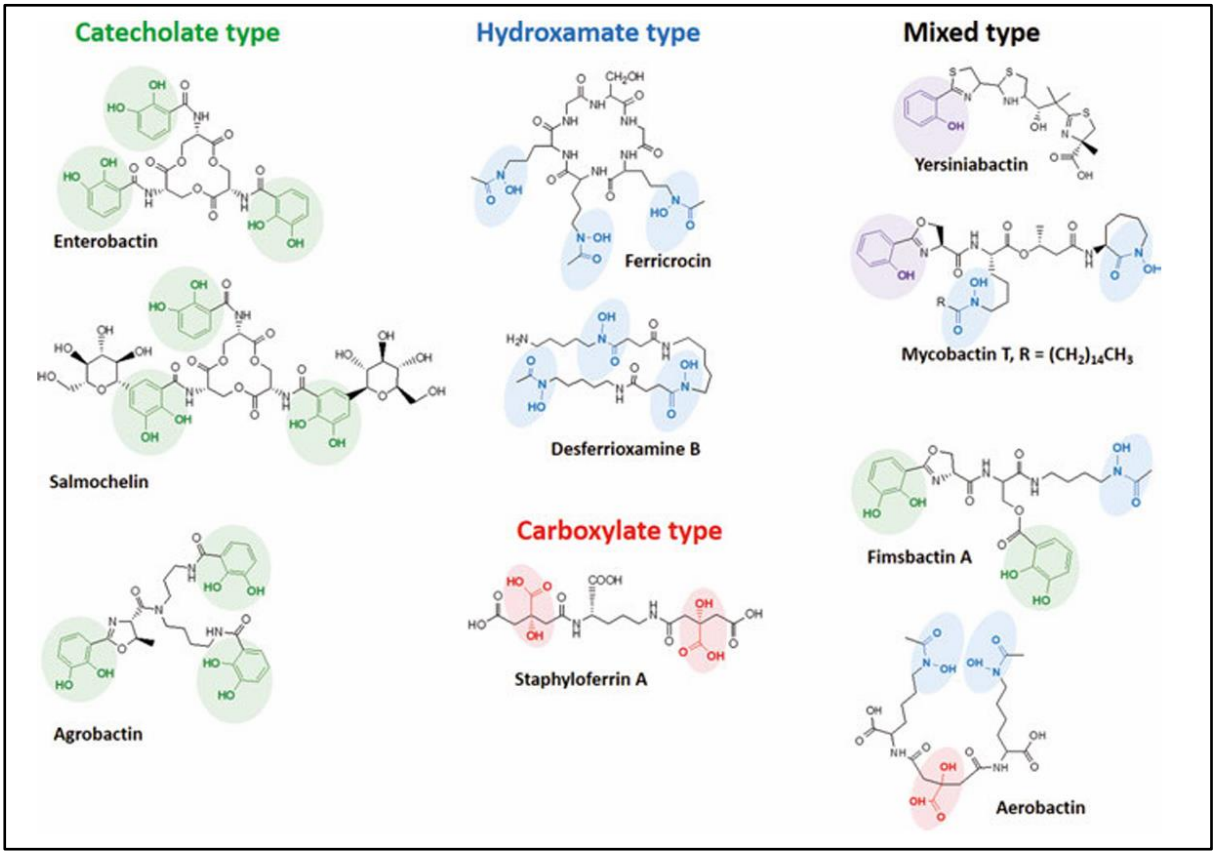

Fig. 1.1: Main structural type of siderophores (Ref: (Gumienna-Kontecka & Carver, 2019))

1.2.1 Catecholate

Catecholate-type siderophores include Fe(III) ions coupled to hydroxyl or catecholate groups. When chelated with Fe(III), a hexadentate-octahedral complex is produced, with two oxygen atoms from each catecholate group participating (Ghosh et al., 2020). All catecholate are derived from salicylic or 2,3-dihydroxybenzoic acid (2,3-DHBA) (Mishra & Baek, 2021).

Azospirillum brasilense produces the catecholate-type siderophore, known as *spirilobactin*, in a Fe-depleted media (Bachhawat & Ghosh, 1987). *Azospirillum lipoferum* produces 2,3-dihydroxybenzoic acid (2,3-DHBA) and 3,5-DHBA conjugates with threonine and lysine, which also exhibit siderophore activity (Shah et al., 1992). Enterobactin, found in harmful bacteria such as *E. coli*, is made up of three monomers of 2,3-dihydroxybenzoyl-L-serine.

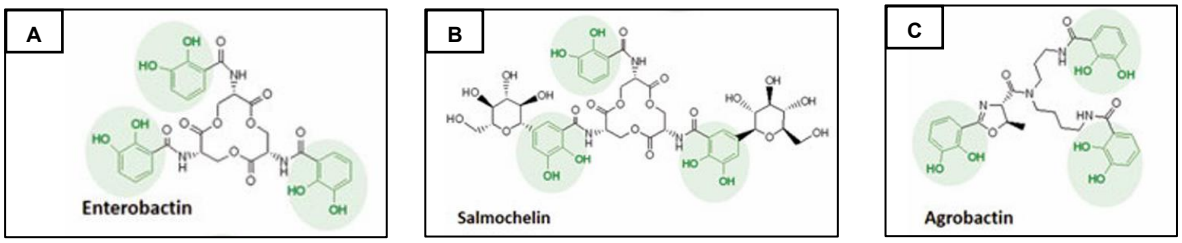

Fig. 1.2 Structures of Catecholate siderophore A) Enterobactin B) Salmochelin C) Agrobactin

1.2.2 Hydroxamate

Hydroxamate siderophores have the structure C(=O)N-(OH)R, where R is an amino acid or its derivative containing two oxygen atoms that form a bidentate ligand with Fe ions. Each siderophore may produce hexadentate ligands and octahedral complex molecules using Fe(III) ions (Ustiatik et al., 2021). When hydroxamate reacts with Fe(III), its functional group loses a proton from the hydroxylamine group (-NOH), forming a bidentate ligand (Feistner et al., 1993). The basic structure consists of hydroxamic acids. The standard tests used to detect hydroxamate type of siderophores are: Neiland‘s spectrophotometric assay (Neilands, 1981).

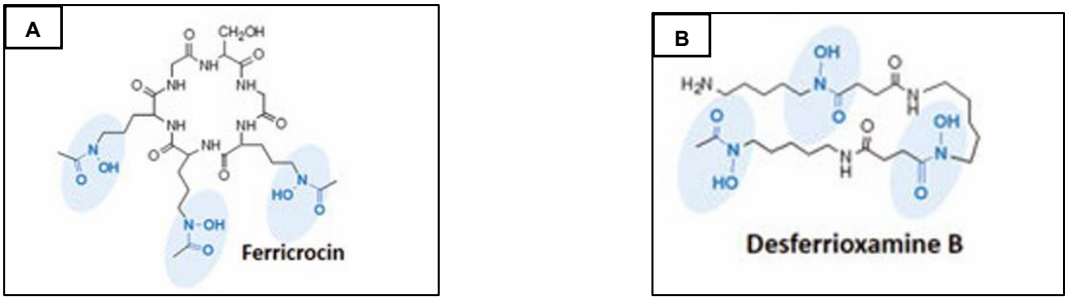

Fig. 1.3 Structure of hydroxamate siderophore A) Ferricrocin B) Desferrioxamine B

1.2.3 α-Hydroxycarboxylate, carboxylate, and mixed functional Group

The iron chelating group in carboxylate type of siderophores is a carboxylic acid (Sandy & Butler, 2009). Vogel's chemical test can detect the presence of carboxamate-type siderophores (Yeole et al. 2001) or Shenker's test (Shenker 1992).

The siderophores produced by fluorescent strains of Pseudomonas are pyoverdines (Budzikiewicz, 1993). Attached to the chromophore, each pyoverdine molecule comprises a quinoline chromophore, a peptide, and a dicarboxylic acid (or its corresponding amide). While the peptide remains consistent within bacteria of identical strains, variations can occur across different strains and species (Ringel & Brüser, 2018). For instance, Pseudomonas fluorescens has yielded three distinct pyoverdines, namely pyoverdine, pyoverdine 0, and pyoverdine A (also known as ferribactin), following isolation procedures (Philson & Llinás, 1982). *Azotobacter vinelandii*'s initial discovery of a siderophore was azotobactin, categorized as a pyoverdine-type siderophore (Bulen & LeComte, 1962). Pyoverdins are believed to play a role in the biological regulation of phytopathogenic microorganisms within the rhizosphere by forming stable complexes with soil iron, thereby rendering this crucial element inaccessible to harmful rhizosphere microorganisms (Ambrosi et al., 2000).

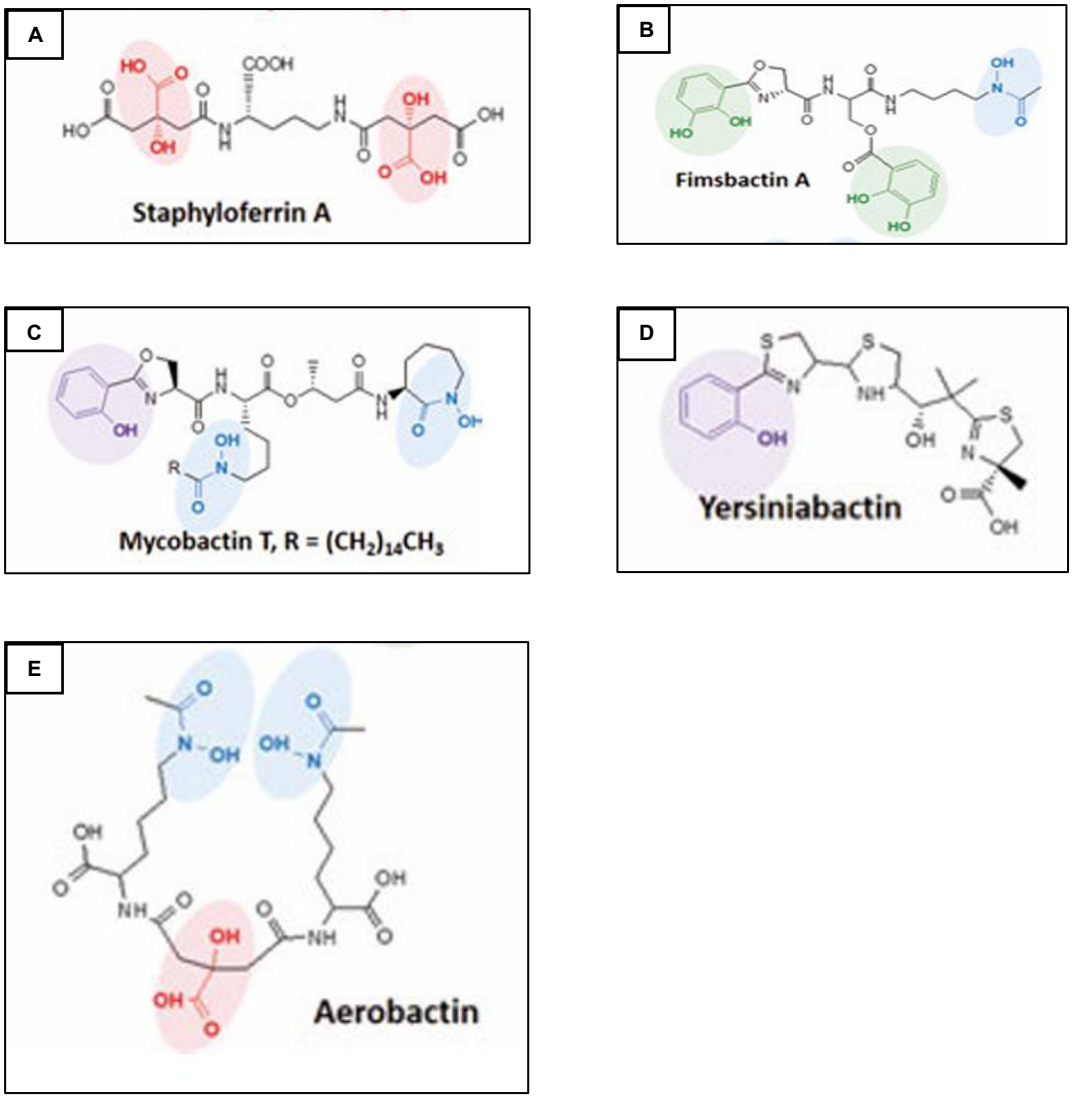

Fig. 1.4 A) Structure of Staphyloferrin A carboxylate siderophore. Mixed type siderophore B) Fimsbactin A C) Mycobactin T D) Yersiniabactin E) Aerobactin

1.3 Iron acquisition and ferric siderophore transport

Iron-binding molecules known as siderophores exhibit a strong affinity for iron, facilitating the extraction of iron from various chemical and organic complexes through equilibrium displacement. Both xenosiderophores and endogenously produced siderophores are utilized by numerous bacteria. Within cells, siderophores transport iron-bound forms. Gram-positive

bacteria recognize ferric siderophores at the cell surface through specific membrane-anchored binding proteins, whereas Gram-negative bacteria employ specific outer membrane receptors for this purpose. Transport of this iron-siderophore complex occurs through pores formed by β-barrel proteins, whose conformational state adjusts based on the microbial cell's substrate requirements. To date, three receptors have been subjected to crystallization: FepA (Buchanan 1999) FhuB (Ferguson 1998, Locher et al. 1998) and FecA (Ferguson et al. 2002, Yue et al. 2003) which are the receptors for enterobactin, ferrichrome, and ferric dicitrate.

The interaction between these outer membrane receptors and a cytoplasmic membrane-anchored protein is mediated by TonB (Postle, 1993). The ExbBD protein complex, which is also located in the inner membrane, is associated with TonB. Conformational changes in TonB are facilitated by the ExbBD complex, which harnesses the proton motive force maintained across the cytoplasmic membrane (Larsen et al., 1999).

In bacterial systems, specific high-affinity binding proteins are utilized to interact with their respective ligands, such as sugars, amino acids, oligopeptides, ions, and diverse compounds. Periplasmic binding proteins (PBPs) are pivotal in the process of binding and transporting ferric siderophore complexes across the periplasmic space. Subsequently, the PBPs deliver these ligands to specific ATP-dependent transporters or chemotaxis receptors embedded within the inner membrane. Upon association with loaded PBPs, inner-membrane transporters facilitate the translocation of their ligands into the cytoplasm (Davidson & Chen, 2004).

Two theories have been suggested for iron release from siderophores. The first mechanism relies on the observation that siderophores exhibit a significantly lower affinity for ferrous iron compared to ferric iron. Consequently, following iron reduction catalyzed by free intracellular electron donors or siderophore reductases, iron release occurs spontaneously, allowing for the

recycling of the unbound siderophore. In contrast, the second mechanism entails intracellular degradation of the siderophore, suggesting a unidirectional utilization where siderophores are employed only once and cannot be recycled.

The conversion of Fe^3+ to Fe^2+ occurs either within the cytoplasm or on the inner membrane's cytoplasmic side. Although several studies have suggested the presence of extracellular reductases that could provide cells with easily assimilable Fe^2+, none of these enzymes have been cloned or purified successfully so far. (Schröder et al., 2003). Moreover, these reductases need cofactors to shuttle electrons and endogenous electron shuttles such as NAD(P)H, FAD, FMN and GSH which do not freely diffuse through membranes. Such extra cytoplasmic reductases could hence use either exogenous electron donors present or soluble quinones that freely diffuse through the envelope. Quinolic compounds have been characterized in *Shewanella putrefaciens*, and are found to be utilized in extracellular electron transfer, enabling Fe^{+3} reduction with iron as terminal electron acceptors (Newman & Kolter, 2000). Whether the resulting Fe^{+2} is used as an iron source by *S. putrefaciens* or other symbiotic organisms is not known.

Under anaerobic or reducing conditions, Fe^{2+} predominates as the primary form of iron available to microorganisms. This highly soluble ion freely diffuses through the outer membrane porins of Gram-negative bacteria. Subsequently, it is transported across the cytoplasmic membrane via an ABC Fe^{2+} transporter that is conserved across many species (Kammler et al., 1993).

$$Fe^{3+} + e^{-} \rightarrow Fe^{2+}$$

1.4 Marine Siderophore

Observations indicate that marine bacteria require micromolar concentrations of iron.; however, the iron levels in surface ocean waters typically range from only 0.01 to 2 nanomoles

per litre (Sandy & Butler, 2009). Therefore, many marine bacteria from the alpha and gamma proteobacteria groups are recognized for synthesizing siderophores. Marine siderophores can be broadly classified into two main groups: (a) Siderophores that are generated as suites of amphiphiles and vary in the length of a fatty acid appendage (Homann et al., 2009; Y. Ito & Butler, 2005; Martin et al., 2006), and (b) siderophores produced containing an α-hydroxy carboxylic acid moiety, which exhibits photo-reactivity upon coordination with Fe^{3+} (Barbeau et al., 2003; Hickford et al., 2004; Küpper et al., 2006). The aquachelins, marinobactins, ochrobactins, and synechobactins bind Fe^{3+} through both oxygen atoms of each hydroxamate group and both oxygen atoms of the α-hydroxy carboxylate group. Amphibactins, on the other hand, coordinate Fe^{3+} using the three hydroxamate groups. The siderophores all feature either one or two α-hydroxy carboxylate groups, such as β-hydroxy aspartic acid in the alterobactins and pseudoalterobactins, or citric acid in aerobactin and petrobactins. The alterobactins and pseudoalterobactins bind Fe^{3+} via the two β-hydroxy aspartate moieties and one catecholate group, while petrobactin and petrobactin sulfonate coordinate Fe^{3+} with the two catecholates and the α-hydroxy acid portion of the citrate backbone.

The defining and distinctive trait of marine siderophores is their photoreactivity (Barbeau et al., 2003). Siderophores incorporating an α-hydroxycarboxylate moiety undergo oxidation when complexed with Fe^{3+}, leading to the reduction of Fe^{3+} to Fe^{2+}. Marine siderophores containing fatty acid moieties possess a crucial property of amphiphilicity, with the extent varying across different types. These variations in amphiphilicity arise from differences in head-group composition relative to the length of the fatty acid chain. Amphibactins and ochrobactins exhibit high hydrophobicity and are typically extracted from the bacterial pellet (Martin et al., 2006). In contrast, aquachelins are isolated from the aqueous supernatant, indicating their hydrophilic nature.

1.5 Applications of siderophores

The emergence of bacterial resistance to antimicrobial agents poses significant challenges in the treatment of various bacterial infections. To counteract permeability-mediated drug resistance, the 'Trojan horse' strategy has been employed. This strategy exploits bacterial iron uptake systems to infiltrate and eliminate bacteria. In this approach, the siderophore-drug complex is recognized by specific siderophore receptors, facilitating its active transport across the outer membrane. The recent recognition of the efficacy of this strategy has spurred the synthesis of a range of siderophore-based antibiotics. Numerous studies have demonstrated that siderophore-drug conjugates enable the design of antibiotics with enhanced cellular transport and a reduced likelihood of resistance mutations. The increasing interest in siderophore-drug conjugates for treating human diseases, including iron overload, cancer, and malaria, has propelled efforts to discover novel siderophore-drug complexes. This strategy holds particular significance for the development of therapeutics based on iron oxide nanoparticles (Górska et al., 2014).

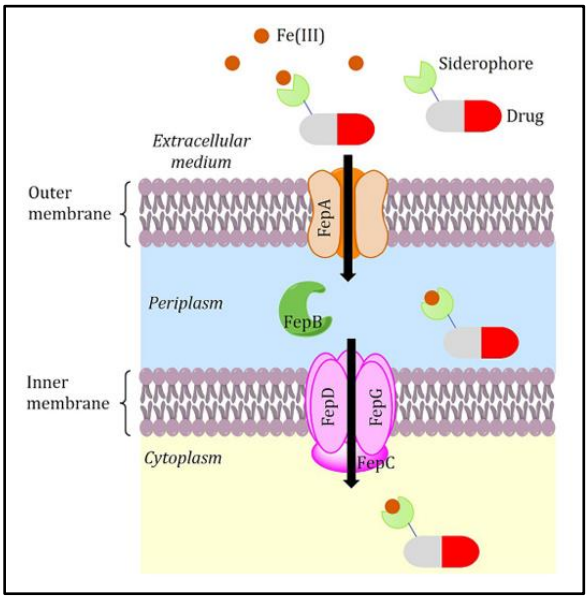

Fig. 1.5 Schematic representation of the antibiotic-siderophore conjugate transport for precise delivery into the cell. (Ref. (Rodríguez & González-Bello, 2023))

Chapter 2

Siderophore producing bacteria from mangrove ecosystem: isolation, screening, and identification of selected isolate.

2. Materials and Methods

2.1 Culturing organisms from mangrove sediment sample

Bacterial strains were isolated from sediment samples collected from two sites of mangroves located in Ribander, Goa, Site 1 (Lat 15.504356°, Long 73.862673°) and Site 2 (Lat 15.501845°, Long 73.876639°) **(Fig. 3.1).** The sediment samples were obtained from two specific locations. The total count of culturable bacteria in each sample was determined by serially diluting 100 µl of the sample into various dilutions (10^1, 10^2, and 10^3) with a 2.5% saline solution, and then spreading them on Zobell Marine Agar. Subsequently, the plates were incubated at room temperature (20-25°C). All identifiable bacterial colonies were purified through streaking on ZMA plates. The isolated cultures were preserved at 4°C and were subsequently employed for the screening of siderophore-producing bacteria.

2.2 Screening of siderophore producing bacteria

Siderophore producing bacterial isolates were separated from total culturable colonies by Chrome Azurole S (CAS) agar plate method. Cas Agar plate was incubated for 2 days at room temperature.

2.3 Isolation of siderophore producing bacteria

Following 48 hours of incubation, the CAS plates were examined for any noticeable color changes. Orange halos around the colony on the plates were selected using a sterile loop, and the colony were then inoculated on Zobell Marine Broth (ZMB) to isolate the organism. This method facilitates the isolation of a pure strain for subsequent characterization. ZMB broth was then incubated at 30°C for 3 days.

2.4 Purification and maintenance of the isolates

The predominant colonies were selected and the isolates were purified through repeated streaking on the respective media. To preserve them, the isolates were streaked onto slants, and stored at 4°C.

2.5 Characterization of the type of siderophore

Microbial siderophore consist of four main types. Hydroxamates produced by bacteria and fungi. Catecholate, Carboxylate and Mixed type produced by bacteria. Out of these Catecholate or Hydroxamate is generally produced by the bacteria. (Ahmed & Holmström, 2014)

2.5.1 Assay for hydroxamate siderophore

The Hydroxamate siderophore assay involved Csáky tests.

For confirmation of Hydroxamate siderophore production, the Csáky assay was employed. In this assay, 1 mL of culture supernatant was hydrolyzed with 1 mL of 6 M H_2SO_4 and autoclaved for 30 minutes. To this mixture, 3 mL of sodium acetate (35%), 1 mL of sulfanilic acid (1% in 30% acetic acid), and 0.5 mL of iodine solution (1.3% in 30% acetic acid) were added. After a 5-minute incubation, excess iodine was neutralized with 1 mL of sodium arsenite solution (2% w/v). Subsequently, 1 mL of naphthylamine (0.3% in 30% acetic acid) was added, and the mixture was allowed to develop a pink color for 20 minutes.

2.5.2 Assay for Catecholate siderophore

Arnow's assays were employed to confirm catecholate siderophore production.

Arnow's assay served as a confirmatory test for catecholate-type siderophore production. To 1 mL of culture supernatant, 1 mL of HCl was added, followed by 1 mL of nitrite-molybdate solution. Subsequently, 1 mL of 1 N NaOH was added to develop the color, with a change to red indicating a positive result.

2.6 Identification of the selected isolate

(i) Morphological characterization

Gram staining technique was used to identify the Gram characteristics and morphology of the isolates.

(ii) Molecular characterization

a) DNA extraction

The genomic DNA was extracted using Phenol Chloroform extraction method.

1. Transfer 1.5 ml of the overnight E. coli culture (grown in LB medium) to a 1.5 ml Eppendorf tube and centrifuge at max speed for 1min to pellet the cells.

2. Discard the supernatant. Note: Remove as much of the supernatant as you can without disturbing the cell pellet.

3. Resuspend the cell pellet in 600 µl lysis buffer and vortex to completely resuspend cell pellet.

4. Incubate 1 h at 37 °C.

5. Add an equal volume of phenol/chloroform and mix well by inverting the tube until the phases are completely mixed.

Note: Do not vertex the tube—it can shear the DNA.

CAUTION: Phenol is a very strong acid that causes severe burns. Chloroform is a carcinogen. Wear gloves, goggles and lab coat, and keep tubes capped tightly. To be safe, work in the hood if possible

6. Spin at max speed for 5 min at RT (all spins are performed at RT, unless indicated otherwise). There is a white layer (protein layer) in the aqueous: phenol/chloroform interface.

7. Carefully transfer the upper aqueous phase to a new tube by using 1 ml pipette (to avoid sucking the interface, use 1 ml tip with wider mouth-cut 1 ml tip-mouth about ~2 mm shorter).

8. Steps 4-6 can be repeated until the white protein layer disappears.

9. To remove phenol, add an equal volume of chloroform to the aqueous layer. Again, mix well by inverting the tube.

10. Spin at max speed for 5 min.

11. Remove aqueous layer to new tube.

12. To precipitate the DNA, add one tenth volume of sodium acetate and 2 volume of ice-cold ethanol (store ethanol at -20 °C freezer) and mix gently (DNA precipitation can be visible). Note: DNA precipitation may simply diffuse, which is normal. Keep the tube at -20 degree for at least 30 min (the longer the better) and then spin it down (see Steps 13-14). You should see DNA pellet. It looks transparency when it is wet and turns to white when it becomes dry.

13. Incubate the tube at -20 °C for 30 min or more.

14. Spin at max speed for 15 min at 4 °C.

15. Discard the supernatant and rinse the DNA pellet with 1 ml 70% ethanol (stored at RT).

16. Spin at max speed for 2 min. Carefully discard the supernatant and air-dry the DNA pellet (tilt the tube a little bit on paper towel). To be faster, dry the tube at 37 °C incubator.

17. Resuspend DNA in 20 microlitre of TE buffer.

18. Check isolated Genomic DNA on an agarose gel. Note: we expect to see bands with smear patterns from high to low MW range

b) 16s rRNA gene amplification

The isolated DNA was sent to Bio-Lab, Goa for the purification and 16s rRNA gene amplification.

c) BLAST and alignment of 16s rRNA gene sequence

The 16S rRNA gene sequence was analysed by comparing it with sequences available in the GenBank database utilizing the BLAST search program (Altschul et al., 1990). The 16S rRNA gene sequences were aligned using the multiple alignments Mega program (Kumar et al., 2008).

Chapter 3

Result and Discussion

3. Result and Discussion

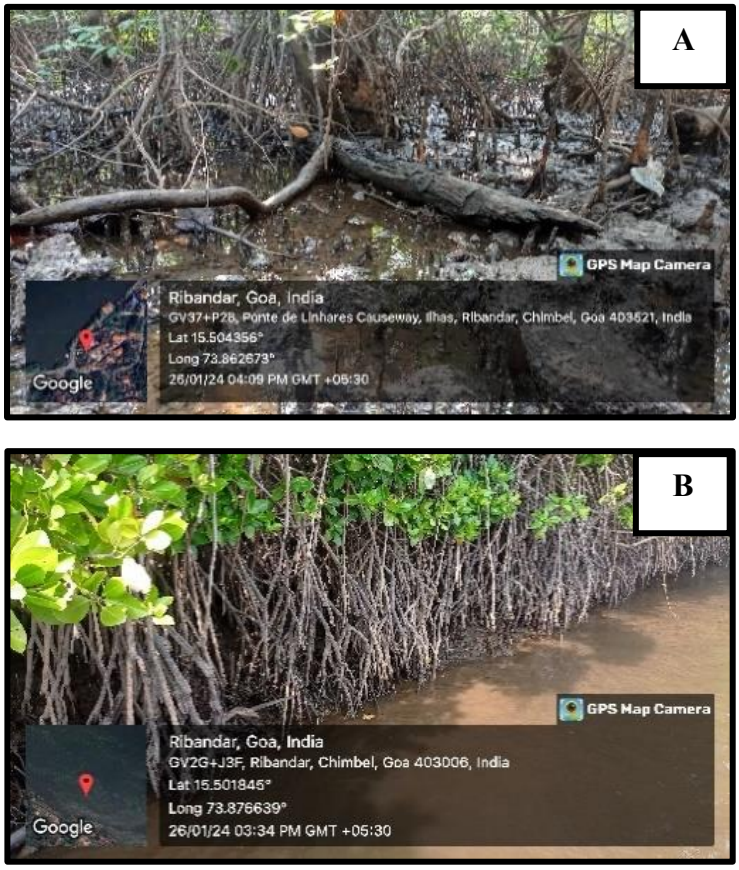

Fig 3.1 Sampling sites of Mangrove ecosystem at Ribander (A) Site 1 (B) Site 2

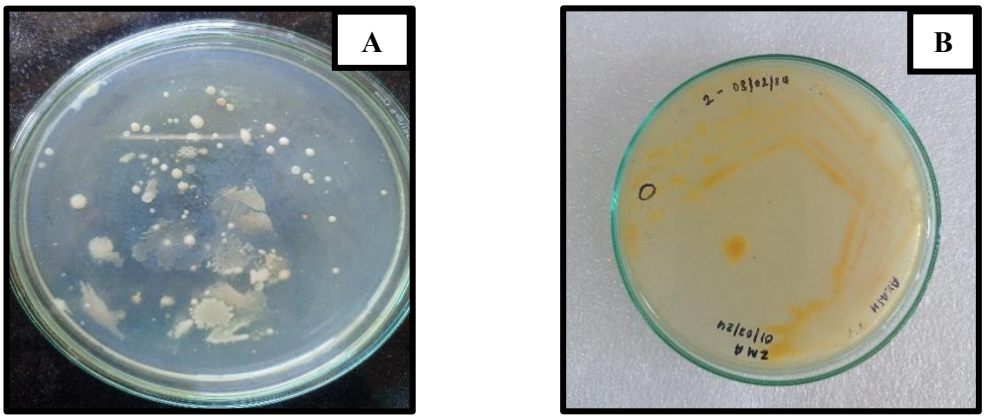

Fig 3.2 (A) Culture grown on 10^1 dilution ZMA plate (B) Pure culture of bacteria producing siderophore.

3.1 Screening of siderophore producing bacteria

Siderophore producing bacterial isolates were separated from total culturable colonies by Chrome Azurole S (CAS) agar plate method. Cas Agar plate was incubated for 2 days at room temperature. The positive isolates depict a yellow zone on CAS agar **(Fig 2.3)**.

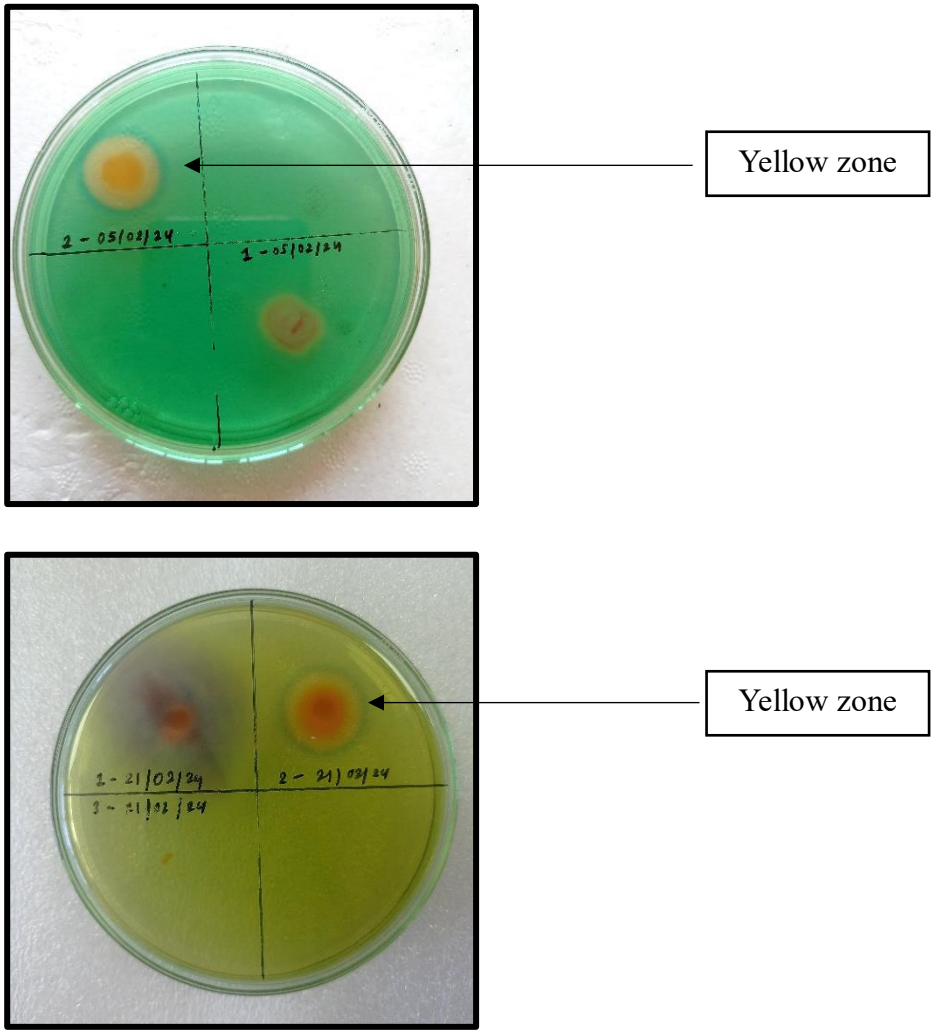

Fig 3.3 Formation of yellow zone around the colony of siderophore producing bacteria on CAS agar plate in duplicates

The test is based on the siderophores' capacity to bind to ferric iron with high affinity. The agar contains Chrome Azurol S (CAS) dye, which becomes blue/green when complexed with Fe3+. If Inoculated organisms produce siderophores, which remove ferric iron from the dye and alter the media's color from blue/green to orange or yellow. Coloration around the inoculation site indicates the presence of siderophores (Elgazzar, n.d.).

3.2 Isolation of siderophore producing bacteria

After incubating for 48 hours, The CAS plates were inspected for any alterations in color. Colonies surrounded by orange halos on the plates were chosen using a sterile loop, and the colony were then inoculated on Zobell Marine Broth (ZMB) to isolate the organism. This method allows for obtaining a pure strain, facilitating further characterization. The ZMB broth was subsequently incubated at 30°C for a period of 3 days. The isolated sample was assigned a unique identifier for further analysis. Purified colonies underwent gram staining to determine their gram response and physical characteristics, distinguishing between gram-positive and gram-negative bacteria, including bacilli and cocci.

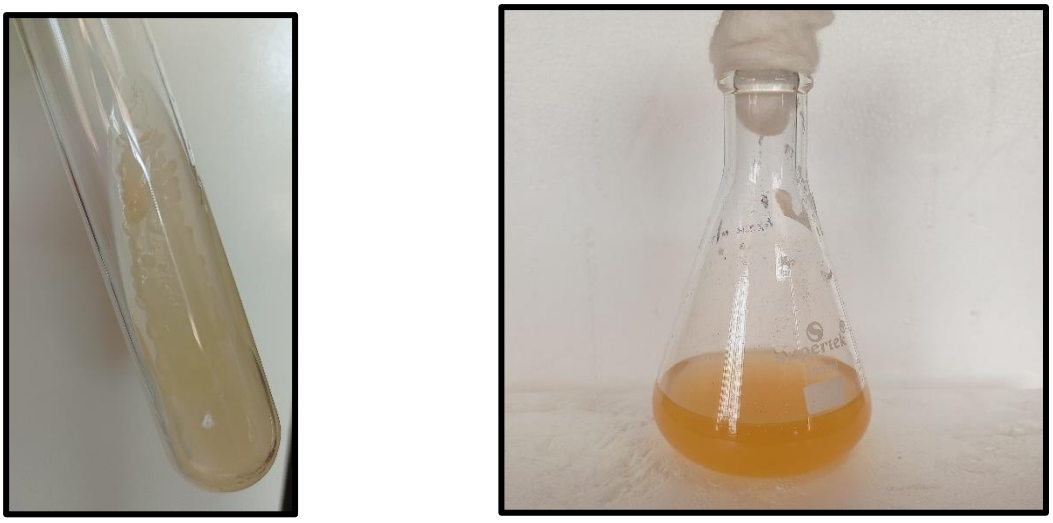

Fig. 3.4 (A)Maintenance of the culture by streaking on slants and stored at 4°C

(B) Culture inoculated in ZMB

3.3 Identification of the selected isolate

Based on morphological characterization, the siderophore-producing bacteria were identified as Gram negative and cocci.

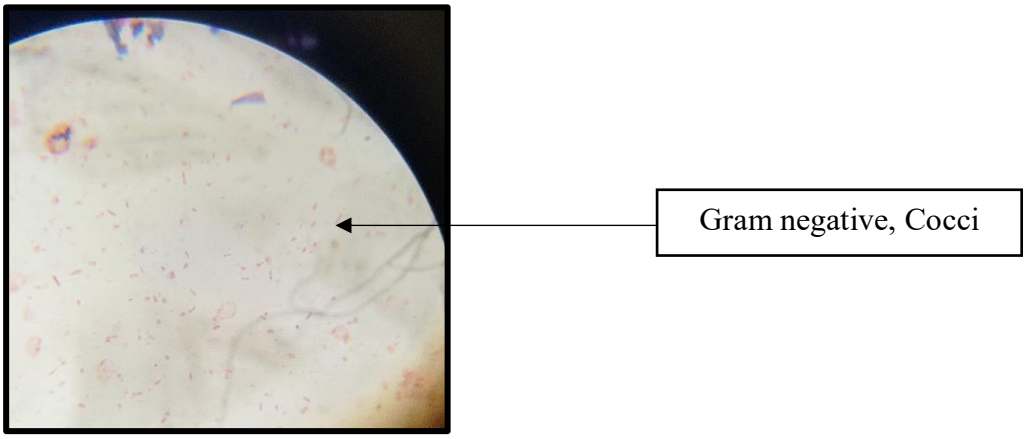

Fig. 3.5 Bacteria were identified as Gram negative after performing Gram staining.

Chapter 4

Application of siderophore: siderophore-antibiotic conjugate drug

4.1 Introduction

Antibiotics are super important for fighting bacterial infections. But if we use them too much, it can mess up the balance of bacteria in our bodies, making the antibiotics less effective over time (Murray et al., 2022). One challenge in creating new antibiotics is making sure they can get inside bacteria efficiently. This is especially tricky when treating infections caused by certain types of bacteria called gram-negative pathogens (Jones, 2017). Recently, scientists have been focusing more on developing precise methods to transport antibiotics effectively into bacteria. This area of research has become increasingly important in the development of new antibiotic drugs (Richter et al., 2017). The first antibiotic approved based on this idea is cefiderocol. It involves adding specific functional groups to the antibiotic structure, which have a strong attraction to and selectivity for Fe(III), also known as siderophores. Siderophores are produced by bacteria and act as iron chelators. This modification ensures that the antibiotic is effectively and precisely absorbed by the bacteria. These compounds can form stable iron complexes, making it easier for them to get inside the bacterial cells through pathways used by bacteria to take in iron (El-Lababidi & Rizk, 2020; Górska et al., 2014; Raymond & Carrano, 1979; Schalk & Mislin, 2017). Using siderophores to transport antibacterial agents is a promising strategy that can enhance the effectiveness of drugs, especially against gram-negative pathogens. This method has been particularly helpful in delivering β-lactam antibiotics, which make up around 70% of prescribed antibiotics today. This review providesan overview of how this approach works at the molecular level and discusses recent successful examples (Rodríguez & González-Bello, 2023).

The idea of using antibiotic-siderophore conjugates to deliver antibacterial agents draws inspiration from nature, particularly from how bacteria acquire iron from the host's

environmental sources (Miller & Liu, 2021). In November 2019, the FDA approved the first antibiotic-siderophore conjugate, called cefiderocol (previously known as S-649266), developed by Shionogi & Co. Ltd. This approval was specifically for intravenous treatment of severe infections caused by gram-negative bacteria. In September 2020, cefiderocol received further approval for treating hospital-acquired bacterial pneumonia and ventilator-associated bacterial pneumonia caused by susceptible gram-negative bacteria. The structure of Cefiderocol consist of Cephalosporin and Enterobactin catecolate-siderophore.

4.2 Cefiderocol

Cefiderocol (S-649266) is a new combination of a catechol-type siderophore and a cephalosporin antibiotic developed by Shionogi & Co. Ltd. It utilizes the siderophore-iron complex pathway to enter the outer membrane of Gram-negative bacteria, alongside the typical passive diffusion through membrane porins. Its chemical structure comprises a cephalosporin core with side chains resembling those of ceftazidime and cefepime. Notably, the aminothiazole ring and carboxypropyl-oxyimino group attached to the 7-position side chain provide increased effectiveness against Gram-negative bacilli. Once inside the periplasmic space, cefiderocol separates from the iron and attaches to penicillin-binding proteins (PBP), particularly PBP3. This binding action inhibits the synthesis of peptidoglycan, a crucial component of bacterial cell walls (A. Ito et al., 2018; Zhanel et al., 2019).

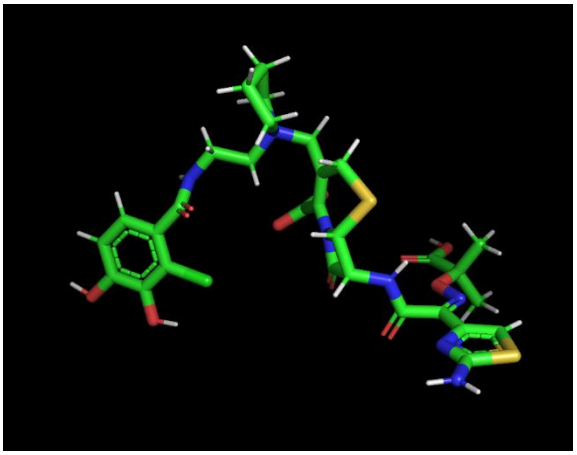

Fig. 4.1 3D representation of the structure of cefiderocol (FDA approved) visualized using PyMOL

4.3 Cephalosporin

Cephalosporins are a type of beta-lactam antimicrobial medication commonly prescribed to treat a broad spectrum of infections caused by both gram-positive and gram-negative bacteria. There are five generations of cephalosporins, each with unique characteristics and applications. They are effective against various infections such as skin infections, bacteria that are resistant to other antibiotics, meningitis, and many others.

This overview will cover the indications, contraindications, and potential adverse effects associated with cephalosporins. Additionally, it will discuss their mechanism of action, common adverse reactions, monitoring requirements, routes of administration, and other essential considerations (Hsieh & Ho, 1975).

Bacteria produce a cell wall that is reinforced by connecting peptidoglycan units with the help of penicillin-binding proteins (PBPs), also known as peptidoglycan transpeptidases. Cephalosporins belong to a large group of bactericidal antimicrobial drugs that function through their beta-lactam rings. These rings attach to penicillin-binding proteins, disrupting

their normal activity. As a result, bacteria are unable to synthesize their cell wall, leading to their eventual death (Bui & Preuss, 2024).

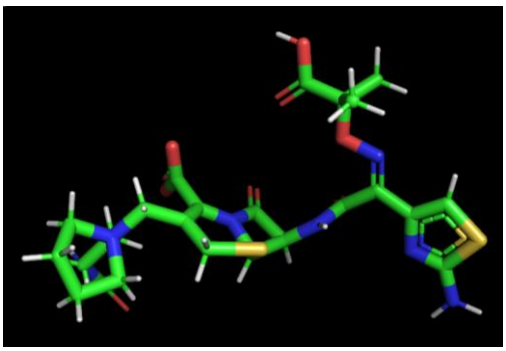

Fig. 4.2 3D representation of the structure of cephalosporin visualized using PyMOL

4.4 Enterobactin catecholate siderophore

Enterobactin (also referred to as enterochelin) is the distinctive siderophore utilized by bacteria belonging to the Enterobacteriaceae family (Pollack & Neilands, 1970). This catecholate-type siderophore, composed of a cyclic trimer of 2,3-dihydroxy-N-benzoylserine, is produced by the majority of genera within the Enterobacteriaceae family (Payne & Neilands, 1988). Bacillibactin, another siderophore, shares a similar structure to enterobactin.

Enterobactin is the primary tris-catechol-containing siderophore found in enteric gram-negative bacteria such as Escherichia coli, Salmonella typhimurium, and Klebsiella pneumoniae. Being the first catechol-based siderophore identified, enterobactin has significantly contributed to our understanding of the fundamental coordination chemistry of siderophores (Codd, 2023).

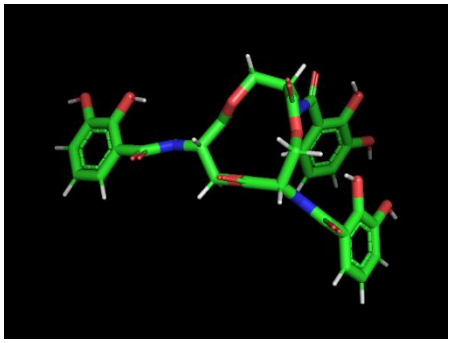

Fig. 4.3 3D representation of the structure of enterobactin catecolate-siderophore visualized using PyMOL

4.5 Siderophore-drug conjugate

In this research, we conducted compound docking simulations using PyRx software to explore the interactions between catecholate siderophores and cephalosporin antibiotics. Catecholate siderophores, exemplified by enterobactin, are vital molecules produced by various gram-negative bacteria like *Escherichia coli* and *Klebsiella pneumoniae*. These siderophores play a critical role in acquiring iron, a crucial nutrient for bacterial growth and survival. By studying their interactions with cephalosporin antibiotics, such as cefiderocol, we aim to gain insights into potential synergistic effects or novel mechanisms of action.

The identification of enterobactin, the inaugural catechol-based siderophore, has not only broadened our comprehension of bacterial iron procurement tactics but has also laid the groundwork for investigating the coordination chemistry of siderophores. Through the computational simulations, we strive to contribute to this wealth of knowledge by elucidating the structural and molecular basis of interactions between catecholate siderophores and cephalosporin antibiotics.

4.5.1 Selection and the preparation of siderophore group of enterobactin

The siderophore group of enterobactin where iron used to bind was selected within PyMOL software and used as the ligand for docking with antibiotic. The ligand was prepared in Discovery Studio Visualization software by adding hydrogens for proper binding with the receptor. **(Fig. 4.4 A).**

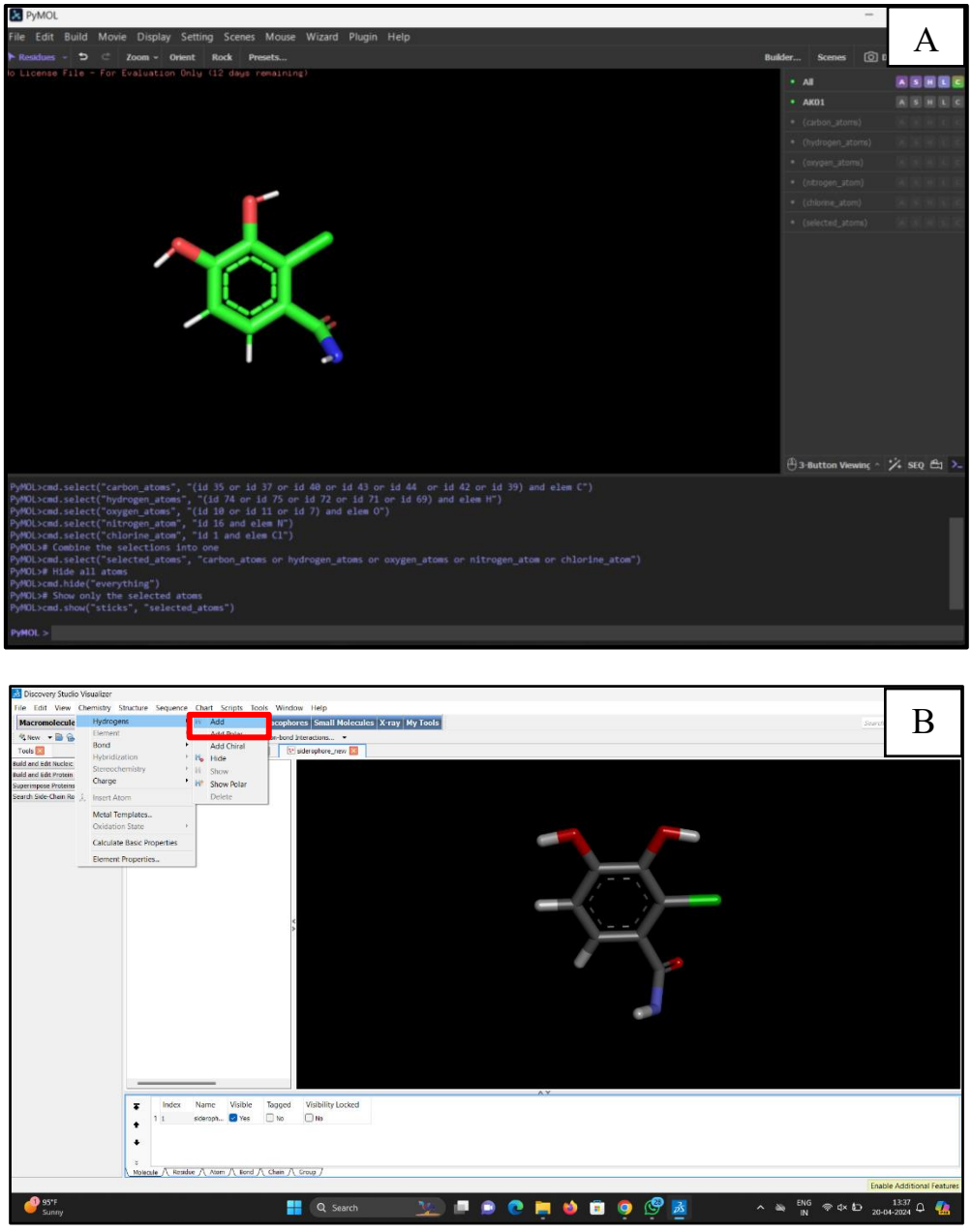

Fig. 4.4 (A)Selection of the enterobactin siderophore group in PyMOL(B) Preparation of the siderophore group for docking in Discovery Studio Visualization

4.5.2 Preparation of cephalosporin antibiotic for docking

The cephalosporin antibiotic was downloaded from the PubChem website and then prepared for docking using Discovery Studio Visualizer software. To prepare the cephalosporin as a receptor, hydrogens and charges were added to the cephalosporin structure to ensure proper binding with the ligand.

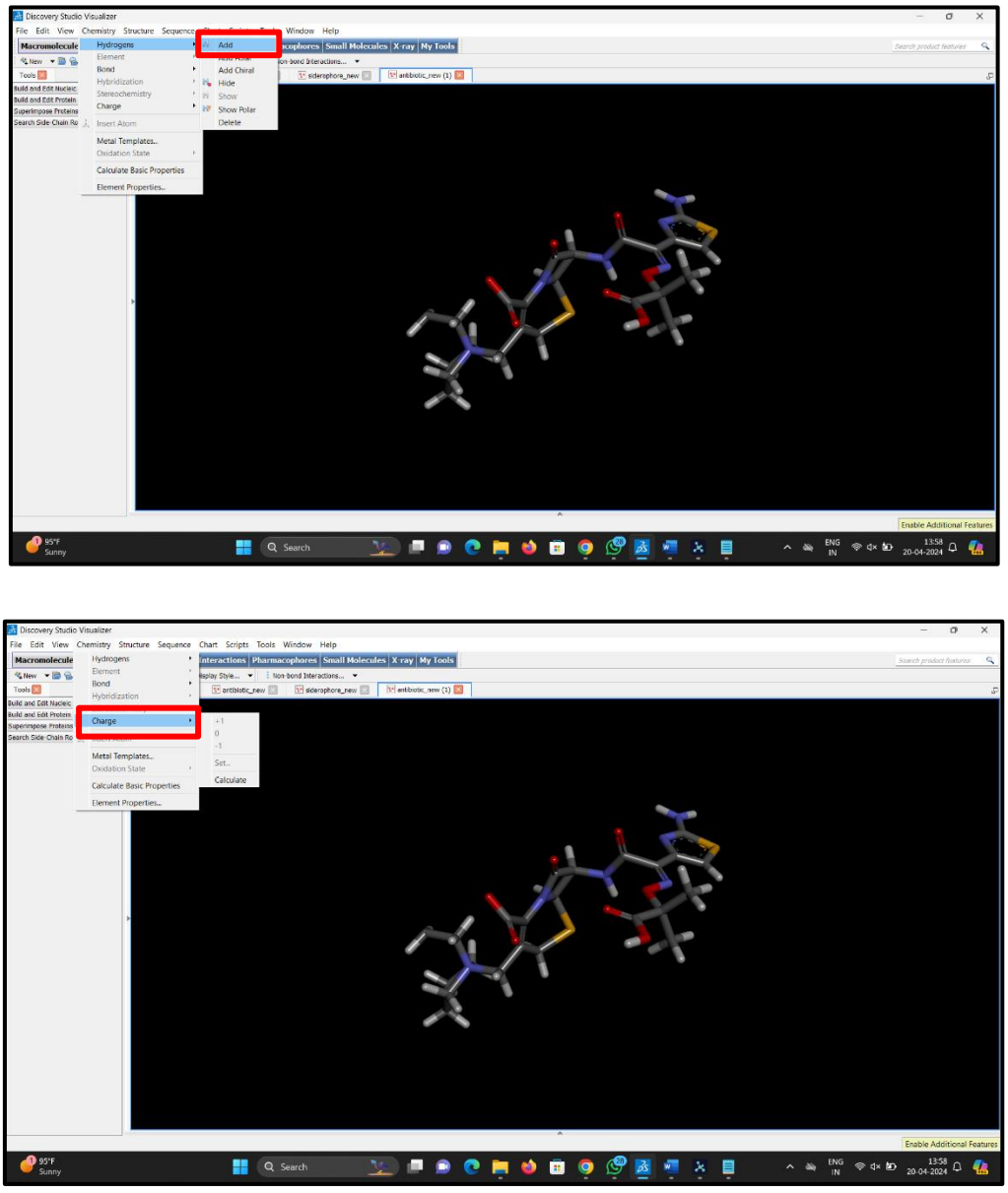

Fig. 4.5 Preparation the cephalosporin structure for docking in Discovery Studio Visualization software.

4.5.3 Docking of the compounds

The compound docking was performed using the "PyRx - Virtual Screening Tool" software. Both the siderophore and cephalosporin were imported into the PyRx - Virtual Screening Tool software, and both compounds were prepared as ligands and macromolecules for the docking process.

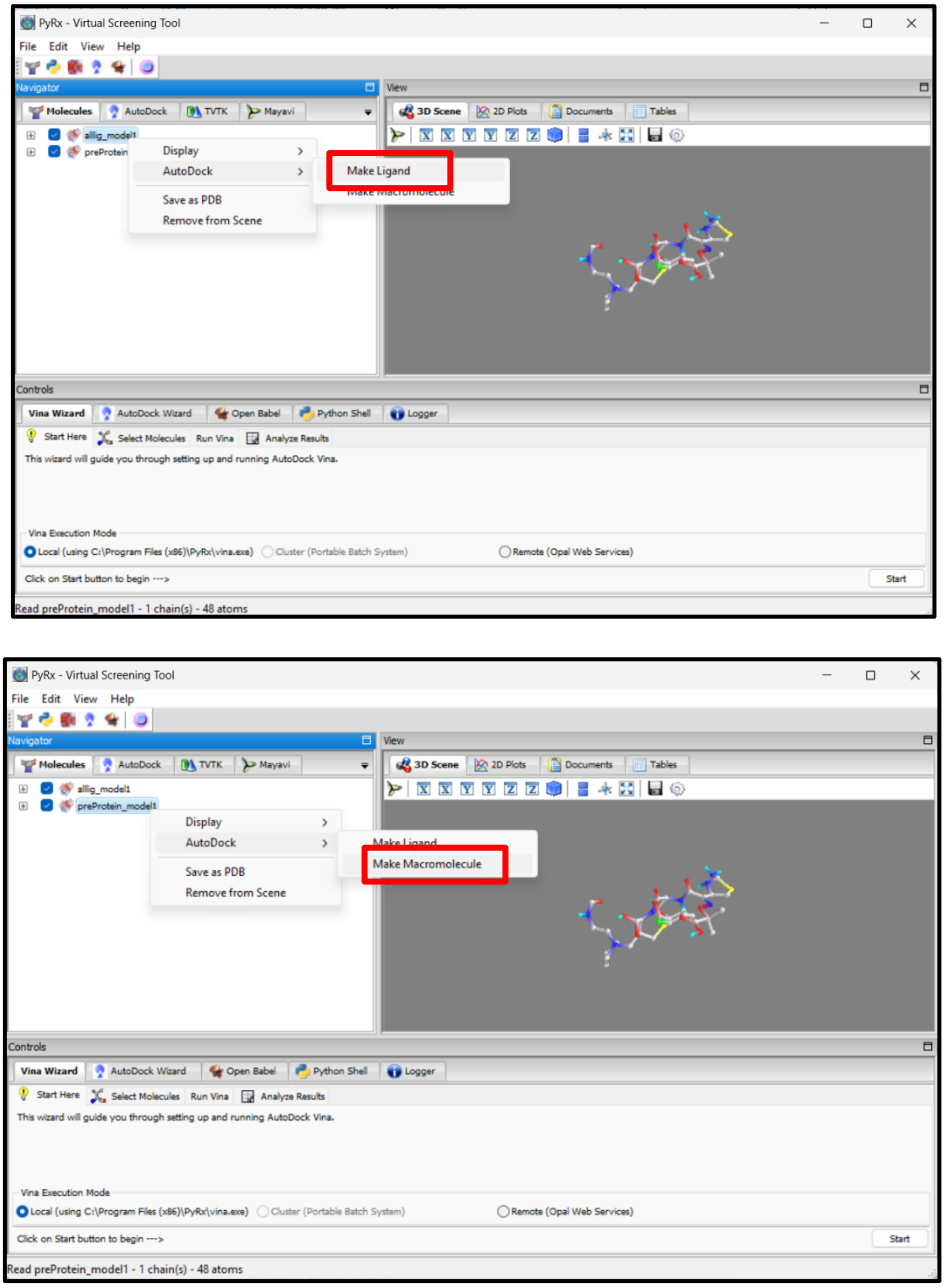

Fig. 4.6 Preparation of the compound as ligand and receptor for the docking in PyRx.

Before conducting targeted docking, blind docking was executed to identify binding sites within the macromolecule (cephalosporin). Binding affinity within the range of -5.0 to -8.0 kcal/mol indicates high affinity. Once a specific site with strong binding affinity was identified, a grid was generated in that particular site for targeted docking.

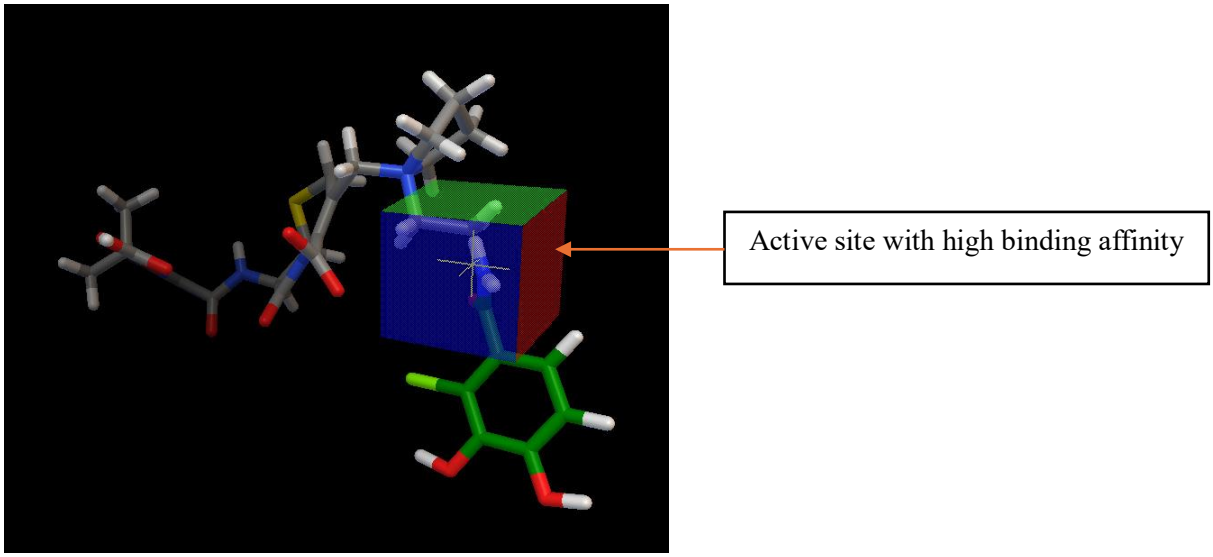

Fig. 4.7 Compound docking of siderophore and antibiotic

This research bridges the gap between fundamental coordination chemistry and practical applications in antibiotic development. By comprehensively analyzing the docking results, we aim to uncover potential avenues for enhancing the efficacy of cephalosporin antibiotics, especially against multidrug-resistant gram-negative pathogens. Ultimately, our discoveries could lay the groundwork for devising therapeutic approaches to combat infectious diseases and tackle the worldwide issue of antibiotic resistance.

Summary

Iron is an essential mineral that is required for the growth, development, and metabolism of all living things. However, iron is often scarce in many environments, and this can lead to iron deficiency. Iron deficiency can have a number of negative consequences for organisms, including affecting the activity of some enzymes, impairing growth and development, and increasing susceptibility to disease.

In order to overcome iron deficiency, many organisms have evolved mechanisms to scavenge and utilize iron more efficiently. One such mechanism is the production of siderophores. Siderophores are small, iron-binding molecules that are produced by a wide variety of bacteria, fungi, and plants. Siderophores chelate iron, making it more available for the organism that produced them. This can help to improve the growth and survival of organisms in iron-poor environments.

The bacteria that produce siderophores are called siderophores. Siderophores are classified as hydroxamates, catecholates, carboxylates, or mixed types depending on the chemical makeup of their coordination sites. Bacterial siderophores are either hydroxamate or catecholate-type. Siderophore production is influenced by iron bioavailability and growth conditions such as temperature, nutrition, carbon supply, and so on.

Siderophores have a strong affinity for ferric iron, but they may also form complexes with metals other than Fe^{3+}, although with a lesser affinity.

In recent years, there has been a great deal of interest in the use of siderophores for antibiotic delivery. Antibiotics are drugs that are used to treat bacterial infections. However, many bacteria are becoming resistant to antibiotics. One way to overcome this resistance is to use siderophores to deliver antibiotics to bacteria. Siderophores can bind to antibiotics and carry them into bacteria. This can make the antibiotics more effective against bacteria that are resistant to them.

Cefiderocol is a new antibiotic that uses siderophores for delivery. Cefiderocol is catechol-type siderophore and a cephalosporin antibiotic. It utilizes the siderophore-iron complex pathway to enter the outer membrane of Gram-negative bacteria, alongside the typical passive diffusion through membrane porins. This makes cefiderocol more effective against Gram-negative bacteria than other antibiotics.

The use of siderophores for antibiotic delivery is a promising new area of research. Siderophores can help to make antibiotics more effective against bacteria that are resistant to them. This could lead to new and improved treatments for bacterial infections.

Future perspective

The research presented in this dissertation has provided a comprehensive understanding of the diversity of siderophores produced by bacteria isolated from mangrove ecosystems. The isolation and characterization of these siderophores has paved the way for future studies aimed at their potential applications in drug delivery. In particular, the results of this study suggest that siderophores could be used as carriers for the targeted delivery of antibiotics to bacterial cells. This could lead to the development of new and more effective therapeutic strategies for a variety of diseases.

Furthermore, the identification of siderophore-producing bacteria from mangrove ecosystems has opened up new possibilities for the discovery of new bioactive compounds. These compounds could have a wide range of applications, including in the fields of medicine, agriculture, and environmental science.

The future perspective of this research is bright. With continued research, it is possible to develop more effective and targeted ways to use siderophores for drug delivery. This could lead to a new era of antibiotic therapy that is more effective and less likely to cause side effects. Additionally, the discovery of new siderophore-producing bacteria from mangrove ecosystems could lead to the discovery of new bioactive compounds with a wide range of applications. This research has the potential to make a significant contribution to the fields of medicine, agriculture, and environmental science.

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Appendix

Appendix – A

Media Composition

A.1 Kings B medium base

Ingredients	g/L
Peptone	20
Dipotassium hydrogen phosphate	1.50
Magnesium sulphate	1.50
Glycerol	15
Agar	15
Distilled water	900
pH	6.8±0.2

Appendix – B

Composition of reagents and buffers

B.1 Reagents

B.1.1 Reagents for Csaky Test

i) Sulphanilic acid solution

Sulphanilic acid	1 g
Acetic acid (30%) (make to final volume)	100 ml

ii) Iodine solution

Iodine	1 g
Glacial acetic acid (make to final volume)	100 ml

iii) Sodium arsenite

Sodium arsenite	2 g
Distilled water (make to final volume)	100 ml

iv) Sodium acetate

Sodium acetate	35 g
Distilled water (make to final volume)	1000 ml

v) α - napthylamine

α - napthylamine	3 g
Acetic acid (30%) (make to final volume)	1000 ml

B.1.2 Nitrite Molybdate reagent

Sodium nitrite	10 g
Sodium molybdate	10 g
Distilled water (make to final volume)	100 ml

B.1.3 CAS

CAS (in 50 ml distilled water)	60.5 mg
CTAB	72.9 mg
Fe (III) solution	10 ml

B.2 Buffers

B.2.1 Normal saline

NaCl	0.9 g
Distilled water (make to final volume)	100 ml

B.2.2 Tris acetate EDTA buffer (pH 8)

Tris – base	24.2 g
Glacial acetic acid	5.71 ml
EDTA (0.5 M, pH 8)	10 ml
Distilled water (make to final volume)	1000 ml