

ANTI-BIOFILM METABOLITES FROM HIMALAYAN ACTINOBACTERIA

Submitted in partial fulfilment of the requirements for the award of Master
of Technology degree in Biotechnology

by

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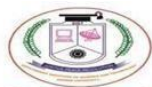
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This is to certify that this Project Report is the bonafide work of Ashmitha.k (41810003) who carried out the project entitled "**ANTI-BIOFILM METABOLITES FROM HIMALAYAN ACTINOBACTERIA**" under our supervision from January 2023 to May 2023.

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ABSTRACT

The production of antibiofilm metabolites from such rare ecosystem gives a great remedy for clinical biofilm which is a serious global health concern, resistant to antibiotics, host defense systems and other external stresses leads to chronic infections. Therefore, it is essential to produce new bioactive metabolites against biofilm pathogens from rare habitat Actinobacteria. The present study aimed to evaluate the *Streptomyces* for Antibiofilm properties against biofilm forming bacterial pathogens viz., *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. For the study about 20 actinobacterial previously isolated from Indian Himalayan region were obtained from the Actinobacterial Research lab, Center for Drug Discovery and Development, Sathyabama Institute of science and technology, Chennai, Tamil Nadu. Actinobacterial extracts were prepared by agar surface fermentation using ethyl acetate and evaluated for biofilm inhibition against biofilm pathogens by crystal violet – microtitre plate (CV-MtP) method, which revealed that 14 extracts showed prominent inhibition against *P. aeruginosa* and *K. pneumonia* for about 50 – 80% of inhibition at 100 µg/mL concentration. Among that, the extract of HS2C7 was showed the eradication of biofilm for about 87% against *K. pneumonia* in CV-MtP method at 100 µg/mL concentration. During optimization Lactose, Yeast extract and CaCl₂ at pH 7 with Distilled water was found to influence the metabolite production. Based on phenotypic and molecular characterization the potential strain HS2C7 was identified as *Streptomyces mutabilis*. Partial purification was done which shows five bands in the solvent of Ethyl acetate: chloroform (0.7:0.3). In Further studies like confocal Analysis and SEM/TEM analysis of the compound will be done.

Key words: Anti-Biofilm, CV-MtP method, Himalaya, Streptomyces.

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LIST OF ABBREVIATION

EPS	Extracellular polymeric substances
pH	Potential of Hydrogen
QS	Quorum sensing
UV	Ultraviolet
DNA	Deoxyribonucleic acid
c-di-GMP	Cyclic diguanosine monophosphate
CDC	Centre for Disease Control
WHO	World health organization
Ag	silver Nano particles
rRNA	ribosomal RNA
SCA	Starch M-protein Agar for actinomycetes
AIA	Actinomycete Isolation Agar
NA	Nutrient Agar
Mtp	Microtitre plate method
TSB	Tryptic Soy Broth
OD	Optical Density
ISP-2	International Streptomyces Project-2 Medium
RF	Retardation Factor
PBS	Phosphate Buffered Saline
TLC	Thin Layer Chromatography

CHAPTER 1

INTRODUCTION

A population of microorganisms that is well-organized and cooperative is called a biofilm. The release of an external polymer matrix, decreased growth rate, and gene regulation distinguish these linked cells from the floating ones (Donlan, 2002). Biofilm production requires intracellular signaling and transcription processes that are different from those seen in planktonic cells and go through multiple phases. The protection provided by the biofilm state against environmental challenges such as pH changes, Ultraviolet rays, osmotic stress and desiccation, penetration of antimicrobial agents, the development of new genetic characteristics, and nutrition availability is crucial for the community's survival (Kokare *et al.*, 2009). The morphological, physiologic, and molecular states of cells change as a result of this fundamental alteration in the environment for life, which results in ongoing alterations in the expression of several genes. Cells are able to withstand severe situations thanks to their residence in a biofilm. Adherence, maturation, separation, and dispersion are all stages in the development of biofilms. Microbial adhesion is the initial and most important phase. Currently, medical research is focused on preventing microbial adherence and blocking quorum sensing (Friedlander *et al.*, 2015; Garrett *et al.*, 2008; Pirog *et al.*, 2014; Sadiki *et al.*, 2017; Adak *et al.*, 2011). Healthcare-associated infections are difficult to treat because of biofilms, especially when medical devices such as urine catheters, intravascular catheterization, and orthopaedic implants are implanted (Francolini and Donelli 2010). Adherence, maturation, separation, and dispersion are all stages in the development of biofilms. Microbial adhesion is the initial and most important phase. Currently, medical research is focused on preventing microbial adherence and blocking quorum sensing (Friedlander *et al.*, 2015; T.R. Garrett *et al.*, 2008; Pirog *et al.*, 2014; Sadiki *et al.*, 2017). (Adak *et al.*, 2011). Healthcare-associated infections are difficult to treat because of biofilms, especially when medical devices such as urine catheters, intravascular catheterization, and orthopaedic implants are implanted (Francolini and Donelli 2010). One of the biggest phyla in the realm of bacteria is the phylum Actinobacteria, which has 23 orders (Ludwig *et al.*, 2012). One of the biggest phyla in the realm of bacteria is the phylum Actinobacteria, which has 23 orders (Ludwig *et al.*, 2012). With the continuing

discovery of new genera, there have been 342 genera in existence in December 2015. Usually, they have favourable Gram results. The actinomycetes life cycle is distinctive. The cycle begins with the germination of "dispersal form" seeds and development in branching hyphae termed green fungus, which differentiates into specific generative structures called aerial mycorrhizal fungi (Wink *et al.*, 2017). Attacking the physiology of bacterial growth and cell division in Actinobacteria has received a lot of attention (Arasu *et al.*, 2008; Ningthouja *et al.*, 2009; Kavitha *et al.*, 2010; Sanchez *et al.*, 2006).

Even though Actinobacteria are widely distributed, there aren't many studies regarding the phylum's biofilm development. This phylum is significant because it includes many organisms with a broad range of phenotypic diversity, vast distribution in nature, and varying oxygen, nutrient, temperature, and pH needs for development (Polkade *et al.*, 2016). They play a significant role in biotechnology thanks to their wide range of physiological possibilities. They have a wide range of uses, including in the pharmaceutical business, the bioremediation sector, and many other fields (Hamedi *et al.*, 2017; Harir *et al.*, 2018; Zhang *et al.*, 2014). They are essential to natural geochemical cycles, particularly because of their capacity to break down organic material. Additionally prevalent in the rhizosphere, actinobacteria generate a variety of physiologically active compounds that have an impact on plant growth (Dharmaraj *et al.*, 2010). Actinobacteria are well-known plant and animal diseases (Miao and Davies 2010). The generation of a large variety of secondary metabolites, including antibiotics and other substances of biotechnological relevance, is one of Actinobacteria most significant potentials, though, and it has been widely utilised (Singh *et al.*, 2015). They are essential to natural geochemical cycles, particularly because of their capacity to break down organic material. Additionally prevalent in the rhizosphere, actinobacteria generate a variety of physiologically active compounds that have an impact on plant growth (Dharmaraj *et al.*, 2010). Actinobacteria are well-known plant and animal diseases (Miao and Davies 2010). However, the creation of a large variety of secondary metabolites, including antibiotics and other substances of biotechnological interest, that have been used, is among Actinobacteria most significant potential (Singh *et al.*, 2015). The development of actinomycetes in fermenters and shaken-flask cultures has been extensively studied (Whitaker 1992). Although surface-associated growth is undoubtedly a distinct phenotype and an intensive in their life cycle, with probable morphological and physiological changes

that may affect metabolite synthesis, cover growth has been given less attention (Alonso *et al.*, 2008). Bacteria exist in multispecies groups in nature where they compete with one another for nutrients and living area (Hibbing *et al.* 2010; Stubbendieck *et al.*, 2016). The majority of bacteria are able to recognize changes in their surroundings and adapt to thrive in new settings. In order to survive in unfavourable environmental circumstances, bacteria have evolved the ability to build biofilms (Ikuma *et al.*, 2013; Dang and Lovell 2016). The incidence of biofilm-associated illnesses in people has been facilitated by this, nevertheless. Infections caused by biofilms are typically difficult to cure because the body's immune system cannot reach the biofilms to remove the germs. Additionally, the currently accessible medicines only work against planktonic cells (Hall and Mah 2017). To tackle bacterial biofilms, new medications and methods are desperately needed. Anti-biofilm substances should not be toxic in order to prevent the development of drug resistance because biofilms may also impart resistance to antimicrobials (Hengzhuang *et al.*, 2011; Penesyan *et al.*, 2015). Knowing biology, genetics, and biochemistry is also crucial for the creation of anti-biofilm chemicals. This includes understanding the process by which biofilms become resistant to antimicrobial drugs, whether at the societal or cellular level. It is widely documented that research on microbes is a possible source of novel and powerful therapeutic drugs with various mechanisms of action (Cragg and Newman 2013). Actinobacteria, the most prevalent phylum of bacteria, is one of them and is a rich source of novel bioactive substances (Azman *et al.*, 2017). (Raveh *et al.*, 2013; Claveras *et al.*, 2015; Spadari *et al.*, 2015; Abd-Elanby *et al.* 2016; Azman *et al.* 2017) Actinobacteria have been shown to generate a variety of bioactive chemicals of clinical significance, comprising anti-bacterial, fungicidal, antiviral, cytotoxic, and neuroprotective substances. The primary producers of metabolites are actinobacterial species. Their genetic make-up and metabolic capacity show considerable promise as a source of medicines that inhibit biofilms. Their physiologic potential for producing different bioactive natural products, such as antibiotics, anticancer agents, antivirals, anthelmintics, immunosuppressive agents, herbicides, and enzymatic activities with various industrial uses is reflected in their diverse array of habitat types, such as terrestrial and aquatic ecosystems (van Bergeijk *et al.*, 2020). This study highlights the mechanism of biofilm formation, Antibiofilm properties of Actinobacteria and need for new bioactive metabolites against biofilm pathogens from rare habitat Actinobacteria.

CHAPTER 2

REVIEW OF LITERATURE

2.1 BIOFILMS

The formation of a biofilm by bacteria allows them to enter a habitat that not only protects them from the harsh environment but also encourages the storage of essential micronutrients (Flemming *et al.*, 2016). They are described as heterogeneous and homogeneous microbial communities that live in an extracellular polymeric substance (EPS) matrix that they self-produced. This matrix allows for the exchange of a variety of crucial secondary metabolites, stress-resistant genetic material, and signaling molecules. About 90% of the overall biofilm is made up of EPS, whereas less than 10% of the dry bulk is made up of microbial cells. The interactions between the EPS components, which control how the bacteria in the biofilm behave physiologically, provide the basis for the EPS's self-organization in the matrix. The biofilm's matrix guards bacteria from human immune systems in addition to environmental stresses such as desiccation, UV radiation, metals, antibiotics, and oxidising chemicals (Wingender *et al.*, 2010). So colonies of grouped bacterial cells encased in external polymer matrices constitute biofilms (Singh *et al.*, 2017). They have also been poetically described as a "city of bacteria," whereby the EPS stands in for the "home of the biofilm" (Flemming *et al.*, 2007). Biofilms may develop on a wide range of surfaces, including living tissues, industrial/potable water pipelines, natural aquatic systems, and home medical equipment. Gram-positive and Gram-negative bacteria can both form biofilms. For example, life-threatening infections are linked to *Proteus vulgaris*, *Escherichia coli*, *Staphylococcus aureus*, *E. faecalis*, *Klebsiella pneumoniae*, *Mycobacterium tuberculosis*, Fatal neurodegenerative gonorrhea, *Bacillus subtilis* and *Helicobacter* (Khatoon *et al.*, 2018).

2.2 MECHANISM OF BIOFILM FORMATION

The process of forming a biofilm is intricate and multifaceted. It necessitates the transcription of a unique set of genes that are distinct from the genes expressed in the planktonic state of a similar species of bacteria, as well as the stimulation of a specific sort of signaling known as signalling molecules (Jamal *et al.*, 2018). When bacteria detect environmental stress factors, either biotically or abiotically, they are prompted

to cling to the surface and the creation of a biofilm occurs. Different bacteria respond differently to certain ecological settings. While developing, some bacteria, like *P. aeruginosa*, can create biofilms practically everywhere, but other strains of *E. coli* can only do so under nutrition circumstances (Kaplan *et al.*, 2000). Although the method of biofilm development by different microbes is complicated, it typically involves four steps: the creation of microcolonies, the maturity of the biofilm, the maturation of the adhesion to the abiotic or biotic surface, and the dispersion of planktonic bacteria (Jamal *et al.*, 2018).

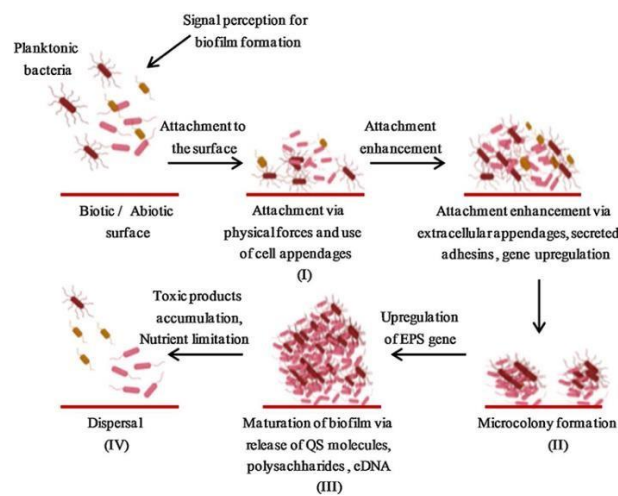


Fig.2.1 The process through which biofilms are created. The four phases of biofilm development are: Using cell appendages to temporarily attach free-living bacteria to a suitable surface, followed by producing adhesins to permanently adhere the bacteria. (II) Through the upregulation of genes necessary for maintaining attachment, discrete colonies, also known as microcolonies, with approximately a hundred bacteria per cluster, are formed. (III) Biofilm maturation includes EPS gene upregulation, polysaccharide release (IV) The dispersion of bacteria living in biofilm happens when nutrients get exhausted and harmful substances start to build up over time. (Goel *et al.*, 2021)

2.2.1 Initial attachment

Adhesion, the process by which germs first adhere to a surface, is at least somewhat stochastic and is influenced by hydrostatic pressure, Brownian motion, and gravitational forces. Physical forces and their cell appendages like pili, fimbriae, or

flagella help microbial cells adhere to the surface (Beloin *et al.*, 2008; Kostakioti *et al.*, 2013). Depending on the characteristics of the medium, the number of nutrients, the strength of the ions, the pH, the temperature, and the makeup of the cell surface, the bacteria cells experience both attracting and repulsive forces within the environment (Donlan 2002). Many diseases have motile flagella that aid them in overcoming hydrodynamic and repulsive forces. For several bacteria, including *P. aeruginosa*, *Coli*, *L. monocytogenes*, and *Vibrio cholerae*, the significance of flagella has been well studied (Kaplan *et al.*, 2000; Kostakioti *et al.*, 2013).

The modest contacts between both the membrane and the bacterium cause the first attachment to be dynamic and reversible. During this time, if the forces of repulsion are stronger or in reaction to the availability of nutrients, bacteria may separate and become planktonic. Extracellular sticky appendages and produced adhesins play a role in irreversible attachment to surfaces. For instance, in addition to using cilia to maintain adhesion and move across the attaching surface, *P. aeruginosa* also employs type IV-pili-mediated twitching motility (Kostakioti *et al.*, 2013). Hydrophobic surfaces are preferred by microorganisms for adhesion because they tend to weaken repulsions between the bacterium and the surface (Jamal *et al.*, 2018).

2.2.2 Microcolony formation

Responses that result in altered gene expression and overexpression of proteins that sustain adhesion are brought on by attachment to the surface. Bacteria usually start to grow and form distinct cell clusters or tiny aggregates known as biofilm communities by creating small amounts of the biofilm. A typical microcolony has roughly 100 microorganisms (Kostakioti *et al.*, 2013; Monds *et al.*, 2009). Depending on the circumstances under which the biofilm forms, the microcolonies that make up the biofilm may include a single bacterial species or several diverse bacterial species. Two kinds of mutants in *P. aeruginosa* were investigated for their ability to generate microcolonies. One class lacked motility and flagella, making it impossible for it to cling to surfaces, and the other class lacked type IV pili. The fact that *P. aeruginosa* type IV pili mutant cannot produce microcolonies suggests that type IV pili are crucial for microcolony development. Specific genes necessary for the development of the extracellular polymeric substances matrix components of the biofilm are activated during micro colony formation (Costerton *et al.*, 1999).

2.2.3 Maturation of biofilm

Cells interact with one another at this stage of biofilm development by producing autoinducer molecules, which then trigger the activation of several genes necessary for the biofilm matrix to form (Khatoon *et al.*, 2018). Quorum sensing, which aids in measuring the cell density in the immediate vicinity, is made possible by the autoinducer signaling molecules. *P. aeruginosa* releases three polysaccharides at this point in the biofilm development process, alginate, Pel, and PSL, which provide biofilm stability. The concentration of the signal controls the management of these three polysaccharides (Kostakioti *et al.*, 2013). Extracellular DNA, which has been the subject of several investigations, has also been shown to have a role in the development of cell-to-cell communication and the maintenance of the biofilm matrix. In general, there are two steps in biofilm maturation: the first stage includes cell-to-cell contact and the creation of autoinducer chemicals and the second is the transformation of microcolonies into microcolonies by triggering all genes necessary for EPS matrix development (Khatoon *et al.*, 2018).

2.2.4 Dispersal

In order to colonize a new location, bacteria must transition from their sessile condition to their mobile state via dispersion from the biofilm. This marks the beginning of a new lifecycle for biofilm development (Costerton *et al.*, 1999). In addition to passive dispersal brought on by shear pressures, bacteria have evolved mechanisms for deciding whether to stay in the current biofilm or initiate the creation of a new one (Kostakioti *et al.*, 2013). As the biofilm ages, harmful substances accumulate, oxygen levels fluctuate, and nutrients become scarce (Khatoon *et al.*, 2018). Involved sensory systems track the number of tiny chemicals in the atmosphere and trigger the activation of certain genes, promoting dispersion in response to stress. For instance, *P. aeruginosa* responds to an increase in carbon and nitrogen sources by dispersing. Additionally, an increase in c-di-GMP molecules is a sign that a microcolony is forming, whereas a decrease in the molecule's level results in an upregulation of motility. Polymeric hydrolase in *P. aeruginosa* is one example of an enzyme that is said to assist in biofilm dispersion. Biofilm dispersion is decreased by an elevation in surfactant molecules like rhamnolipid, which inhibits the contact of bacteria with the surface (Kostakioti *et al.*, 2013). In contrast to bacterium cells that are eliminated as a

consequence of an active dispersion mechanism, bacteria that scatter as a consequence of shear force by sloughing off aggregation may lose their biofilm features, such as resistance to antibiotics (Donlan 2002).

Serious risks to human health have been posed by microorganisms' ability to form biofilm on both abiotic and biotic surfaces. According to the CDC, biofilm development caused around 1.7 million hospital-acquired illnesses, which resulted in more than a million fatalities per year (Banerjee *et al.*, 2019). According to the National Institutes of Health (NIH), biofilm development is the cause of over 80% of all microbial illnesses in humans (Khatoon *et al.*, 2018). Life-threatening infections are caused by biofilm growth on medical equipment such as breast implants, catheters, artificial hearts, corrective lenses, artificial hearts, and spinal fluid shunts. Some of the most noteworthy bacteria that may develop biofilm on medical equipment. Because antibiotics have a reduced ability to combat bacteria found in biofilm, conditions including osteomyelitis, cystic fibrosis, infective endocarditis, chronic wounds, and periodontitis are difficult to treat (Muhammad *et al.*, 2020; Khatoon *et al.*, 2018). According to the NIH, biofilm-forming microbes are thought to be the cause of 65% of microbiological illnesses (Jamal *et al.*, 2018).

2.3 ACTINOBACTERIA: A SOURCE OF BIOFILM INHIBITORS

One of the most numerous and diverse groups of microorganisms in nature are actinobacteria. They span the gamut of spore- and non-spore-forming, motile, anaerobic, and aerobic bacteria. They have a high guanine-cytosine concentration and are Gram-positive in nature. They were once thought to be soil bacteria, but as shown in Fig. 2.2, now it is understood that they are a part of almost every ecosystem. Producers of metabolites like antibacterial, antiviral, and anticancer compounds include actinobacterial species. About 70% of the biological active chemicals are now being used in therapeutic settings. More than 50 gene clusters have been found in *Streptomyces* sp., according to genome sequencing research, indicating that this organism has the ability to produce the predicted medicines in the future. While the majority of the antibiotic classes have been traced back to *Streptomyces*, the potential of additional bacterial isolate species cannot be discounted. On a variety of bacterial strains, the bioactive has demonstrated the ability to inhibit biofilm. A mycelium-

forming actinobacteria called *Frankia casuarinae* was discovered in Tamil Nadu, India, inside the rhizosphere soil of the *Casuarina* species.

The unique findings indicated that manufacturers of anti-biofilm compounds versus *P. aeruginosa* and *Candida* sp. biofilm are *F. casuarina* (Marappa *et al.*, 2020). Additionally, *Streptomyces californicus* ADR1 secondary metabolites, at particular concentrations, inhibited 90% of the biofilm development highlighting the significance of actino- bacteria as a promising approach for the discovery of novel anti-biofilm agents.

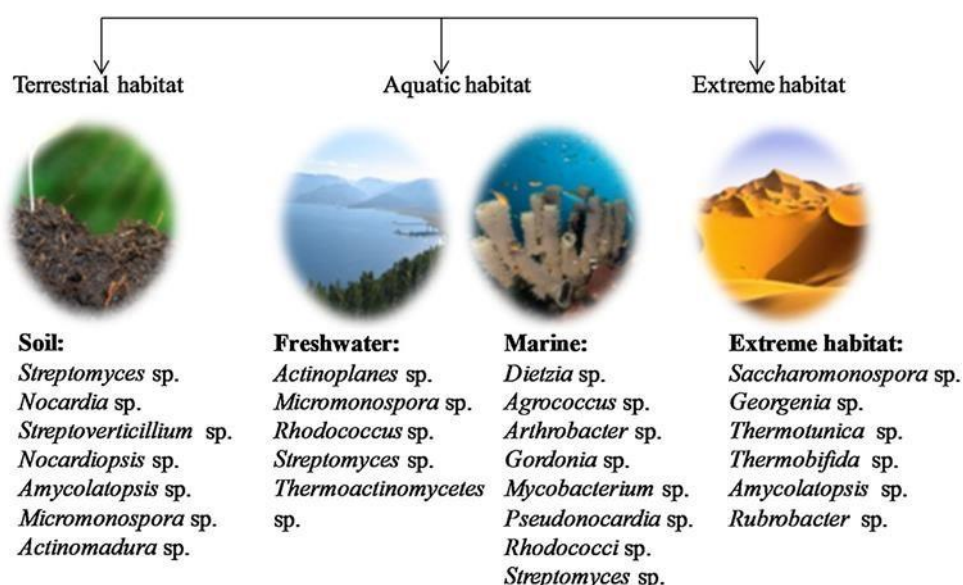


Fig. 2.2 Distribution of Actinobacteria in the environment (Barka *et al.*, 2015)

The biggest phylum among the significant lineages now acknowledged within the bacteria domain is Actinobacteria or Actinomycetes. Actinobacteria are typically Gram-positive bacteria with extraordinarily high genome content. Actinobacteria are considered a category between bacteria and fungi because they are filamentous bacteria that developed from branching hyphae (Barka *et al.* 2016). One of the vital microorganisms for producing useful goods in the medicinal, agricultural, biotechnology, and food sectors are the well-known bioactive chemical producer actinobacteria. (Mohammadipanah and Wink 2015). A minimum of 34,000 bioactive substances from bacteria have been identified as of 2010, and 40% of such bioactive components are generated by Actinobacteria. 20% of actinobacterial chemicals are identified from non-*Streptomyces*, whereas 80% are produced by *Streptomyces*. (Berdy 2012).

2.4 ANTIBIOFILM ACTIVITY OF ACTINOBACTERIAL COMPOUNDS

Earlier research has demonstrated that cyanobacteria play a significant role in the formation of antibiofilm compounds, and actinobacteria have been considered as possible sources of anti-biofilm chemicals as well (Venugopal *et al.*, 2013). You and colleagues (2007) showed how *Vibrio* was inhibited. For the development of innovative medications versus highly resistant *Staphylococcal* biofilms, coral-associated actinobacteria (CAA) appear to be a viable source of anti-biofilm chemicals. A coral-associated actinomycete was tested for its impact on *S. aureus* biofilm communities both in *in vivo* and *in vitro* by Bakkiyaraj and Pandian in 2010. *In-vitro* biofilm development and clinical isolates of *S. aureus* were reduced by methanolic extracts of CAA-3. According to research by Paramasivam *et al.* (2010), CAA extracts considerably reduce the growth of biofilms by 60-80%. All of the M serotypes that form biofilm were effectively inhibited by the extraction of *Streptomyces akiyoshinensis*. According to a 2011 study by Bavva *et al.*, an actinobacteria strain might prevent the development of biofilms. The biofilm-preventing ability of *Streptomyces filamentous* was strong. A promising organism to combat the biofilm that *Vibrio* species form is marine *Streptomyces*. Without altering the proliferation of zooplankton *P. aeruginosa* cells, *Streptomyces* sp. reduced the production of *P. aeruginosa* biofilm by 90%. A hydrophilic component (s), perhaps external acids or peptides from *Streptomyces* species, may be responsible for the decrease of *P. aeruginosa*'s biofilm, according to research on the extraction method, and thermal treatment (Kim *et al.*, 2012).

Actinobacteria that live on land can also prevent the development of biofilms. Manickam *et al.* (2014) highlighted the importance of crude fat extract from *Streptomyces* species in preventing *S. pyogenes* ATCC 19615 from forming biofilms. Hemachandran *et al.* attempted to investigate the *in-vitro* suppression of extended-spectrum beta-lactamase pathogens that generate biofilms, including *Escherichia coli*, *Pseudomonas* sp., and *Klebsiella* species (2011). All of the ESBL infections that formed biofilms were inhibited by the extracts that were obtained from the actinobacterial strains. We predict that these novel tactics and approaches will ultimately be created from the actinobacterial world for use in the therapy of troublesome biofilm-related illnesses in clinical settings and industries, as the recent findings of anti-biofilm research revealed. Finding alternate methods to address the issue is necessary due to the rising prevalence of germs that are resistant to

antibiotics. Antibiofilm activity has emerged as the most popular alternative target to stop their spread since pathogenicity and the capacity to produce biofilms are connected. Traditional antibiotics are inherently ineffective against biofilms (Corona *et al.*, 2013; Bjarnsholt *et al.*, 2010). This shows the need for fresh antibacterial medications that work against biofilms as well as planktonic bacteria. Antibiofilm action against Gram-positive infections has been demonstrated by a number of bioactive substances (Correa *et al.*, 2019; Miquel *et al.*, 2016). New chemical discovery continues to be crucial. The bulk of bioactive chemicals are primarily produced by actinomycetes. Actinomycetes have the ability to break down biofilms in a variety of ways, including by preventing microbial adherence (Siddharthan *et al.*, 2020; Srivastava *et al.*, 2016) or by blocking QS (Miao *et al.*, 2017; Younis *et al.*, 2016). Against *S. pyogenes* biofilms demonstrated antibiofilm activity. Up to 80% of biofilm growth is significantly prevented by extract application. Five extracts from *Streptomyces* decreased the cell membrane hydrophobicity, which is essential for *Staphylococcus pyogenes* biofilm formation (Nithyanand and Thenmozhi 2010).

The oil sector has a serious issue with sulfate-reducing bacterial biofilms, which is why the *Streptomyces* strain develops an active antibacterial chemical against them. Results indicated that biofilm development was decreased dramatically at the supra-minimal concentration. The oil sector may be very interested in using this antibiofilm chemical as a possible biocide to limit the development of sulfate-reducing bacteria (Pacheco *et al.*, 2016). ADP4 strain of *Streptomyces chrestomyceticus* prevents the growth of biofilms. (Siddharthan *et al.*, 2020). The adherence of *Candida* spp. and subsequent transition to the mycelium state were severely inhibited by *Streptomyces* strain ADP4's anti-biofilm activities. These *Streptomyces chrestomyceticus* strain ADP4-produced compounds showed potent anti-*Candida* action. They impede cell transformation into biofilms, making them viable medications for the treatment of biofilm-mediated infections (Siddharthan *et al.*, 2020). Actinomycetes produced from marine sponges were tested by Balasubramanian *et al.* for their capacity to prevent the growth of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* in biofilms. An organic extract from the ocean sponge-like culture type of bacteria *Streptomyces* sp. SBT343 significantly inhibited staphylococcal biofilm formation on polythene, glass, and corrective lens surfaces without affecting bacterial

growth, according to the results of invitro biofilm formation assays, scanning electron, and confocal microscopy (Balasubramanian *et al.*, 2017).

Studies have revealed that the majority of *Streptomyces* species display anti-biofilm behaviors. On plastic, glass, and contact lens surfaces, an ethyl acetate extract from the *Streptomyces* species SBT343 was recently discovered to strongly prevent the development of *Staphylococcus epidermidis* RP62A biofilms without impacting the growth of bacteria (Balasubramanian *et al.* 2017). Similar antagonistic effects were also seen in the ethyl acetate extract against the development of *S. aureus* biofilms, but no inhibitory effects were seen in the extract against *Pseudomonas* biofilms. The production of the polymerizable polysaccharide intercellular adhesion promotes the development of *S. epidermidis* biofilm (PIA). *S. epidermidis* may transition between PIA-dependent and PIA-independent forms of biofilm development under specific circumstances. (Hennig *et al.* 2007).

Streptomyces sp. SBT343's ethyl acetate extract, however, inhibited the biofilms from transitioning between the PIA-dependent and independent habitats (Balasubramanian *et al.* 2017). Another study found that the civilization aqueous phase of Actinobacteria species strains, isolated from lakes, rivers, and paddy fields, had strong anti-biofilm properties against pathogenic biofilms including those containing *P. aeruginosa* enter toxin-producing- *Escherichia*, *Vibrio parahaemoliticus*, *Vibrio cholera*, *Streptococcus pneumonia*, *Streptococci* Nucleotide sequence, proteins, and polysaccharide were discovered in the bioactive substances after further analysis, interrupting the cell membrane and intercellular communication, which is necessary for the development of biofilms (Waturangi *et al.* 2016).

When applied to surfaces, polysaccharides can have an anti-adherence impact on microorganisms (AbuSayemetal.2011; Renduelesetal.2012), whereas extracellular DNA can bind to the adhesive structure of planktonic cells required for surface attachment (Berne *et al.* 2011). *Streptomyces sp.* produces a protein (extracellular enzyme) that is also capable of interfering with additional cytoplasmic proteins (such as surface-exposed proteins), which are important for bacterial adhesion to inorganic surfaces (O'Toole *et al.* 2000; Waturangi *et al.* 2016).

The effectiveness of certain *Streptomyces* cultures against pathogens bacteria biofilms, such as *Bacillus*, *Acinetobacter*, and *Moraxella* was examined in a study

(Saleem et al. 2015). *B. cereus*, however, does not naturally exist in the oral microbiota. However, *B. cereus* spores or vegetative bacteria travelling through food infected with *B. cereus* may cause colonization of the oral cavity (Bottone 2010). *B. cereus* participates in a multispecies biofilm as a secondary colonizer of the dental plaque, where it benefits from the establishment of the biofilm by the primary colonizers and helps the biofilm to survive and grow (Majed et al. 2016).

The methanolic extract of *Streptomyces* sp. significantly reduced the biofilm development of *B. cereus*, whereas the methanol extract of *Streptomyces* significantly reduced the biofilm formation of *Moraxella* sp (Saleem et al. 2015). In another experiment, 90% of *P. aeruginosa*'s PAO1 biofilm formation was inhibited by the culture-wasted media, without influencing the corresponding planktonic bacterial growth. Within the same work, further transcriptome analysis revealed that *P. aeruginosa* exhibited enhanced expression of the genes for pyoverdine, phenazine, pyochelin synthesis, and iron absorption in response to the wasted medium of *Streptomyces* sp. BFI 230. The decrease in biofilm development was shown to be caused by *Streptomyces*. BFI 230 interferes with *P. aeruginosa*'s ability to acquire iron. Additionally, the extracellular peptides or proteins from *Streptomyces* sp. BFI230 responsible for the biofilm suppression was revealed by the results of the extraction method, protein kinase, and thermo mechanical experiments. (Kim et al., 2012).

Actinobacteria with anti-biofilm action are being studied in other Actinobacteria genera as well as uncommon or non-*Streptomyces* actinobacteria. *Rhodococcus* sp., a plant pathogen, was used to produce indole-3 acetaldehyde and indole-3 acetic acid (Lee et al., 2012). The bioactivity of these two substances revealed that they might decrease the production of *E. coli* O157:H7 biofilms without having an impact on the proliferation of planktonic cells. Curli, a significant proteinaceous component of a complicated extracellular matrix generated by several Enterobacteriaceae, and exopolysaccharides including cellulosic and colanic acid are associated with biofilm development in *E. coli* (Uhlich et al. 2006). Based on transcriptome research, indole-3-acetaldehyde suppressed the curli introns, which prevented the development of biofilms.

Additionally, 1% of *Rhodococcus* waste media dramatically decreased the development of biofilm (Lee et al., 2012). Two strains of *Streptomyces* and one strain

of *Nocardiopsis* and *Streptomyces* sp. A745 were three Actinobacteria strains obtained from the Arctic that significantly reduced the production of *V. cholerae* biofilms (Augustine *et al.*, 2012). In comparison to strains A733 and A731, strain A745 had the highest level of inhibition, with 20% of its supernatant inhibiting the development of biofilms by nearly 88%. Another research looked for any anti-biofilm activities against *S. aureus* in a collection of 458 Actinobacteria. (Park *et al.*, 2012).

The Actinobacteria catalogue that was employed in the study has a long history of helping researchers find antibiotics, anticancer medicines, and anti-inflammatory chemicals. Two chosen powerful cultures *Streptomyces* and *Kribbella* were utilized for additional research. Results from this study showed that Actinobacteria have strong protease activity, which greatly decreased biofilm development and increased *S. aureus* ability to disperse its biofilms (Park *et al.* 2012). Conclusion: Actinobacterial protease can be a viable substitute for managing and getting rid of *S. aureus* antibiotic-resistant biofilms since it can dissolve the biofilm by hydrolysing both its matrix proteins and adhesins (Leroy *et al.* 2008). The signaling peptides of Gram-positive bacteria's intercellular communication can also be broken by protease. (Schallenberger *et al.* 2012).

2.5 ANTIBIOFILM METABOLITES FROM ACTINOBACTERIA

A study was conducted to identify the key ingredients of active extracts, evaluate the antibiofilm activities of GMR22 versus *C. albicans*, and look into the biosynthesis genes involved in encoding the enzymes involved in metabolic pathways. On *Candida albicans* ATCC 10231, in vitro experiments were used to determine the antifungal and antibiofilm effects. These findings demonstrated the amazing biotechnological potential of the soil-dwelling *Streptomyces* sp. GMR22 bacterium, which produces palmitic acids as an antibiofilm drug against *Candida albicans* (Nirwati *et al.* 2022). A research found that strong positive, multi-drug resistant gram-negative bacteria and yeasts are to blame for catheter-associated Urinary infections. These organisms are also capable of producing strong bacterial growth on surfaces like Microtitre plates, cover glass, and urinary catheters. This study's usage of a crude extract from the *Streptomyces* strains showed that it had broad-spectrum anti-quorum sensing action that can weaken the QS-dependent biofilm and is a promising candidate to treat urinary catheter infections (Younis *et al.*, 2016).

Marine soil samples that had about 30 actinomycetes was collected from Thondi, Ramanathapuram for the study of novel secondary metabolites. Out of 30 one good efficient strain was identified and drug-resistant clinical pathogens were discovered utilizing main and secondary screening techniques. This research demonstrates that the recovered *Streptomyces* species is a promising drug strain, and the metabolite generated from this strain has strong antibacterial and antibiofilm action against antibiotic-resistant clinical pathogens and might be exploited for many biotechnological purposes (Dhandapani *et al.*, 2022).

Actinomycetes that were isolated from marine crustaceans were examined for their capacity to prevent the production of biofilms. The results of the investigation indicated the existence of 31 chemicals, the majority of which are alkanes. The discovery of interesting candidates for the control of biofilm-mediated infection will be aided by a further investigation into how effective these bioactive chemicals are against the *Candida albicans* biofilm (Siddharthan *et al.*, 2020)

One of Egypt's most contaminated coastal marine ecosystems, Lake Mariout is thought to be a storehouse for harmful effluents from various human activities. Local microbial populations are compelled by this selection pressure to develop new favorable themes. Two *Streptomyces* isolates were therefore tested from the silt of Lake Mariout in this investigation. It is envisaged that the isolation of actinobacteria from a contaminated marine ecosystem would be thought to be helpful in the finding of distinctive strains that demonstrate a double and concurrent role as producer of secondary metabolites. It investigated how different dietary characteristics affected the production of Ag nanoparticles. Additionally, the antibiofilm action was dose-dependently shown. Moreover, the coupling of Ag nanoparticles with the crude bioactive byproducts of both biological Nano factories increased the antibacterial efficacy, reflecting a synergistic effect against the pathogens (free-living and biofilm) under study. (Eltarahony *et al.* 2021).

Singh and Dubey discovered a brand-new kind of epiphytic actinobacteria from the plant *Datura metel*, which generated secondary metabolites with strong anti-infective properties. *Streptomyces californicus* strain was determined to be the isolate. The antibacterial properties of the isolate's metabolites against significant pathogens had been studied. Against a variety of Gram-positive bacteria, including those listed by the

World Health Organization as high-priority pathogens, had very low MIC90 values. As a result, it was hypothesized that it may be a fantastic source for potent anti-infective that might be used to treat a variety of difficult bacteria. The biofilms of methicillin strains of *S. aureus* were shown to be effectively inhibited by the ADR1 metabolites. These components could be responsible for the pharmacological effects of the extract, either singly or in combination (Singh and Dubey 2020).

A study was conducted to evaluate the antibacterial and anti-biofilm effectiveness of bioactive molecules (SMs) produced by actinobacteria recovered from the rhizosphere soil. This work provides preliminary information on the toxicity profile and possible bioactive chemicals of a *Microbacterium* extract that may be useful for future research to advance the development of novel, therapeutic treatment of biofilm-associated illnesses (Naushin Bano *et al.*, 2022).

In aquaculture, biofilm may be a serious issue in addition to producing foodborne illnesses. There is a need for further characterization of the actinomycetes extracts since a study shows antibiofilm action against a variety of fish and foodborne diseases. Four extracts' chemical compositions were identified, together with their antibiofilm abilities, and their genetic resemblance to other known *Streptomyces* isolates was determined. It was discovered that numerous extracts included various antibiofilm substances and that the antibiofilm actions of all extracted were most persistent at pH 6. The biofilms created by fish and foodborne pathogens on stainless coupons were successfully removed by the crude extracts. Additionally, their antibiofilm properties were verified by examining treated biofilms with scanning electron microscopy and energy-dispersive X-ray spectroscopy. We suggested actinomycetes extracts as viable candidates for antibiofilm compounds to be employed in the agriculture and aquaculture industries (Miller *et al.*, 2022).

CHAPTER 3

AIM:

To produce anti-biofilm metabolites from Himalayan Actinobacteria.

OBJECTIVE:

1. Preparation of organic extracts from selected actinobacteria isolated from Indian Himalayan region
2. In vitro screening of actinobacterial extracts for anti-biofilm property against Gram negative bacterial pathogens
3. Selection of potential strain and production of anti-biofilm metabolites.
4. Identification of potential actinobacterial strain by 16s rRNA sequencing.
5. Partial purification of active metabolites and its testing for anti-biofilm activity.

CHAPTER 4

MATERIALS AND METHODS

4.1 DESCRIPTION OF ACTINOBACTERIA

For this present study, about 20 actinobacterial cultures were obtained from the Actinobacterial Research Lab, Centre for Drug Discovery and Development, Col Dr. Jeppiaar Research Park, Sathyabama Institute of Science and Technology, Chennai, Tamilnadu. All the cultures were previously isolated from soil samples collected from different less explored Himalayan ecosystems in India and maintained in AIA (Actinomycete Isolation Agar) , SCA (Starch M-protein Agar for actinomycetes) , NA (Nutrient Agar) agar slants as well as in 30% glycerol broth.

Table 4.1 List of actinobacterial strains used for antimicrobial study

S.No.	Strain name	Source	Ecosystem
1.	HS2D9	soil	Terrestrial(Himalayan)
2.	HS2D25	soil	Terrestrial(Himalayan)
3.	HS2D30	soil	Terrestrial(Himalayan)
4.	HS2D31	soil	Terrestrial(Himalayan)
5.	HS2D36	soil	Terrestrial(Himalayan)
6.	HS2Z11	soil	Terrestrial(Himalayan)
7.	HS2Z12	soil	Terrestrial(Himalayan)
8.	HS2Z14	soil	Terrestrial(Himalayan)
9.	HS2Z16	soil	Terrestrial(Himalayan)
10.	HS2Z19	soil	Terrestrial(Himalayan)
11.	HS2C7	soil	Terrestrial(Himalayan)
12.	HS2C5	soil	Terrestrial(Himalayan)
13.	HS2C6	soil	Terrestrial(Himalayan)
14.	HS2D4	soil	Terrestrial(Himalayan)
15.	HS2D3	soil	Terrestrial(Himalayan)
16.	HS2D1	soil	Terrestrial(Himalayan)
17.	HS2D2	soil	Terrestrial(Himalayan)
18.	HS2D31	soil	Terrestrial(Himalayan)
19.	HS2D35	soil	Terrestrial(Himalayan)
20.	HS2D20	soil	Terrestrial(Himalayan)

4.2 CHARACTERIZATION OF ACTINOBACTERIAL STRAINS

Cultural characterization of actinobacterial strains were studied using SCA, NA, AIA agar medium. Agar was prepared and sterilized by autoclaving at 121°C for 15 minutes and poured into sterile petriplates under aseptic condition and allowed to solidify. Then all the actinobacterial cultures were inoculated into corresponding agar plates and

incubated at 28°C for 7-14 days. After incubation, all the plates were observed for growth, consistency, aerial mycelia colour, reverse side pigment and soluble pigment production.

4.3 PRODUCTION OF BIOACTIVE METABOLITES BY AGAR SURFACE FERMENTATION

Actinobacterial cultures were fully streaked on respective media and Incubated for 10 days at 30°C. After incubation the powdery spores were scraped using sterile spatula, and agar were finely chopped into pieces, transferred and soaked in ethyl acetate for 48 hrs. Collecting the solvent portion and concentrated using rotary evaporator and quantified.

4.4 SCREENING OF ANTI-BIOFILM ACTIVITY USING MICROTITRE PLATE METHOD (Mtp)

4.4.1 Inoculum preparation

The biofilm forming gram negative pathogens viz., *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* used for this study were sub cultured into NA (Nutrient Agar) and incubated for 24 hrs at 37°C. Then the fresh single colonies were inoculated into Tryptic Soy Broth (TSB) + 1% Glucose and incubated at 37°C for 24hrs. To prepare standardized inoculum, the broth culture was adjusted to find concentration of about 1×10^8 CFU/ML ($OD_{600} = 0.08$ to 0.1) by comparing with 0.5 McFarland standard.

4.4.2 Biofilm inhibition assay by Mtp method

In Microtitre plate, 100 μ L of TSB is added in all wells. 100 ml of 400 μ g/ ml of extract was added and 2 fold dilution was done until the deserved concentration is achieved. In control wells TSB serves as blank, in positive control well broth and test pathogens; in negative control well broth, test pathogens and antibiotic (Ciprofloxacin- 15 mcg) is added. 50 μ L of standard pathogen was added to all wells and incubated at 37°C for 24 hrs in static condition. The next day plates were rinsed with $1 \times$ PBS at pH 7.3 twice and air dried. Then 200 μ L of 0.1% crystal violet was added and left for 30 mins, washed and dried completely. The strained wells were resolublized with 200 μ L of 95% Ethanol or methanol or 33% glacial acetic acid and Optical Density were taken at 570nm using Microplate spectrophotometer (BioTek EPOCH 2).

The percentage of BF Inhibition was calculated using the formula

$$\text{Percentage of BF Inhibition (\%)} = \frac{\text{OD}_{570} \text{ of Positive control} - \text{OD}_{570} \text{ of Test}}{\text{OD}_{570} \text{ of Positive control}} \times 100$$

4.4.3 Biofilm eradication Assay

In a Microtitre plate 100µL of standard was added to 96 well plate and OD is taken at 600nm and the plates were incubated at 37°C for 24 hrs. In control wells TSB serves as blank, in positive control well broth and test pathogens; in negative control well broth, test pathogens and antibiotic (Ciprofloxacin- 15 mcg) is added. 50 µL of standard pathogen was added to all wells and incubated at 37°C for 24 hrs in static condition. The next day the spent media is gently discarded and the wells are rinsed with 150µL, sterile TSB media twice. Then 180µL TSB medium was added to all the wells and 20µL of extract at deserved concentration is added and incubated at 37°C for 24 hrs at static condition. Spent media was discarded and washed with 1×PBS at pH 7.3 twice and air dried. 200µL of 0.1% Crystal Violet was added and left for 30 mins, washed and air dried completely. The wells were resolubilized with 200 µL of 95% Ethanol or methanol or 33% glacial acetic acid and Optical Density were taken at 570nm using Microplate spectrophotometer (BioTek EPOCH 2).

The percentage of BF Eradication was calculated using the formula

$$\text{Percentage of BF Eradication (\%)} = \frac{\text{OD}_{570} \text{ of Positive control} - \text{OD}_{570} \text{ of Test}}{\text{OD}_{570} \text{ of Positive control}} \times 100$$

4.4.4 Statistical analysis

All the experiments were done in triplicate (n=3)

4.5 CHARACTERIZATION AND IDENTIFICATION OF POTENTIAL

4.5.1 Micromorphology

Micromorphological characteristics of potential strains were studied by adopting slide Culture method. 2 ml of melted ISP2 agar medium was mixed with the spores of

Actinobacterial strain HS2C7. It was poured on the surface of sterile microscopic slide

as a thin layer and kept in a sterile Petri plate for incubation at 28 °C for 7 days. The slide was observed under bright field microscope at 45X magnification. Gram stain was performed and observed under oil immersion.

4.5.2 Cultural Characterization

Cultural characteristics were studied by inoculating the Actinobacterial strains into International Streptomyces Project-2 Medium (ISP 2), SCA, ISP 3, ISP5, ISP7 and AIA. The plates were incubated for 7 days at 28 °C.

4.5.3 Physiological characterization

The selected isolate HS2C7 was optimized at different criteria's to and the suitable media for maximum metabolite production. The composition of the fermentation media used for optimization is: Yeast 0.4%, NaCl 1%, Glucose 0.4% and Agar Agar 2%, pH maintained at 7.

4.5.3.1 Assimilation of carbon source

To study the effect of carbon source the selected isolate was-quadrant streaked on individual petri plates containing fermentation media with different 1% of carbon sources namely glucose, Lactose, Xylose, Starch, Mannitol, Inositol, and Raffinose. The plates were further incubated, at 28 °C for 7 days and checked for antibacterial activity.

4.5.3.2 Assimilation of nitrogen source

The effect of nitrogen source the selected isolate was quadrant streaked on petri plates containing fermentation media and replacing the nitrogen source alone. The nitrogen sources used are Soya bean, Malt, Peptone, Yeast and KNO₃. The plates were further incubated at room temperature.

4.5.3.3 Assimilation of Mineral source

The effect of Mineral source the selected isolate was quadrant streaked on petri plates containing fermentation media and replacing the mineral source alone. The mineral source used are NaCl, MgSO₄, FeSO₄, MnCl₂, and CaCl₂. The plates were further incubated at room temperature.

4.5.3.4 Effect of pH

To study the effect of pH the isolate was quadrant streaked on fermentation medium which the pH was adjusted as 5,6,7,8 and 9 and incubated to identify the optimum pH required for metabolite production. The plates were further incubated at room temperature.

4.5.4 MOLECULAR CHARACTERIZATION OF POTENTIAL ORGANISM

4.5.4.1 16s rRNA Sequencing

Also called as Sanger's sequencing is the method that's used to obtain the DNA sequence of any organism. Using the sequence obtained one can understand the genetic structure of an organism. 16rRNA sequencing is a culture free method to identify and compare bacterial diversity from complex microbiomes or environments that are difficult to study. It is commonly used to identify bacteria present within a given sample down to the genus and/or species level. In general the comparison of the 16s rRNA gene sequences allows differentiation between organism at the genus level across all major phyla of bacteria, in addition to classifying strains at multiple levels, including what we now call the species and subspecies level.

4.5.4.2 Phylogenetic Analysis

16s rRNA gene sequences of the isolates were compared with 16s rRNA sequences available in the BLAST search in NCBI, Gene bank Database. The 16s rRNA sequences of potential strains AS-1-19 were deposited in the GenBank database.

4.6 PARTIAL PURIFICATION AND BIOASSAY OF CRUDE EXTRACT

4.6.1 Thin Layer Chromatography Analysis

A TLC sheet/ plate was taken which is generally coated with silica gel and alumina; it was cut into small strips. A small amount of extract (HS2C7) was spotted on the TLC sheet using a capillary tube. The extract is usually spotted near the bottom of the sheet. Chromatogram was run by using different solvent systems such as chloroform:ethyl acetate, Hexane: ethyl acetate, Dichloromethane: ethyl acetate, Hexane: Ethyl acetate: chloroform in different ratios.

The TLC sheet was then placed in the beaker containing the mixture of the solvents. The solvent moves up the sheet by capillary action carrying the extract with it. The

solvent front was allowed to move to few centimetres off the top of the sheet. The TLC sheet was removed and allowed to air dry. The separated spots were then visualized by placing the sheet in UV spectrophotometer or by placing them in a reagent such as iodine.

$$R_f = \frac{\text{Distance travelled by component}}{\text{Distance travelled by liquid}}$$

CHAPTER 5

RESULTS AND DISCUSSION

5.1 DESCRIPTION AND MORPHOLOGICAL CHARACTERIZATION OF SELECTED ACTINOBACTERIAL STRAINS

5.1.1 Collection and characterization of Actinobacteria

For this present study, about 20 actinobacterial cultures were obtained from the Actinobacterial Research Lab, Centre for Drug Discovery and Development, Col Dr. Jeppiaar Research Park, Sathyabama Institute of Science and Technology, Chennai, Tamil Nadu. All the cultures were previously isolated from soil samples collected from less explored ecosystems of Himalayan region in India and maintained in ISP2 agar slants as well as in 30% glycerol broth.

5.2 CHARACTERIZATION OF COLLECTED ACTINOBACTERIA

The growth and cultural characteristics of the actinobacterial strains are given in the table. All the 20 actinobacterial culture showed good growth on SCA, AIA, and NA agar medium. About 50% of the culture produced light and dark grey colour aerial mycelium growth on corresponding agar. All the plates were observed for growth, consistency, aerial mycelia, reverse side pigment and soluble pigment production which are listed below (Table 5.1).



Fig 5.1 Characterization of collected Actinobacteria

Table 5.1 Details of actinobacterial cultures used in this study

S.No	Strain No	Media	Growth	Aerial Mass Color	Soluble Pigmentation	Consistency	Reverse Pigmentation
1	HS2D 9	NA	Good	Light grey	NO	Leathery	Grey
2	HS2D 25	SCA	Good	White	NO	Powdery	White
3	HS2D 30	SCA	Good	White	YES	Powdery	Brown
4	HS2D 31	SCA	Good	Grey	YES	Powdery	Light grey
5	HS2D 36	AIA	Good	Dark grey	NO	Powdery	Light grey
6	HS2Z 11	AIA	Good	Grey	YES	Leathery	Brown
7	HS2Z 12	SCA	Good	Dark grey	YES	Powdery	yellow
8	HS2Z 14	AIA	Good	White	YES	Powdery	light brown
9	HS2Z 16	AIA	Good	Grey	NO	Powdery	Light grey
10	HS2Z 19	SCA	Good	Grey	NO	Powdery	White
11	HS2C7	NA	Good	white	NO	Leathery	white
12	HS2C5	NA	Good	brown	NO	Powdery	yellow
13	HS2C6	NA	Good	grey	NO	Powdery	light brown
14	HS2D4	NA	Good	light grey	NO	Powdery	yellow
15	HS2D3	NA	Good	light grey	NO	Powdery	yellow
16	HS2D1	NA	Good	white	NO	Powdery	white
17	HS2D2	NA	Good	white	NO	Powdery	yellow
18	HS2C8	NA	Good	grey	NO	Powdery	white
19	HS2D35	SCA	Good	dark grey	NO	Leathery	white
20	HS2D20	AIA	Good	White	NO	Leathery	Yellow

5.3 PRODUCTION OF BIOACTIVE METABOLITES BY AGAR SURFACE FERMENTATION

All the 20 actinobacterial cultures were fully streaked, incubated and soaked in ethyl acetate and crude extracts were collected and quantified. The values of weight (mg) are given below (Table 5.2).

Table 5.2 Weight of crude extracts

S.NO	STRAIN NO	Weight (mg)
1	HS2D30	5
2	HS2D9	11
3	HS2Z14	4
4	HS2D36	12
5	HS2Z11	11
6	HS2Z16	11
7	HS2D1	17
8	HS2C5	13
9	HS2C7	22
10	HS2D3	28
11	HS2D4	18
12	HS2D2	21
13	HS2C6	18
14	HS2D31	11
15	HS2D25	9
16	HS2Z12	14
17	HS2D35	8
18	HS2D20	22
19	HS2C8	7
20	HS2C1	9

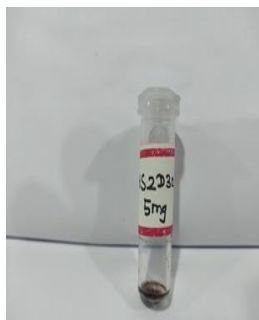


Fig 5.2 Production of bioactive metabolites by agar surface fermentation

5.4 SCREENING OF ANTI-BIOFILM ACTIVITY USING MICROTITRE PLATE METHOD (Mtp)

5.4.1 Biofilm inhibition assay by Mtp method

In this study, 14 well grown actinobacterial strains were screened for antibiofilm activity against gram negative pathogens. Of the 14 tested, strains HS2C7 showed maximum percentage of antibiofilm inhibition against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* pathogens for about 50-80%.

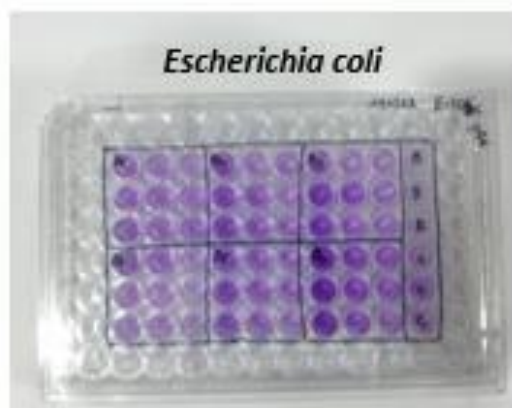
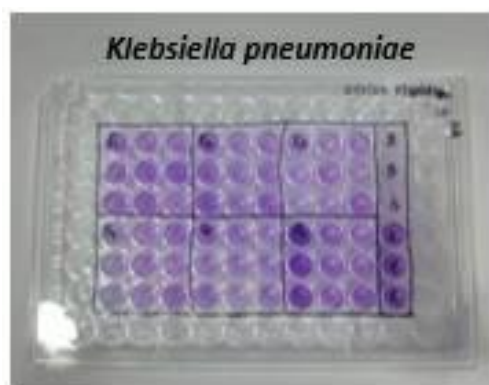


Fig 5.3 Screening of Anti-biofilm activity using Microtitre plate method

Table 5.3 *Klebsiella pneumoniae* BF Inhibition Assay

S.NO	Strain No	Biofilm inhibition (%)		
		200µg/mL	100 µg/mL	50 µg/mL
1	HS2D30	10±0.01	12±0.08	21±0.21
2	HS2D9	1±0.06	43±0.01	28±0.09
3	HS2Z14	57±0.06	49±0.08	41±0.09
4	HS2D36	57±0.01	47±0.05	30±0.09
5	HS2Z11	40±0.07	58±0.06	54±0.03
6	HS2Z16	0	36±0.11	44±0.08
7	HS2C7	67.4±0.02	39.6±0.134	40.68±0.042
8	HS2D1	64.32±0.03	52.5±0.11	55.83±0.18
9	HS2C5	53.42±0.131	47.9±0.202	33.3±0.061
10	HS2D3	46.02±0.119	42.12±0.22	14.33±0.187
11	HS2D4	53.15±0.26	46.12±0.133	49.4±0.180
12	HS2D2	28.6±0.167	36.4±0.35	33.73±0.32
13	HS2C6	62.5±0.142	35.15±0.064	35.7±0.198
14	HS2D31	64.41±0.019	20.56±0.252	18.31±0.173

*Note: All values represent the mean values given with SEs (n = 3)

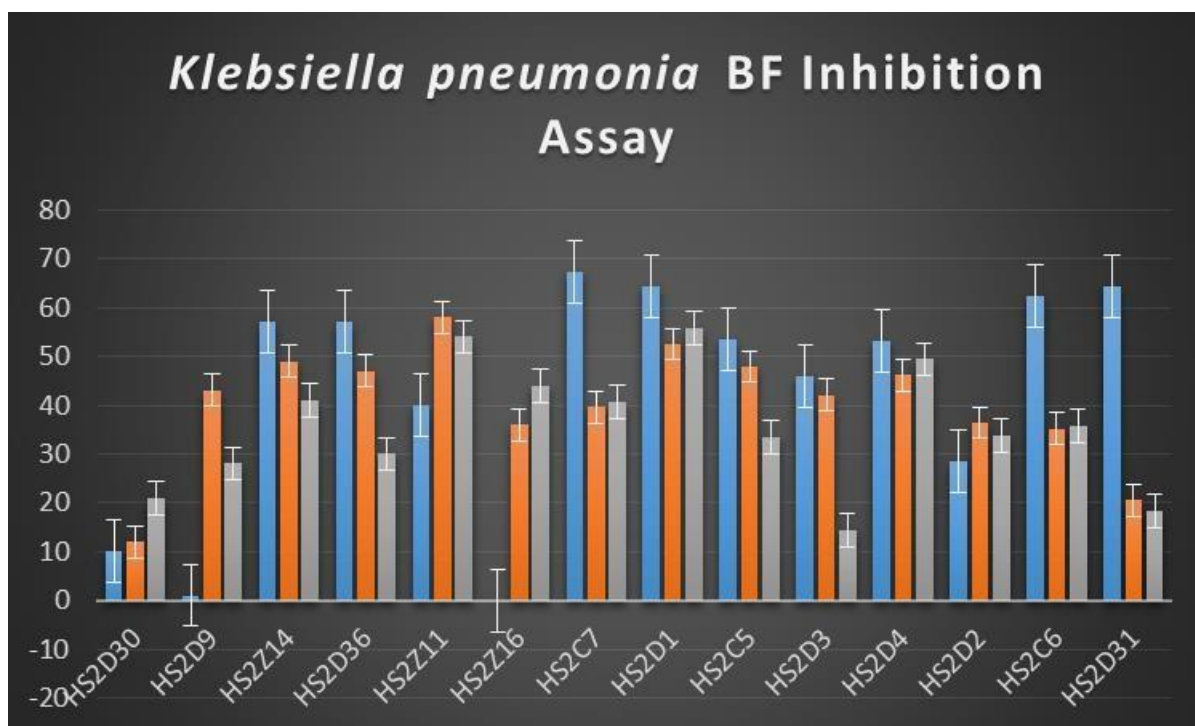


Fig 5.4 Biofilm inhibition assay Graph (*Klebsiella pneumonia*)

Table 5.4 *Pseudomonas aeruginosa* BF Inhibition Assay

S.NO	Strain No	Biofilm inhibition (%)		
		200µg/mL	100µg/mL	50µg/mL
1	HS2D30	18±0.09	0	0
2	HS2D9	50±0.116	50±0.101	55±0.03
3	HS2Z14	75±0.02	71±0.04	43±0.08
4	HS2D36	0	19±0.09	25±0.144
5	HS2Z11	0	7±0.43	45±0.07
6	HS2Z16	42±0.38	25±0.08	30±0.10
7	HS2C7	0	61.35±0.002	59.88±0.023
8	HS2D1	22.62±0.03	44.7±0.06	46.95±0.04
9	HS2C5	0	0	0
10	HS2D3	36.53±0.019	40±0.045	20.47±0.03
11	HS2D4	2.7±0.067	18.21±0.007	19.7±0.08
12	HS2D2	16.4±0.029	12.8±0.117	45.135±0.031
13	HS2C6	0	0	0
14	HS2D31	0	0	23.077±0.053

*Note: All values represent the mean values given with SEs (n = 3)

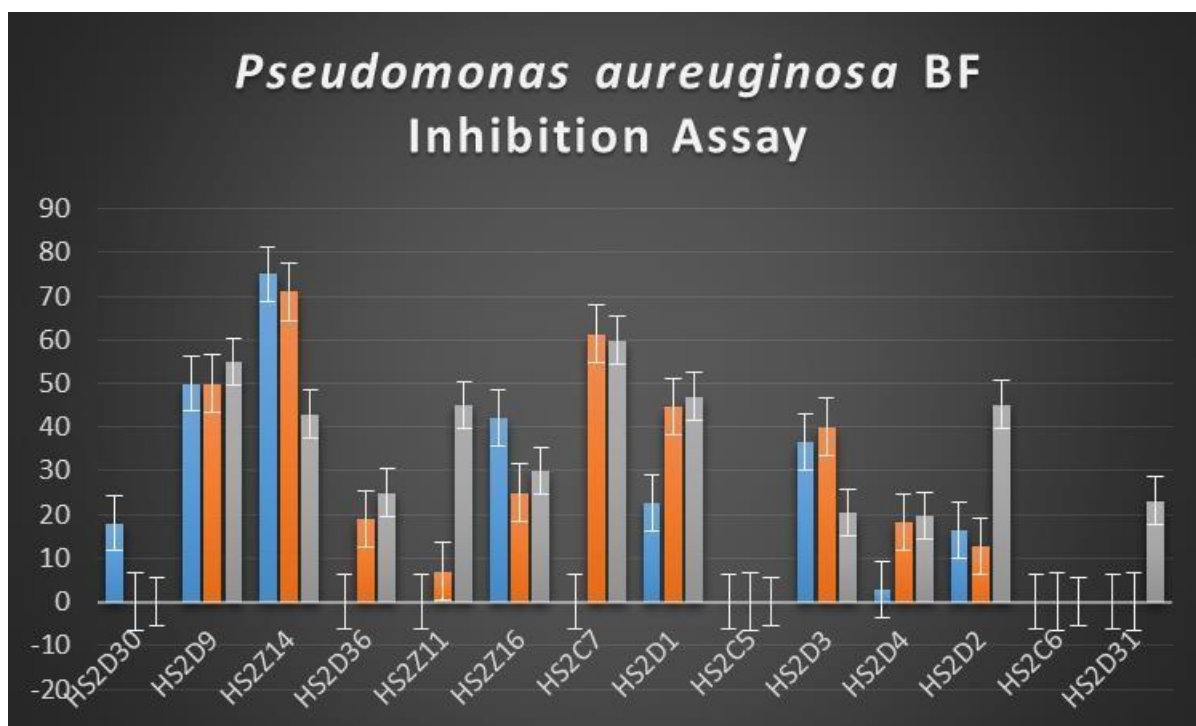


Fig 5.5 Biofilm inhibition assay Graph (*Pseudomonas aeruginosa*)

Table 5.5 *Acinetobacter baumannii* BF Inhibition Assay

S.NO	Strain No	Biofilm Inhibition (%)		
		200 µg/mL	100 µg/mL	50 µg/mL
1	HS2D30	0	0	0
2	HS2D9	0	0	0
3	HS2Z14	27±0.20	4±0.21	0
4	HS2D36	21±0.15	0	0
5	HS2Z11	0	0	0
6	HS2Z16	0	3±	7±0.13
7	HS2C7	0	21.04±0.099	16.71±0.06
8	HS2D1	0	17.57±0.06	0
9	HS2C5	13.56±0.04	0	16.58±0.016
10	HS2D3	17.25±0.05	0	16.2±0.02
11	HS2D4	0	0	16.76±0.031
12	HS2D2	6.621±0.075	0	2.16±0.064
13	HS2C6	0	0	0
14	HS2D31	0	0	0

*Note: All values represent the mean values given with SEs (n = 3)

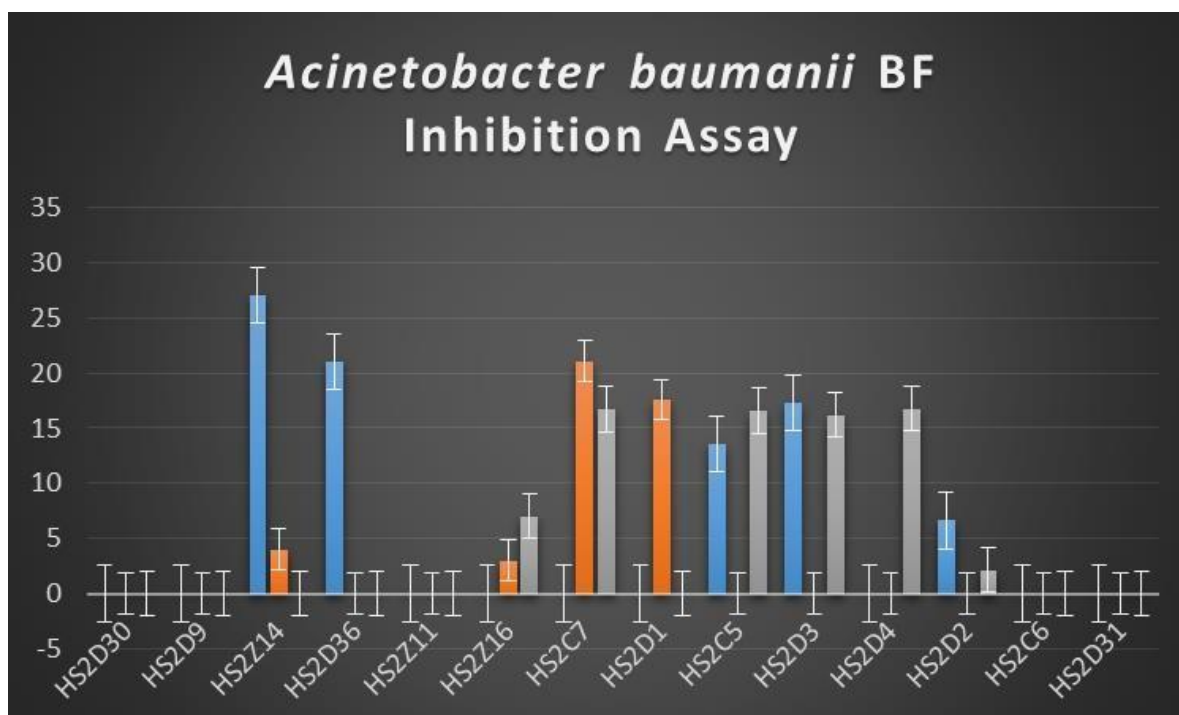


Fig 5.6 Biofilm inhibition assay Graph (*Acinetobacter baumannii*)

Table 5.6 *Escherichia coli* BF Inhibition Assay

S.NO	Strain No	Biofilm Inhibition (%)		
		200 µg/mL	100 µg/mL	50 µg/mL
1	HS2D30	0	0	0
2	HS2D9	0	0	0
3	HS2Z14	0	0	0
4	HS2D36	0	0	21±0.03
5	HS2Z11	0	0	0
6	HS2Z16	0	0	0
7	HS2C7	0	0	0
8	HS2D1	0	0	0
9	HS2C5	0	0	0
10	HS2D3	0	0	0
11	HS2D4	0	8.4±0.08	0
12	HS2D2	0	0	0
13	HS2C6	4.8±0.04	0	0
14	HS2D31	0	0	0

*Note: All values represent the mean values given with SEs (n = 3)

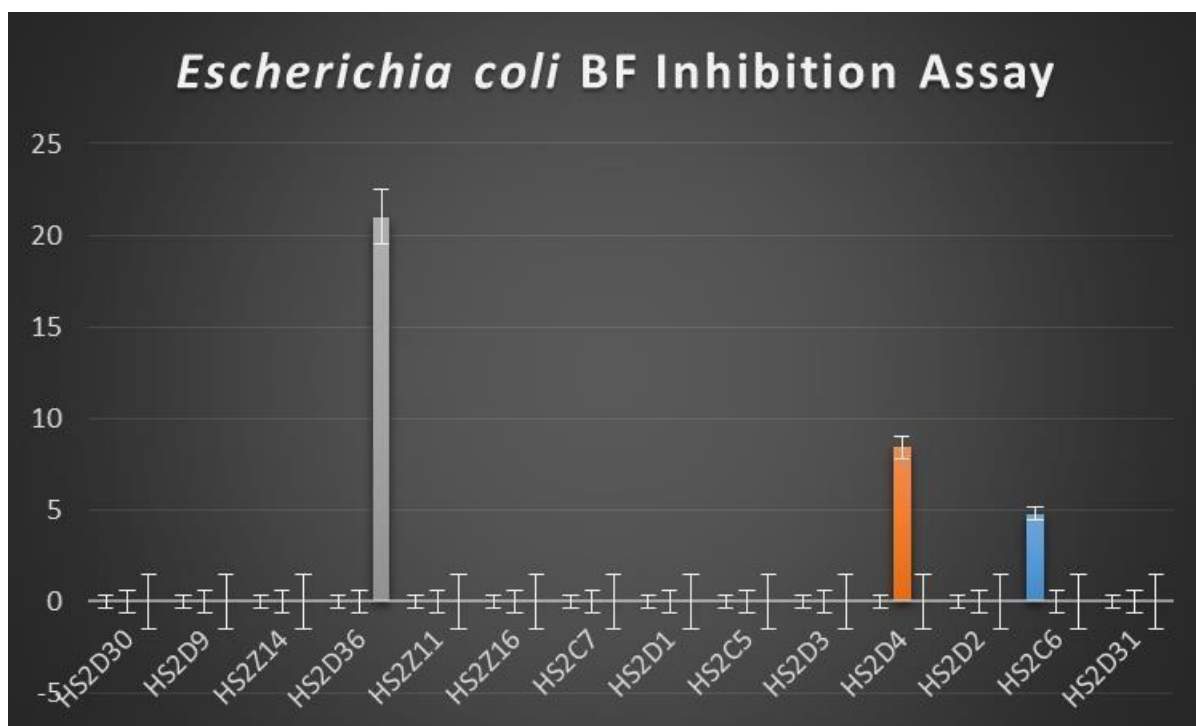


Fig 5.7 Biofilm inhibition assay Graph (*Escherichia coli*)

5.4.2 Biofilm Eradication assay by Mtp method

In this study, 11 well antibiofilm inhibiting actinobacterial strains were screened for antibiofilm eradication activity against gram negative pathogen (*Klebsiella pneumonia*). Of the 11 tested, strains HS2C7 showed maximum percentage of antibiofilm eradication against *Klebsiella pneumonia* pathogen for about 86.8%.

Table 5.7 Biofilm eradication assay (*Klebsiella pneumonia*)

S.NO	STRAIN NO	Biofilm Eradication (%)		
		200 µg/mL	100 µg/mL	50 µg/mL
1	HS2C7	86.8±0.09	86.6±0.07	57.1±0.76
2	HS2D1	47.15±0.30	71.4±0.20	74.5±0.139
3	HS2C5	73.2±0.3	70.5±0.257	48.72±0.07
4	HS2D4	78.53±0.13	78.4±0.052	73.72±0.14
5	HS2D2	45.83±0.77	60.87±0.52	75.32±0.157
6	HS2C6	58.9±0.33	72.1±0.2	68.08±0.03
7	HS2D31	42.3±0.776	60.3±0.43	41.35±0.85
8	HS2D9	45.51±0.97	36.2±0.82	51.2±0.523
9	HS2Z14	56.3±0.386	71.2±0.135	48.2±0.65
10	HS2D36	78.5±0.082	73.3±0.295	73.6±0.093
11	HS2Z11	68.1±0.07	67.3±0.13	71.2±0.0414

*Note: All values represent the mean values given with SEs (n = 3)

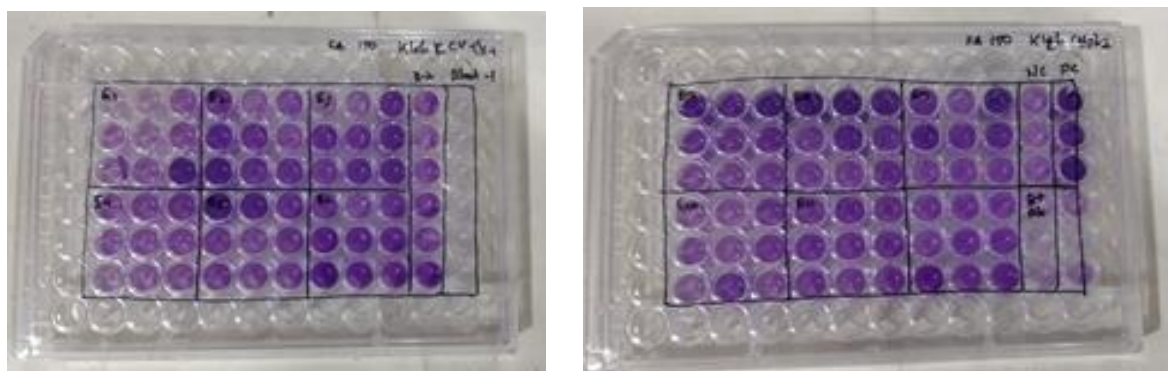


Fig 5.8 Screening of Anti-biofilm eradication activity using Microtitre plate method

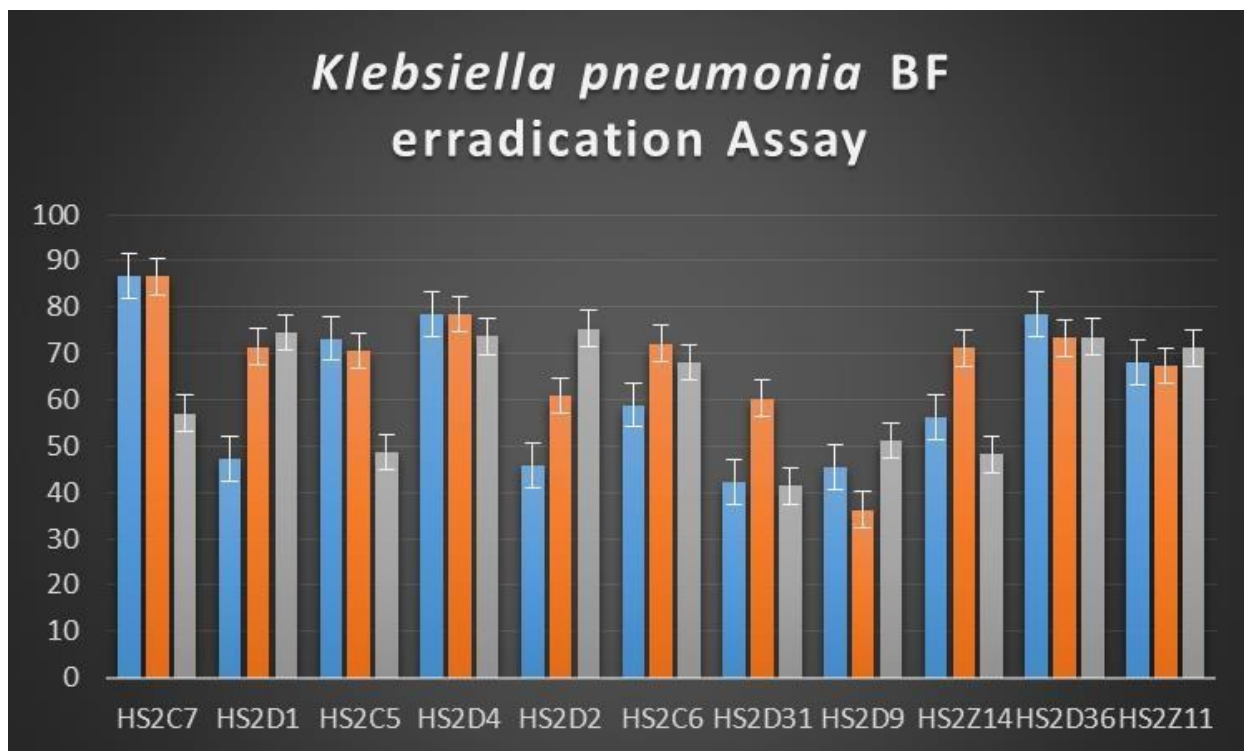


Fig 5.9 Biofilm Eradication assay Graph (*Klebsiella pneumonia*)

5.5 CHARACTERIZATION AND TAXONOMY OF POTENTIAL ACTINOBACTERIAL STRAIN

5.5.1 Micromorphology

Under bright field microscopic observation, the strain HS2C7 showed the presence of aerial and substrate mycelium with fragmentation.

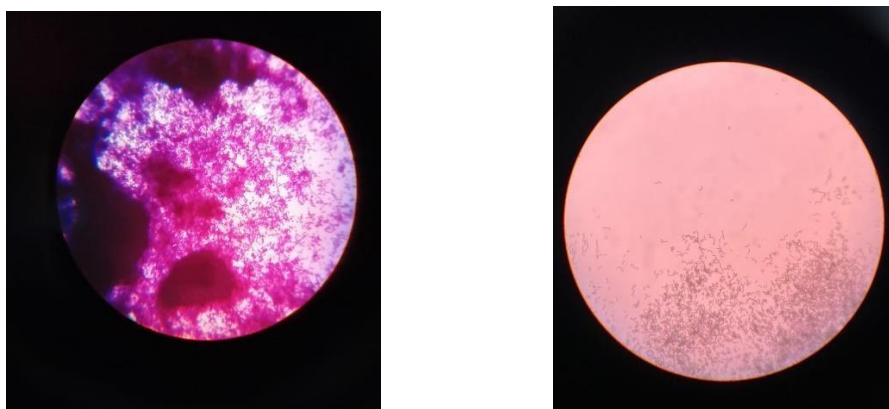


Figure 5.10 Bright field microscopic observation of strain HS2C7

5.5.2 Cultural Characteristics

Cultural morphology of strain HS2C7 showed good growth and produced powdery, greyish white colour aerial growth on different media.

Table 5.8 Culture morphology of HS2C7

S.No	Media	Growth	Aerial Color	Mass	Soluble Pigmentation	Consistency	Reverse Pigmentation
1	AIA	Good	Light grey		NO	Leathery	Grey
2	SCA	Good	White		NO	Powdery	White
3	ISP2	Good	Light grey		NO	Leathery	Brown
4	ISP3	Good	Grey		NO	Leathery	Grey
5	ISP5	Good	Light grey		NO	Powdery	Brown
6	ISP7	Good	Grey		NO	Powdery	Grey

5.5.3 Physiological characterization

Strain HS2C7 was able to grow well and utilize wide range of carbon sources, nitrogen sources, mineral sources and also in different pH listed in Table 5.9

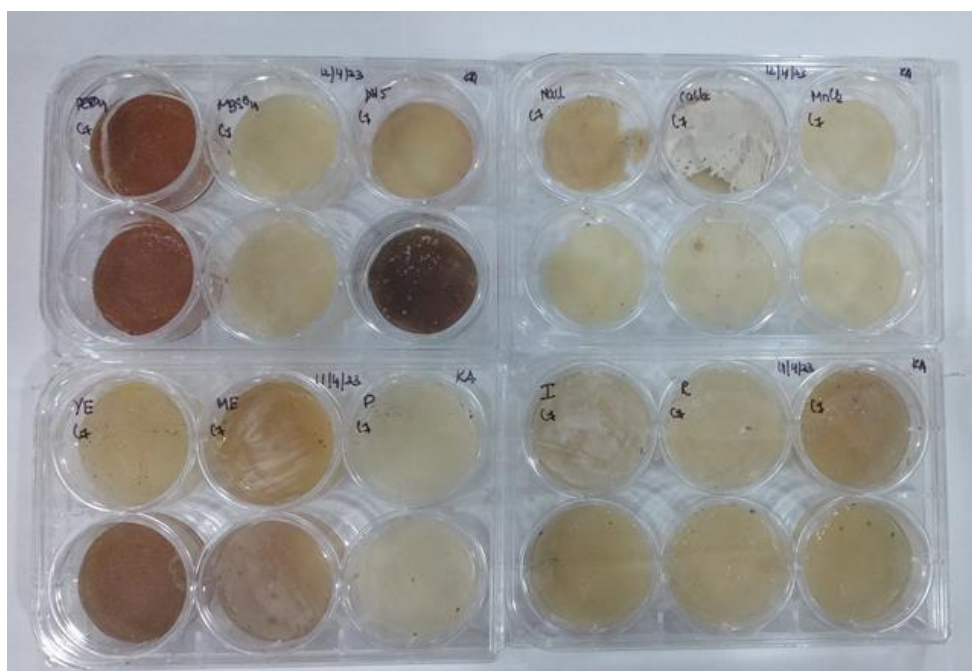


Figure 5.11 Physiological characterization under different sources

Table 5.9 Physiological characterization of strain HS2C7

s.no	Optimization	Growth
	Carbon source	
1	Glucose	Moderate
2	Lactose	Good
3	Xylose	Good
4	Starch	Good
5	Mannitol	Good
6	Inositol	Good
7	Raffinose	Good
	Nitrogen source	
8	Soyabean	Good
9	Malt	Poor
10	Peptone	Moderate
11	Yeast	Poor
12	KNO ₃	Moderate
	Mineral source	
13	NaCl	Moderate
14	MgSO ₄	Good
15	FeSO ₄	Poor
16	MnCl ₂	Poor
17	CaCl ₂	Good
	pH source	
18	pH 5	Poor
19	pH 6	Moderate
20	pH 7	Good
21	pH 8	Moderate
22	pH 9	Moderate

During optimization Lactose, Yeast extract and CaCl₂ at pH 7 with Distilled water was found to influence the metabolite production.

5.6 MOLECULAR CHARACTERIZATION

The PCR Amplification of *Streptomyces* sp. HS2C7 16s rRNA gene yields 971 base pairs. NCBI BLAST analysis of HS2C7 shows 96% similarity to sequence of *Streptomyces mutabilis*. The evolutionary history was inferred by using the maximum likelihood based on the Tamura-Nei model which confirms distinct Phylogenetic tree construction with *Streptomyces* sp. HS2C7 designated as *Streptomyces mutabilis* (figure 5.11).

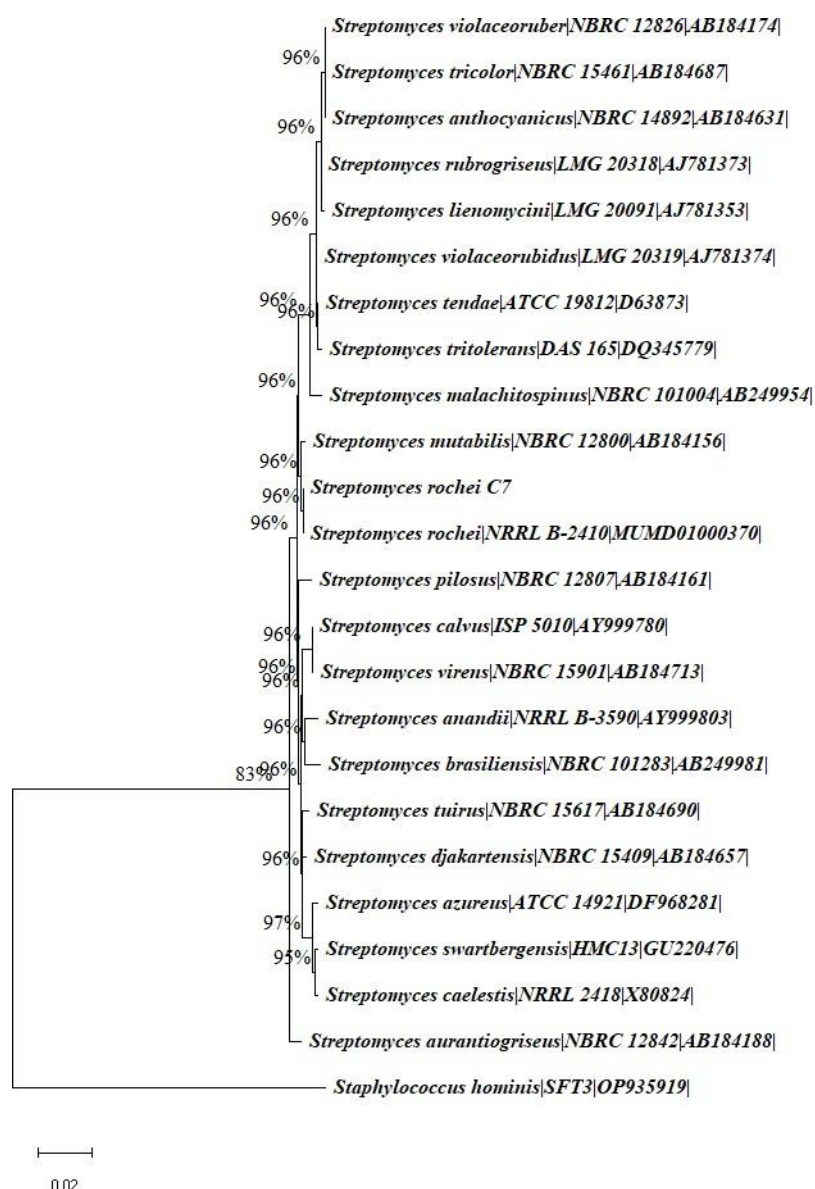


Fig 5.12 Phylogenetic tree of *Streptomyces* sp. HS2C7

5.7 PARTIAL PURIFICATION AND BIOASSAY OF CRUDE EXTRACT

Thin layer chromatography of the crude compound was extracted with ethyl acetate and purified using silica gel as absorbent and solvent system.

5.7.1 Analytical Thin Layer Chromatography

The crude compound showed five spots in Ethyl acetate: Chloroform in the ratio 0.7:0.3 of chromatogram solvents. The spots are fluoresced blue under UV light. The five spots R_f values are tabulated below.

Table 5.10 R_f values of different bands formed by strain HS2C7

S.NO	Band	R_f value
1	A	0.17
2	B	0.25
3	C	0.5
4	D	0.67
5	E	0.83

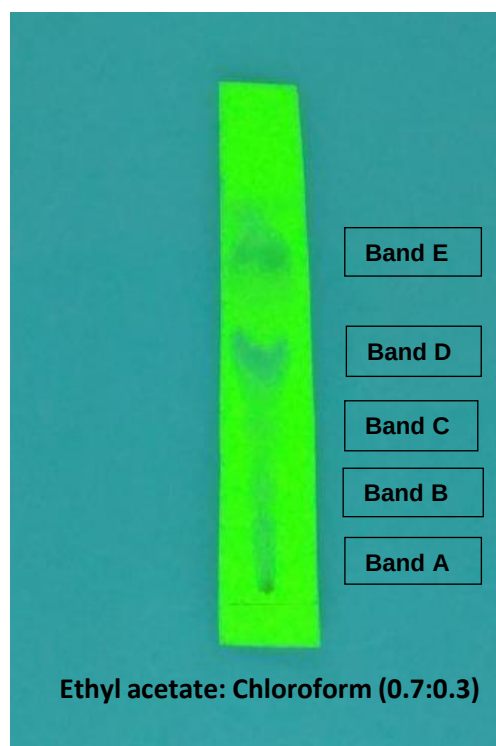
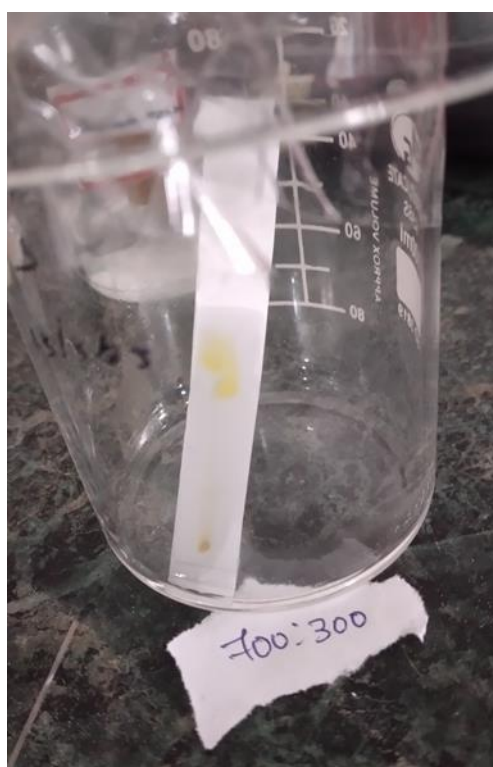


Fig 5.13 Analytical Thin Layer Chromatography

CHAPTER 6

SUMMARY AND CONCLUSION

The present study attempted to explore Actinobacteria for Antibiofilm activity against gram negative pathogens. Totally 20 actinobacterial colonies were obtained from the Bio prospecting division of Centre for Drug Discovery and Development, Sathyabama institute of science and technology. Chennai, Tamil Nadu. All the 20 cultures were retrieved by using SCA, AIA and NA media and studied for cultural characteristics. Bioactive metabolites from 20 samples were collected by Agar surface fermentation. Well grown 14 cultures were subjected to Biofilm inhibition Assay using microtitre plate method against four different pathogens *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*. From the Biofilm inhibition assay 11 extracts showed prominent results against pathogen *Klebsiella pneumonia* which is further subjected to Biofilm eradication assay. Among the 11 extracts HS2C7 showed 86.8% eradication against *Klebsiella pneumonia* which was 0.4% more eradication than the negative control (ciprofloxacin-15mcg). The MIC study revealed that the growth of *Candida* was inhibited at 100 µg/mL concentration of crude extract. Microscopic, culture and physiological characteristics of strains HS2C7 was studied by adopting standard procedures. Various sugars and nitrogen compounds and minerals were found to influence the antibiofilm compound production. Based on phenotypic and molecular characterization the potential strain HS2C7 was identified as *Streptomyces mutabilis*. Partial purification was done which shows five bands in the solvent of Ethyl acetate: chloroform (0.7:0.3). Further studies like confocal Analysis and SEM/TEM Analysis of the compound will be done in future. The findings of the present study concluded that Strain HS2C7 will be a promising source for antibiofilm metabolites against *Klebsiella pneumonia* pathogen. The production of antibiofilm metabolites from such novel species gives a great remedy for clinical biofilm which is a serious global health concern, which are tolerant to antibiotics, host defense systems and other external stresses, therefore they contribute to chronic infections.

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Thanks with regards

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