Chapter 2: Review of Literature

2.1 Natural compound

Natural compounds found in plants, and microorganisms exhibit a wide range of bioactive properties that contribute to human health and disease prevention. Synthetic drugs have many adverse or undesirable side effects and are highly toxic. Due to their toxicities, side effects, and overdose, about 1 lakh people die each year (Karimi et al., 2015). They cause back pain, stomach pain, vomiting, nausea, hair loss, breathing problems, headaches, excessive bleeding, cardiovascular depression, coma, and death. A well-known synthetic antipyretic drug, paracetamol, has lethal side effects and affects liver functioning (Nisar et al., 2018). One of the major drawbacks of synthetic drugs is the resistance developed by pathogenic microorganisms due to the overuse of drugs. The cause of this resistance may be mutation, which causes changes at the sites of drug action and affects the action of drugs. Resistance acquired by pathogens leads to severe forms of diseases and infection, which are difficult to cure, resulting in long-term illness and more chances of death. Treatment failure turns the evolution of multidrug-resistant strains such as Mycobacterium tuberculosis, for which treatment is costly (de Lima Procópio et al., 2012). So, synthetic drugs are being replaced gradually by natural drugs.

Microorganisms are bountiful sources of natural bioactive secondary metabolites and play a significant contribution to the development of anticancer agents, cholesterol-lowering agents, and immunosuppressive agents (Chin et al., 2006). During biosynthesis, they interact continuously with many enzymes, so they gain the ability to bind and interact with other molecules, which is a prerequisite to being an effective drug. They show more drug-likeness and biological friendliness and have better pharmacological and biological activity than conventional synthetic drug molecules (Lahlou, 2013). Thus, due to greater bioavailability, lesser side effects, nontoxic effects, higher selectivity, specificity,

stability, and effectiveness, natural sources are becoming popular in drug development and treating a wide range of human diseases (Michels et al., 1998; Nisar et al., 2018).

2.2 Streptomyces

Streptomyces is the largest genus of the phylum Actinobacteria, whose genetic material is rich in GC content (70%) (Beroigui & Errachidi, 2023). It is an aerobic, facultative, gram-positive, and filamentous soil bacterium with complex morphology (de Lima Procópio et al., 2012; Kapadia et al., 2007). It has the potential to form spores, and due to its ubiquitous nature, it survives in several environmental conditions, like terrestrial and marine regions, symbionts, endophytes, and mangroves (Alam et al., 2022). Over 850 species of *Streptomyces* have been studied (Kemung et al., 2018; Khadayat et al., 2020). *Streptomyces* has the ability to make colonies in different ecological niches and utilize a wide range of carbon and nitrogen sources. The optimal pH for their growth is 6.5 to 8, but some species of *Streptomyces* can tolerate a pH of 9 or more (Al-Enazi et al., 2022; Pacios-Michelena et al., 2021). The genus Streptomyces is known for producing the largest variety of natural products compared to other actinobacteria genera. About 39% of Actinobacteria have been identified as the origin of new natural products, and approximately 80% of these are attributed to the genus Streptomyces. It has been approximated that 45% of metabolites from microbial sources, or about 17% of all known active secondary metabolites, come from this genus, which comprises over 800 species (Donald et al., 2022).

Each strain of *Streptomyces* can potentially produce over 30 secondary metabolites and possesses a linear and moderately large genome (8-10 Mb) with numerous biosynthesis gene clusters (BGCs) that have the potential to synthesize various bioactive compounds for industrial, medical, and agricultural purposes (Hopwood, 2019; Salwan & Sharma, 2020). Environmental conditions and different habitats strongly contribute to the diversity

and production of natural bioactive compounds. *Streptomyces* is one of the main producers of biologically active products with medical, industrial, and agricultural applications. They produce a huge number of secondary metabolites such as antibiotics, biofilm inhibitors, antifungals, antiparasitic, antihelminth, antiviral, antibacterial, phytotoxins, plant growth regulators, herbicides, antitumor, immunosuppressive, anti-inflammatory, and antidiabetic compounds (Barka et al., 2016; Pacios-Michelena et al., 2021).

Streptomyces share the characteristics of both bacteria and fungi through their structural and functional characteristics. While they are fundamentally bacterial, their filamentous growth, mycelium formation, and spore production are reminiscent of fungi. *Streptomyces* are non-motile actinobacteria that utilize their fungus-like mycelium to expand and search for nutrients (Buzón-Durán et al., 2020). Due to this combination of traits and their abundance in soil, *Streptomyces* play an important role in the decomposition of complex mixtures of polymers found in dead plants, animals, and fungi, including cellulose and chitin (Chi et al., 2020). This decomposition process significantly contributes to organic matter turnover and the carbon cycle, replenishing soil nutrients and playing an essential role in humus formation. Due to their decomposition abilities, *Streptomyces* and other actinobacteria are excellent inoculants for compost, boosting the composting process and enhancing the quality of the final product (Feng et al., 2021; T. Wang et al., 2022).

2.3 Life cycle of *Streptomyces*

Genus *Streptomyces* retains a complex lifecycle that includes several distinct stages, like vegetative growth, aerial hyphae formation, and sporulation, and each of these stages is regulated by specific genes (S. E. Jones & Elliot, 2018). The life cycle of *Streptomyces* with various genes involved in different stages of the life cycle has been illustrated in

Figure 2.1. Vegetative Growth involves the growth of substrate mycelium, a network of hyphae that penetrates the growth medium to absorb nutrients. Aerial hyphae formation occurs when nutrients become limited, then *Streptomyces* start to form aerial hyphae that extend above the substrate. This stage is essential for reproduction and is regulated by the bld (bald) genes. These genes control the morphological differentiation that leads to the emergence of aerial structures from the vegetative mycelium. Sporulation is the final stage of the lifecycle that involves the transformation and cell division of aerial hyphae into a chain of pre-spore compartments. These compartments subsequently develop into a chain of resistant spores, which are released into the surrounding environment. This process is regulated by the whi (white) genes, which are essential for the proper division and maturation of the spore cells (Jakimowicz & van Wezel, 2012; Sousa & Olivares, 2016).

Upon encountering favorable conditions and nutrients, these spores germinate by extruding one or two germ tubes and convert into vegetative mycelium thus initiating a new cycle of growth process. Polar growth and hyphal branching in *Streptomyces* involve the elongation of hyphae by adding new cell wall material entirely at the tips. This growth pattern is regulated by several key genes and proteins. One protein is DivIVA, which is crucial in cell wall synthesis at the hyphal tip and part of the large cytoplasmic complex called the polarisome. Another protein is Scy, a cytoskeletal protein that interacts with DivIVA and helps position cell wall synthesis components. Protein FilP maintains the structural integrity of the hyphal tip and ensures its shape and rigidity for effective elongation. SepF regulates the cell division and deposition of new cell wall material at the hyphal tip. Furthermore, a regulatory protein, RsmA, controls the growth, development, and activity of proteins critical for polar growth. All these proteins work

together to make a specific zone at the hyphal tip for cell wall synthesis by cytoskeletal elements, and regulate growth and development (Flärdh, 2003; Fuchino et al., 2013)

During the vegetative phase of growth, the hyphal filament becomes segmented through the formation of cross-wall formation, which requires the tubulin-like cytoskeletal protein FtsZ for cell division. In *Streptomyces*, these cross-walls do not make cell separation and thus maintain a network of interconnected compartments. Due to a lack of nutrients, the airborne hyphae detach and grow separately, initiating the reproductive stage. During this stage, long, multi-genomic hyphae are split into multiple equally sized unigenomic prespore compartments. This division is caused by the simultaneous constriction of several FtsZ rings, leading to the release of dormant, thick-walled spores (Grantcharova et al., 2005; Ramos-León et al., 2021).

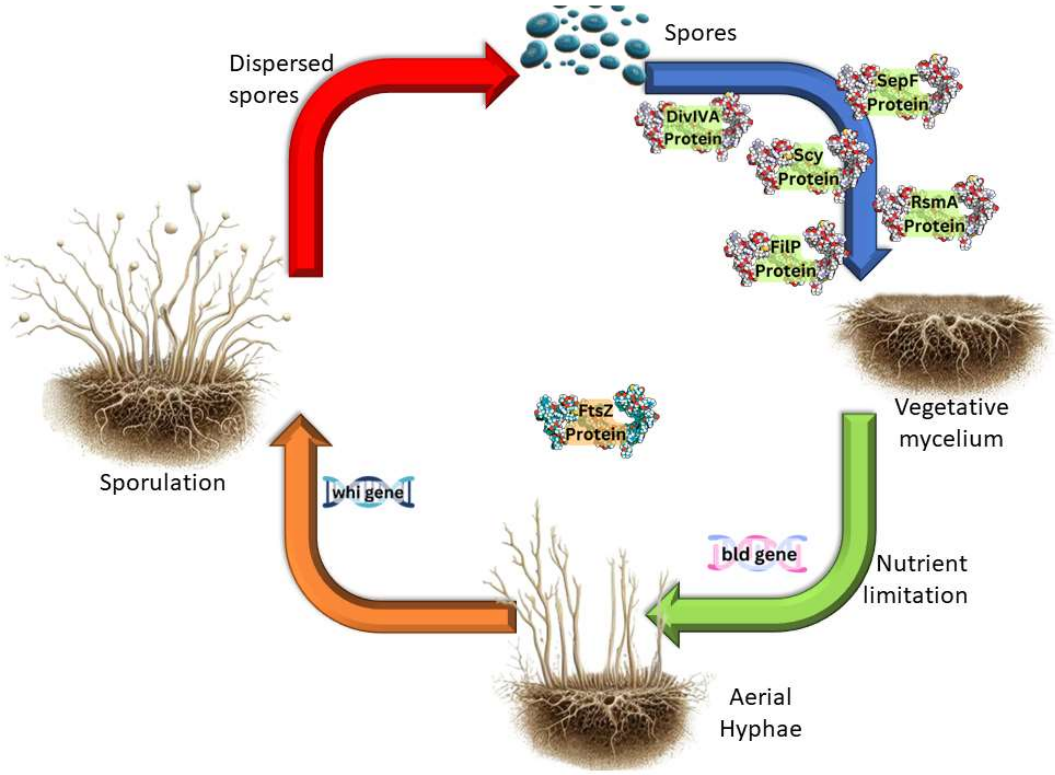

Figure 2.1: The life cycle of *Streptomyces* at different stages.

2.4 Therapeutic Application of *Streptomyces*

The medical field has been greatly influenced by the genus *Streptomyces*, which has contributed a diverse array of antibiotics, immunosuppressants, and anticancer agents (C. Gao et al., 2012). Nearly 50% of clinically effective antibiotics are derived from *Streptomyces* (Z. Zhang et al., 2020). Streptomycin was the first antibiotic produced by *S. griseus*, effectively combating tuberculosis and other bacterial infections (Schatz et al., 2005). Table 2.1 provides a detailed list of various *Streptomyces* species and their specific therapeutic application.

Various antibiotics, including Valinomycin, Tetracyclines, Pristinamycin, Lincomycin, chloramphenicol, spectinomycin, kanamycin, cephamycin, rifamycin, and Hygromycin, have been derived from different species of *Streptomyces* (Thao et al., 2017)

Many antibiotics are produced by one species of Streptomyces, as well as the same antibiotic produced by different species of *Streptomyces*. A new strain of *Streptomyces* that is *S. cheonanensis* VUK-A produces two secondary metabolites that are 2-methyl butyl propyl phthalate and diethyl phthalate, which possess a broad range of antimicrobial activity (Mangamuri et al., 2016).

Beyond antibiotics, *Streptomyces* offers a rich source of immunosuppressants, vital in organ transplantation and autoimmune diseases. An immunomodulator, such as tacrolimus from *Streptomyces tsukubaensis 9993T,* was initially employed in Japan in 1993 for the purpose of decreasing graft rejection following liver transplantation (Bolourian & Mojtahedi, 2018). Various strains of *Streptomyces* that are taxonomically diverse, especially *S. tacrolimus* ATCC 55098T, have been recognized as producers of tacrolimus (Muramatsu & Nagai, 2013). Rapamycin is another immunosuppressant from *Streptomyces hygroscopicus* that helps in organ transplantation and autoimmune disorders (Ganesh & Subathra Devi, 2023).

Streptomyces is known for its ability to produce a wide array of compounds with potential anticancer properties. Over 50% of cytotoxic compounds used in cancer treatment that come from microbes are derived from actinobacteria. They produce potent anticancer compounds like doxorubicin and daunorubicin of the anthracycline family, which are well-known for chemotherapy. Anticancer agents like bleomycin from *Streptomyces verticillus* and doxorubicin from *Streptomyces peucetius var. caesius* exhibit efficacy against various malignancies, including lymphomas, testicular cancers, squamous cell carcinomas, breast cancer, and sarcomas (Karpiński & Adamczak, 2018).

Streptomyces also offers novel antimicrobials like teixobactin, produced by *Streptomyces* sp. myrophorea, to combat drug-resistant pathogens. Additionally, it offers probiotics for gastrointestinal health, including strains like *Streptomyces* rubiginosus (M. B. Jones et al., 2017; Mok et al., 2020).

Table 2.1: List of secondary metabolites produced by different *Streptomyces* species and their therapeutic applications with their year of FDA approval.

Drug	***Streptomyces* Sp.**	**Therapeutic activity**	**year of FDA approval**	**References**
Doxorubicin	*Streptomyces peucetius*	Anticancer	1974	(Di Marco, 2012)
Daunorubicin	*Streptomyces coeruleorubidus*	Anticancer	1979	(Cassinelli, 2016)
Actinomycin D	*Streptomyces sindenensis*	Anticancer	1964	(Cibi & Nair, 2016)
Dactinomycin	*Streptomyces parvulus*	Anticancer	1964	(J.-H. Lee et al., 2016)
Valrubicin	*Streptomyces peucetius*	Anticancer	1998	(Bhattacharya & Mukherjee, 2015)
Pentostatin (deoxycoformycin)	*S. antibioticus*	Anticancer	1993	(Dillman, 2004)
Mithramycin	*Streptomyces argillaceus*	Anticancer	1970	(Saeidnia & Saeidnia, 2015)
Epirubicin	*Streptomyces peucetius*	Anticancer	2006	(Madduri, Kennedy, Rivola, Inventi-Solari, Filippini, &

				Zanuso, G., ... & Hutchinson, 1998)
Bleomycin	*Streptomyces verticillus*	Anticancer	1973	(Blomgren et al., 1977)
Streptozocin	*Streptomyces achromogenes*	Anticancer	1982	(Siedlecka et al., 2020)
Streptomycin	*Streptomyces griseus*	Antibacterial	1947	(Waksman, 1951)
Erythromycin	*Streptomyces erythraeus*	Antibacterial	1952	(Bryskier, 2005)
Neomycin	*Streptomyces fradiae*	Antibiotic (skin, gut decontamination)	1952	(Macdonald & Beck, 1983)
Amphotericin B	*Streptomyces nodosus*	Antifungal	1958	(Cavassin et al., 2021)
Chloramphenicol	*Streptomyces venezuelae*	Antibiotic	1949	(Piraee, 2002)
Vancomycin	*Streptomyces orientalis*	Antibiotic	1958	(Mertz & Doolin, 1973)
Rifampicin	*Streptomyces mediterranei*	Antibiotic (Tuberculosis)	1967	(Selva & Lancini, 2010)
Tetracycline	*Streptomyces aureofaciens*	Antibiotic	1953	(Nelson & Levy, 2011)
Clindamycin	*Streptomyces lincolnensis*	Antibiotic	1970	(Magerlein, 1971)
Daptomycin	*Streptomyces roseosporus*	Antibiotic	2003	(Miao et al., 2005)
Mitomycin C	*Streptomyces caespitosus*	Anticancer	1974	(Schein et al., 1978)
Cycloserine	*Streptomyces orchidaceus*	Antibiotic (Tuberculosis)	1964	(Johnson, 2017)
Kanamycin	*Streptomyces kanamyceticus*	Antibiotic	1958	(Hotta & Kondo, 2018)
Natamycin	*Streptomyces natalensis*	Antifungal	1978	(Meena et al., 2021)
Pristinamycin	*Streptomyces pristinaespiralis*	Antibiotic	1993	(Bonfiglio & Furneri, 2001)
Mepartricin	*Streptomyces aureofaciens*	Treatment for prostatic hyperplasia	1991	(Denis et al., 1998)
Apramycin	*Streptomyces tenebrarius*	Antibiotic	1999	(Sun et al., 2022)
Tobramycin	*Streptomyces tenebrarius*	Antibiotic	1975	(McAllister, 1976)
Capreomycin	*Streptomyces capreolus*	Antibiotic (tuberculosis)	1971	(Skinner & Cundliffe, 1980)
Clavulanic acid	*Streptomyces clavuligerus*	Beta-lactamase inhibitor	1984	(Doran et al., 1990a)

2.5 Industrial Application of *Streptomyces*

Streptomyces are extensively utilized in the industrial production of enzymes such as proteases, lipases, cellulases, amylases, chitinase, laccase, pectinases, and xylanases, which are crucial in the food, textile, and biofuel industries. Table 2.2 provides a detailed list of various *Streptomyces* species and their specific industrial uses. Additionally, some *Streptomyces* species are used in the flavor and fragrance industry to produce geosmin, a compound that imparts a distinctive earthy aroma to various products. *Streptomyces* species have several industrial applications (Beroigui & Errachidi, 2023). For example, the amylase enzyme of *S. erumpons* has several industrial uses like removal of stains, drinking in the paper industry, and the removal of starch from woven fabrics in the textile industry (Harir et al., 2018; Sharma et al., 2021). Another enzyme is chitinase used as bioremediation for the utilization of chitin waste derived from *S. griseus* and *S. antibioctius* (Spasic et al., 2018). Protease is extensively known for its various industrial applications in the fields of cosmetic manufacturing, pharmaceuticals, food processing, chemical applications, detergency, leather processing, and waste management. This enzyme is produced by *Streptomyces flavogriseus*, *Streptomyces nogalotor*, and *Streptomyces mutabilis* (Naveed et al., 2021). Enzyme pectinase produced by *S. lydicus* is used for clarification and mashing in the beverage industry and for scouring in the textile industry (Prakash et al., 2013). Besides this, pectinase is also used in the fruit and vegetable processing industries. Additionally, *Streptomyces* produce several other types of enzymes, namely azoreductase for azo dye biodegradation in the textile and paper industries, and transglutaminase used in food processing to improve the texture and quality of meat products (Beroigui & Errachidi, 2023). The diverse enzymes produced by *Streptomyces* make it an invaluable organism for industrial and biotechnological applications.

Table 2.2: List of compounds produced by various *Streptomyces* and their industrial application.

Compound	Use	Industry	*Streptomyces* Sp	Reference
Phenazine	natural dye	Textile	*Streptomyces aurantiogriseus*	(Harikrishnan et al., 2016)
Hamycin	Antifungal preservative	Food Processing	*Streptomyces pimprina*	(MADDEN, 2017)
Natamycin	Antifungal preservative	Food Processing	*Streptomyces natalensis*	(Meena et al., 2021)
Nystatin	Antifungal preservative	Food Processing	*Streptomyces noursei*	(N. Zhang et al., 2013)
Chitinases	Biodegradation of chitin	Waste Management	*Streptomyces anulatus*	(Mander et al., 2016)
Surfactin	Biosurfactant	Cosmetic Manufacturing	*Streptomyces sp.*	(Ahmadi-Ashtiani et al., 2020)
Filipin	Cholesterol-binding agent	Food Processing	*Streptomyces filipinensis*	(Bukiya et al., 2022)
Lipo-peptides	Emulsifiers and stabilizers	Food Processing	*Streptomyces roseosporus*	(Baltz et al., 2006)
Lipases	Waste oil degradation	Waste Management	*Streptomyces griseus*	(Vishnupriya et al., 2010)
	Removal of stains	Detergency		
	Deinking, cleaning	Textile		
	Cheese flavoring	Dairy		
cellulases	Removal of stains	Detergent	*S. thermobifida, S. halotolerans, S. thermomonospora, S. ruber*	(Harir et al., 2018)
	Denim finishing, softening of Cotton	Textile		
	Deinking, modification of fibers	Paper and pulp		
Proteases	Cheesemaking	Food	*S. pactum, S. thermoviolaceus, Streptomyces sp.*	(Ruwandeepika et al., 2022)
	Clarification: low-calorie beer	Brewing		
	Dehiding	Leather		
	Treatment of blood clots	Medicine		
Amylases	Production of glucose, fructose, syrups	Food Industry	*S. erumpons*	(Harir et al., 2018)
Laccases	Clarification (juice), flavor	Bleaching	*S. brahimensis*	(Couto & Herrera, 2006)

	(beer), cork stopper treatment			
Keratinase	Feather degradation	Waste Management	*Streptomyces pactum*	(Tamreihao et al., 2019)
Collagenase	Leather processing (softening)	Leather Processing	*Streptomyces parvulus.*	(Sakurai et al., 2009)
Lipstatin	Lipase inhibitor (weight management)	Food Processing	*Streptomyces toxytricini*	(P. Kumar & Dubey, 2015)
Granaticin	Natural dye	Cosmetic Manufacturing	*Streptomyces violaceoruber*	(Beroigui & Errachidi, 2023)
Prodigiosin	Natural dye	Cosmetic Manufacturing	*Streptomyces coelicolor*	(Srilekha et al., 2024)
Actinomycin X2	Natural dye	Food Processing	*Streptomyces chrysomallus*	(Bhakyashree & Kannabiran, 2020)
Actinorhodin	Natural dye	Food Processing	*Streptomyces coelicolor*	(Bystrykh et al., 1996)
Erythrosine	Natural dye	Food Processing	*Streptomyces griseus*	(A. Banerjee et al., 2000)
Coelimycin	Natural dye	Textile	*Streptomyces coelicolor*	(Bednarz et al., 2019)
Rishirilide B	Natural dye	Textile	*Streptomyces rishiriensis*	(Schwarzer et al., 2020)
Actinomycin D	Pigment	Cosmetic Manufacturing	*Streptomyces antibioticus*	(Nuanjohn et al., 2023)
Melanin	Pigment	Cosmetic Manufacturing	*Streptomyces kathirae*	(Guo et al., 2014)

2.6 Agricultural Application of *Streptomyces*

In agriculture, *Streptomyces* produce bioactive compounds used as biopesticides, biofertilizers, plant growth regulators, insecticides, and herbicides. Furthermore, *Streptomyces* are utilized in bioremediation processes on an industrial scale to degrade environmental pollutants, including pesticides, heavy metals, hydrocarbons, and aliphatic and aromatic compounds, showcasing their versatility and importance in various industrial applications (Alvarez et al., 2017; Vurukonda et al., 2018). This bioremediation process helps manage waste and reduce environmental pollution. *Streptomyces* play an important role in plant biotechnology due to their ability to combat plant pathogens, making them valuable for biocontrol purposes. Many *Streptomyces* strains have the

potential to transform overlooked agricultural and urban waste materials into valuable chemical products. Proteases from *Streptomyces* spp. have been proven to be efficient in recycling agro-industrial waste materials such as plant waste, feathers, hair, and nails (B. Srivastava et al., 2020). In the last five years, about 60% of newly discovered insecticides and herbicides have been derived from *Streptomyces*. Table 2.3 highlights *Streptomyces* strains with promising potential in bioremediation, biopesticides, biofertilizers, plant growth regulation, insecticides, and herbicides, contributing to disease control, enhanced plant growth, and increased yield. One notable example is kasugamycin, a bactericidal and fungicidal compound isolated from *Streptomyces kasugaensis*. It inhibits protein biosynthesis in microorganisms without affecting mammals, due to its excellent toxicological profile. *Streptomyces* spores enable long-term soil survival under harsh conditions. This resilience makes them ideal for bioremediating polluted environments, including organochlorines, organophosphates, pyrethroids, ureas, and chloroacetanilides. It is used as a biofertilizer due to its ability to degrade agro-wastes and produce enzymes that enhance plant nutrition and growth (Al-Quwaie, 2024; Devi et al., 2022). Some species of *Streptomyces*, such as *Streptomyces thinghirensis* HM3 and *Streptomyces tricolor* HM10, produce plant growth hormones like IAA, siderophores, and solubilize inorganic phosphate. They enhance soil phosphate availability by converting bound phosphate into usable forms through phosphate-solubilizing acids and phytase enzymes (Beroigui & Errachidi, 2023).

Table 2.3: List of secondary metabolites produced by the genus *Streptomyces* with their various agricultural uses.

Compound	Agricultural Uses	*Streptomyces* Sp.	References
Abamectin	Insecticide, Nematicide	*Streptomyces avermitilis*	(Jansson & Dybas, 1998)
Aculeximycin	Insecticide	*Streptomyces albus*	(Kontou et al., 2021)
Bafilomycin	Insecticidal, antifungal	*Streptomyces griseus*	(D. Zhang et al., 2011)

Bialaphos	Herbicide	*Streptomyces hygroscopicus*	(Thompson et al., 1987)
Geldanamycin	HSP90 inhibitor (plant growth regulator)	*Streptomyces hygroscopicus*	(Piper & Millson, 2012)
Helvolic acid	Antifungal	*Streptomyces lavendulae*	(Adhikari et al., 2023)
Milbemycin	Acaricide, Insecticide	*Streptomyces hygroscopicus*	(TAKAHASHI et al., 1993)
Nikkomycin Z	Fungicide, Chitin synthase inhibitor	*Streptomyces tendae*	(M Chaudhary et al., 2013)
Phosphinothricin	Herbicide	*Streptomyces viridochromogenes*	(Schwartz et al., 2004)
Polyoxin D	Fungicide	*Streptomyces cacaoi*	(Chen et al., 2009)
Streptomycin	Control of plant pathogens	*Streptomyces griseus*	(Le et al., 2022)
Validamycin	Fungicide, Plant growth regulator	*Streptomyces hygroscopicus*	(Yu et al., 2005)
Hygromycin B	Antibiotic (used in animal feed)	*Streptomyces hygroscopicus*	(Dhote et al., 2008)
Lincomycin	Antibiotic (used in animal feed)	*Streptomyces lincolnensis*	(D.-B. Choi & Cho, 2004)
Phytase	Feed additive (improving phosphate availability)	*Streptomyces sp.*	(Jain & Singh, 2016)
Antimycin A	Piscicide	*Streptomyces griseus*	(Rasimus-Sahari et al., 2016)
Avermectins	Insecticides	*Streptomyces avermitilis*	(Kim et al., 2017)
Neomycin	Veterinary antibiotic	*Streptomyces fradiae*	(Vastrad & Neelagund, 2014)
Tetronasin	Anticoccidial (Veterinary Medicine)	*Streptomyces longisporoflavus*	(Butaye et al., 2003)

2.7 *Streptomyces clavuligerus* and *Streptomyces fragilis* and their secondary metabolites production.

Streptomyces clavuligerus and *Streptomyces fragilis* are gram-positive, filamentous, soil bacteria of the phylum Actinobacteria. They form a complex, aerial mycelium structure which comprises sympodial, branched hyphae and spores. *Streptomyces clavuligerus* produces dark greyish mycelium, while *Streptomyces fragilis* produces dark brownish mycelium (Bascarán et al., 1990; Nithya et al., 2017). *Streptomyces clavuligerus* generates β-lactam antibiotics including clavulanic acid, cephamycin C, penicillin N, O-

carbamoyl derivative deacetylcephalosporin C, and deacetoxycephalosporin C (Nabais & da Fonseca, 1995; Saudagar et al., 2008). It also forms non-β-lactam secondary metabolites, holomycin, and complex MM 19290 (Baggaley et al., 1997). Clavulanic acid is a novel β-lactamase inhibitor (broad-spectrum antibiotic) effective against gram-positive and gram-negative bacteria. Its salt and ester can enhance the impact of β-lactam antibiotics against bacteria that produce β-lactamase (Reading & Cole, 1977; Saudagar et al., 2008). It also engenders a proteinaceous beta-lactamase inhibitor, BLIP, structurally unrelated to clavulanic acid (Doran et al., 1990b). It also retains the ability to produce kinase and phosphatase inhibitors, which can be lead molecules in the target-based treatment of diseases (Běhal, 2000). *Streptomyces fragilis* produces the tumor-inhibiting antibiotic Azaserine, which targets nucleic acid and demonstrates efficacy against gram-positive and gram-negative bacteria, protozoa, and fungi. It also produces the enzyme α-amylase, proteases, and cellulases. This strain produces several secondary metabolites through fermentation, including antibiotics, antifungals, and anticancer compounds (Law et al., 2019; Pittillo & Hunt, 1967; Van Cura et al., 2023).

2.8 Anthracycline and its mechanism of action

Anthracyclines are effective anticancer drugs produced by *Streptomyces* and composed of an anthraquinone ring (a planar polyaromatic ring system with a quinone moiety) linked to an amino sugar (Coufal & Farnaes, 2010). It is basically a 7,8,9,10-tetrahydro-5,12-naphthacenequinone structure, which is glycosylated with sugar residues (Olano, Méndez, et al., 2009). Its anthraquinone ring is a chromophore that absorbs light in both visible spectra as well as in UV spectra and provides orange-red fluorescence to the compounds. This fluorescence is lost by compounds when they are intercalated in DNA (Ozluer & Kara, 2014).

Anthracyclines utilized in cancer therapy include Doxorubicin, Daunorubicin, Mithramycin, Idarubicin, Epirubicin, Aclarubicin, Amrubicin, Valrubicin, and Pirarubicin (McGowan et al., 2017). These compounds retain potential for the treatment of various types of cancer and are used as individually as well as in combination for the therapy. Doxorubicin is employed in treating Hodgkin's lymphoma, lung cancer, recurring ovarian cancer, multiple myeloma, and breast cancer (Thorn et al., 2011). Mithramycin is used for advanced testicular carcinoma, Paget's disease, and chronic myelogenous leukemia (Kormanec et al., 2020). Epirubicin, a less cardiotoxic analog of doxorubicin and is not only effective against leukaemia and lymphoma but also against solid tumors of diverse origins, including breast, lung, and brain cancers. Idarubicin is indicated for acute myeloid leukaemia, while valrubicin is utilized for intravesical treatment of bladder cancer (Cersosimo & Hong, 1986; Mattioli et al., 2023).

Anthracyclines exhibit anticancer or cytotoxicity effects through various mechanisms of action, which include DNA cleavage mediated by DNA topoisomerase II, inhibition of enzymes such as RNA polymerase and cytochrome c oxidase, DNA intercalation, iron chelation, and the generation of reactive oxygen species, which collectively lead to the induction of apoptosis.

But, in 1984, it was found that anthracyclines mainly exert their effect by interfering with the action of nuclear DNA topoisomerase II (Beretta & Zunino, 2008; Zunino & Capranico, 1990). The action mechanism of the anticancer activity of anthracycline is represented in Figure 2.2. During the binding with DNA and inhibition of the DNA topoisomerase II enzyme, the structure and stereochemistry of the aminosaccharide moiety, as well as the positive charge of the amino group of anthracycline compounds, are critical for the molecular recognition of the cellular target and the biological activity of anthracycline compounds. For example, the conversion of the amino group into an

amide group significantly alters the cytotoxicity potential of anthracyclines and also reduces their binding affinity to DNA. Its quinone part also contributes to anticancer potential through the generation of reactive oxygen species. These reactive species cause damage to the cell membrane, lipid, DNA, and protein, which leads to oxidative stress and cancer cell death through apoptosis (Malik et al., 2021).

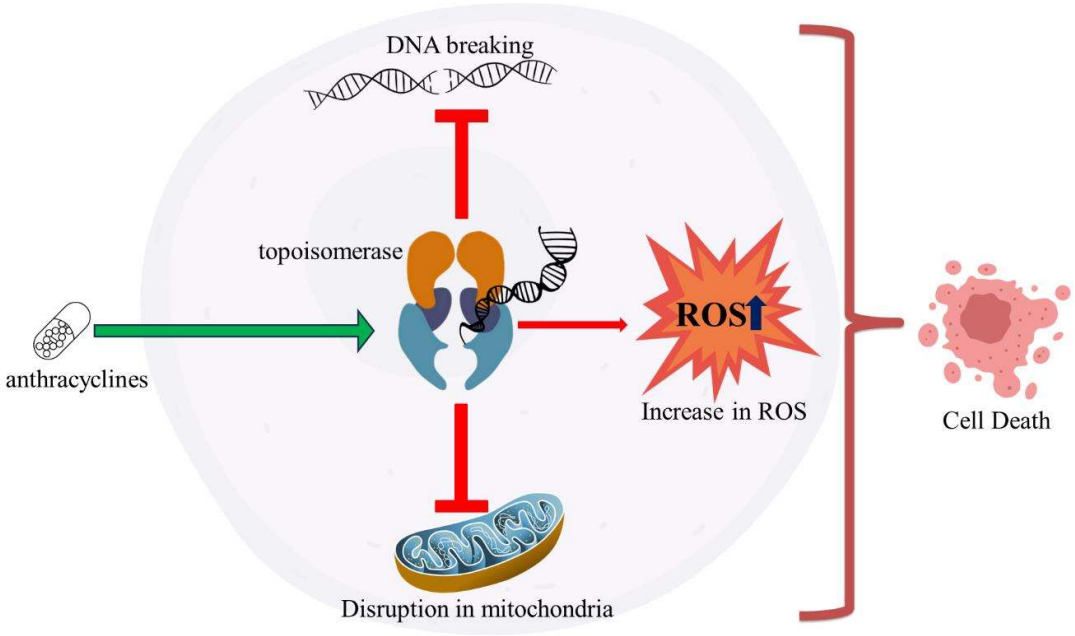

Figure 2.2: Mechanisms of anticancer activity of anthracycline drugs associated with inhibition of DNA topoisomerase II and overproduction of reactive oxygen species and cell apoptosis.

2.9 Doxorubicin and Epirubicin

Doxorubicin, also known as Adriamycin, is a naturally occurring anthracycline widely used in chemotherapy for breast cancer, sarcomas, leukemia, and lymphomas (Carvalho et al., 2009). Epirubicin, on the other hand, is a semisynthetic analogue of doxorubicin with an opposite configuration of the hydroxyl group at the C-4 of the deoxysugar (Han et al., 2011) (Figure 2.3). It emerged with its first clinical trial in 1980 and FDA approval in 1999 for use in cancer treatment, primarily as a component of chemotherapy for breast cancer and other solid tumors (Cozzi et al., 2004).

Despite their similar therapeutic potential and mechanism of action, there are differences in their pharmacokinetic properties and side effect profiles, which lead to differences in their clinical use. Notably, doxorubicin is associated with dose-dependent cardiotoxicity, which leads to cardiac depression, progressive heart failure, the development of resistance by tumor cells, and myelosuppression.

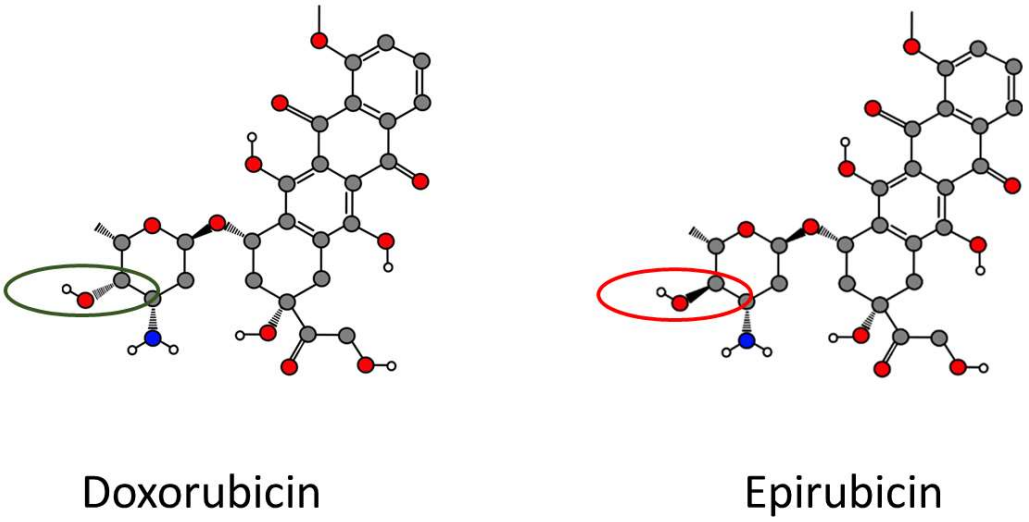

Figure 2.3: 2D image of Doxorubicin and Epirubicin showing the major difference at the C-4 position.

In contrast, epirubicin displays reduced cardiotoxicity while maintaining comparable anticancer potential. The difference in cardiotoxicity between doxorubicin and epirubicin can be largely attributed to their saccharide moieties, which are present in the opposite configuration in epirubicin compared to doxorubicin and play a crucial role in reducing epirubicin's cardiotoxic effects (Schott & Hayes, 2015; Wenningmann et al., 2019). Additionally, epirubicin has an increased volume of distribution and longer half-life than doxorubicin, with t½ of doxorubicin being 1-3 hr and epirubicin having 31-35 hrs. The addition of the 3'-hydroxyl group to the daunosamine sugar moiety leads to the alteration of the pharmacokinetic properties of epirubicin and ultimately enhances its safety profile in comparison to doxorubicin (Camaggi et al., 1988; McGowan et al., 2017).

2.10 Microbial Production of Epirubicin

Microbial production of epirubicin or its precursor involves exploiting the metabolic capabilities of certain bacterial strains to synthesize this intricate molecule or its precursor compounds through the fermentation process. During fermentation, these bacteria are cultivated in bioreactors under controlled conditions like temperature, pH, nutrient availability, and oxygen supply, which are optimized to promote bacterial growth and stimulate the production of epirubicin or its precursors. This approach enhances the efficiency and cost-effectiveness of epirubicin production by significantly boosting its yield (Aquinas et al., 2024).

Another strategy involves genetic and metabolic engineering techniques employed to enhance the expression of key genes involved in this biosynthetic pathway of epirubicin and its precursors by gene knockout, overexpression, or the introduction of genes from other organisms to enhance the metabolic capabilities of the host bacteria. The biosynthesis of epirubicin or its precursor involves the assembly of polyketide scaffolds through the action of various enzymes, including polyketide synthases, glycosyltransferases, cyclases, aromatases, and ketoreductases (Risdian et al., 2019). Genetic manipulation and modification of the biosynthetic pathways of these enzymes have enabled the efficient production of epirubicin and its derivatives. Table 2.4 presents a comprehensive list of microbial strains with the potential to synthesize epirubicin, its precursors, or analogous compounds.

In 1998, Madduri et al. achieved a significant breakthrough by pioneering the microbial fermentation process to produce epirubicin and its analogous compound, 4'-epidaunorubicin. They enhance the production of these compounds by incorporating genes from two bacterial strains, *Streptomyces* avermitilis (avrE) and *Saccharopolyspora erythraea* (eryBIV), into a mutant strain of *Streptomyces* peucetius lacking daunosamine

biosynthesis. This innovative approach demonstrates the production of hybrid antibiotics through combinatorial biosynthesis involving bacterial deoxysugar genes (Madduri, Kennedy, Rivola, Inventi-Solari, Filippini, & Zanuso, G., ... & Hutchinson, 1998).

Following Madduri et al., Shang et al. made significant progress in 2008 by successfully producing 4'-epidaunorubicin from *Streptomyces coeruleorubidus* strain SIPI-1482. This was done by introducing specific genes-aveBIV, oleU, and novS-into the dnmV mutant of *S. coeruleorubidus*. This study marked another milestone in microbial production of 4'-epidaunorubicin, a precursor of epirubicin, by genetic manipulation (Shang et al., 2008).

In 2010, Shao et al. further enhanced the production of 4'-epidaunorubicin by overexpressing *Streptomyces avermitilis* aveBIV in a daunosamine biosynthesis-deficient mutant of *Streptomyces coeruleorubidus* SIPI-A0707 (dnmV mutant). By increasing gene copy number or altering integration techniques, they successfully achieved efficient industrial-scale production of 4'-epidaunorubicin, highlighting the potential for large-scale production of 4'-epidaunorubicin (Shao et al., 2010).

Han et al. developed a highly effective method for producing epirubicin and novel doxorubicin analogs by utilizing mutants of *Streptomyces venezuelae* along with plasmids to create a diverse range of deoxysugars. These deoxysugars were then transferred onto aglycone, resulting in an enhanced production of epirubicin and its analogs. Importantly, this method eliminated the need for chemical synthesis, offering a more sustainable and efficient route for production (Han et al., 2011).

In 2018, Shao et al. achieved a groundbreaking advancement in antibiotic production by achieving the highest yield of 4'-epidaunorubicin to date, reaching 124.1 mg/L. This was accomplished through metabolic engineering conducted in *Streptomyces coeruleorubidus*

SL-7 by replacing the dnmV gene with aveBIV and subsequently knocking out the dnrU and dnrX genes. They also successfully optimized the growth conditions for a microbial strain to enhance the production. This achievement underscored the potential for large-scale commercial production of 4'-epidaunorubicin using fermentation methods (Shao et al., 2018).

Table 2.4: Presents a comprehensive list of microbial strains with the potential to synthesize epirubicin, its precursors, or analogous compounds.

Microbial Strain	Compounds	Reference
Streptomyces coeruleorubidus strain SIPI-A0707	4′-epidaunorubicin	(Shao et al., 2010)
Streptomyces coeruleorubidus strain SIPI-1482	4’ -epidaunorubicin	(Shang et al., 2008)
Streptomyces coeruleorubidus SL-7	4'-epidaunorubicin	(Shao et al., 2018).
Streptomyces venezuelae	Rhodomycin D, Doxorubicin, 4′-epi-rhodomycin D, Epirubicin	(Han et al., 2011)
Streptomyces peucetius	4'-epidaunorubicin	(Madduri, Kennedy, Rivola, Inventi-Solari, Filippini, & Zanuso, G., ... & Hutchinson, 1998)

2.11 Submerged Fermentation and Batch Fermentation

Submerged fermentation for the production of secondary metabolites involves the cultivation of bacteria in a liquid medium or broth containing the desired liquid medium rich in nutrients like sugars, minerals, and nitrogen sources. Submerged fermentation is the most common fermentation process, conducted in closed vessels under controlled conditions of temperature, pH, and agitation to optimize the growth and reproduction of the microorganism (Subramaniyam & Vimala, 2012). This method also has the advantages of optimal control of crucial environmental parameters and nutrient availability, which facilitate the easy scale-up operation and do not cause heat and mass transfer limitations. Additionally, the liquid medium ensures uniform distribution of nutrients and oxygen, promoting consistent drug quality and ease of monitoring.

However, submerged fermentation also has limitations, which include lower concentration and higher dilution of drug product, low yield, high production cost, contamination risk, and higher production of effluents (Doriya et al., 2016). Genus *Streptomyces* mostly prefers submerged conditions for the synthesis of secondary metabolites. To reduce its production costs, various cost-effective carbon sources can be explored compared to synthetic media dextrose**,** which is currently utilized for the submerged production of bioactive compounds.

Batch fermentation is a crucial method for drug production with controlled microbial growth in a closed system with a sterile culture medium and controlled physical parameters to maximize yield (Cinar et al., 2003). During the growth phase, *Streptomyces* rapidly consume nutrients and produce primary metabolites, while secondary metabolites are typically produced during the stationary phase when nutrients become limited. The advantages of batch fermentation include high yield, reduced contamination risk, flexibility, and ease of scale from laboratory to industrial scale (A. Kumar et al., 2020; Schäpper et al., 2009).

2.12 Optimization Approach for Enhancement of Bioactive Compound

2.12.1 Optimization Using Response Surface Methodology Approach

The Response Surface Methodology (RSM) approach is a systematic method used to refine process parameters and enhance product yields or performance. By employing statistical and mathematical techniques, RSM establishes a model that describes the relationship between input variables (factors) and output responses (product properties) (Hadiyat et al., 2022). RSM serves as a powerful tool and is widely utilized across various industries, including pharmaceuticals, chemicals, food, and biotechnology, for enhancing product quality, increasing yields, and improving process efficiency (Yolmeh & Jafari, 2017).

They are used to refine the levels of key process parameters identified during initial screening experiments and are particularly effective for experiments involving curvature effects (Franceschini & Macchietto, 2008). Two widely utilized response surface experimental designs for bioprocess optimization studies are the Central Composite Design (CCD) and the Box-Behnken Design (Boateng & Yang, 2021). In CCD, each process parameter is varied at five levels (-2, -1, 0, +1, +2), whereas in the Box-Behnken Design, each parameter is varied at three levels (-1, 0, +1). Both designs generate second-order quadratic models that can predict outcomes for conditions that have not been experimentally tested. These models are highly effective at measuring variability (pure error) during experiments. Additionally, these experimental designs can estimate the interaction effects among various process parameters in optimization studies. The Box-Behnken experimental design offers a practical advantage over the Central Composite Design (CCD) by requiring fewer experimental runs, with a cost-effective option for the same number of process parameters (Oza et al., 2022). This efficiency is particularly valuable in bioprocess optimization, where resource constraints and the need for precision are critical. Results from both the Box-Behnken and CCD designs can be effectively visualized using response surface plots or contour plots. Data collected from these experiments are then used to develop mathematical models, often quadratic or higher-order polynomial equations, through regression analysis (B. C. Y. Lee et al., 2022; H. M. Singh et al., 2024).

The outcomes obtained from these designs can be analyzed for statistical significance using analysis of variance (ANOVA) (Gelman, 2005). In this context, a p-value of 0.05 or less is generally considered statistically significant and not due to random variation (Adeniran et al., 2021; Sawyer, 2009). This statistical rigor ensures that the conclusions drawn from the experiments are robust and reliable. Utilizing the developed model,

optimization techniques are applied to predict the optimal set of input variables that yield desired responses. This enables efficient identification of optimal operating conditions while minimizing the number of experiments required. Finally, the optimized conditions are validated through confirmation experiments to ensure the predicted responses align with observed outcomes. These experimental designs are implemented using advanced statistical software packages such as Minitab and Design-Expert. These tools provide user-friendly interfaces and powerful analytical capabilities to design experiments, analyze data, and interpret results with greater accuracy and efficiency (Bianchesi et al., 2019; Jena et al., 2021).

2.12.2 Optimization using Artificial Neural Network (ANN)

Artificial Neural Networks (ANNs) have recently been used as a novel methodology or computational approach, which is based on the principles of artificial intelligence and machine learning, to optimize complex processes (Basheer & Hajmeer, 2000). Unlike traditional techniques, ANNs capture complex real-world processes for accurate predictions and improved outcomes (Gupta et al., 2021). It offers adaptability across industries and accommodates diverse data types, including numerical, categorical, and even image or text data. ANNs are computational models that mimic the structure and function of biological neural networks in the human brain (Nwadiugwu, 2020). They consist of interconnected artificial nodes, or neurons, which are organized into three layers: input layer, hidden layers, and output layer (Grossi & Buscema, 2007).

In the fermentation optimization process, feedforward neural networks are the preferred choice due to their efficient flow of information from input to output layers in a unidirectional manner (Nagy, 2007). In this setup, the initial layer comprises input variables or process parameters that need to be optimized. Conversely, the output layer contains the predictions generated by the neural network, such as the estimated yield of

secondary metabolites in this study. Therefore, the input layer neurons encode the input data, while the output layer neurons provide responses corresponding to the input data.

The hidden layers are used to bridge the input and output layers, optimizing their connections to best fit the experimental data (Abdolrasol et al., 2021). Initially, the neural network is trained by providing experimental dataset, which is then divided into three subsets: the training set, validation set, and test set, typically in a ratio of 70:15:15. The training set constitutes the majority of the input data and is used to teach the neural network model important details from the data. Subsequently, a portion of the data is reserved for validating the model's performance during training, known as the validation data. The test dataset is exclusively used to assess the performance of the ANN model after completion of training (Hamed et al., 2004).

For the training of a neural network model, a feed-forward neural network algorithm is utilized, and the results are obtained with predicted values (Bhaskar & Singh, 2012). During training, the values in the training dataset are initialized with certain weights (w), which are then iteratively optimized until reaching optimal values. These optimal weights are achieved when the neural network effectively predicts values closely resembling those obtained experimentally.

Once a successful neural network is developed, capable of predicting outputs akin to experimental values, unknown dataset values are introduced to predict responses. Experimental runs are then conducted on these unknown data points, with close alignment between experimental and predicted values confirming the neural network's accuracy and efficacy.

2.12.3 Optimization Using Machine Learning Approach

Machine Learning (ML) has recently been used as a novel methodology or computational approach that is based on the principles of artificial intelligence to optimize complex

processes. Unlike traditional techniques, ML models capture complex real-world processes for accurate predictions and improved outcomes (Frank et al., 2020). It offers adaptability across industries, accommodating diverse data types including numerical, categorical, and even image or text data. ML models are computational constructs that learn patterns from data and make predictions or decisions without being explicitly programmed. They consist of algorithms that iteratively learn from data to improve their performance (Jordan & Mitchell, 2015).

In fermentation optimization processes, various machine learning algorithms can be employed due to their ability to handle complex relationships between input and output variables. In this setup, the initial step involves input variables or process parameters that need to be optimized. The output from the model consists of predictions such as the estimated yield of secondary metabolites in this study. Therefore, the input data encodes the process parameters, while the output provides responses corresponding to these inputs (Zhu et al., 2020).

The learning process involves splitting the dataset into three subsets: the training set, validation set, and test set, typically in a ratio of 70:15:15. The training set constitutes the majority of the input data and is used to teach the machine learning model important details from the data (L'heureux et al., 2017). Subsequently, a portion of the data is reserved for validating the model's performance during training, known as the validation data. The test dataset is exclusively used to assess the performance of the ML model after completion of training (Raschka, 2018).

During training, machine learning algorithms are utilized to learn from the training dataset and generate predicted values. Initially, the model's parameters are set with certain values, which are then iteratively optimized until reaching optimal values. These optimal parameters are achieved when the machine learning model effectively predicts values

closely resembling the experimentally obtained ones (Uddin et al., 2019). Once a successful machine learning model is developed, capable of predicting outputs akin to experimental values, unknown dataset values are introduced to predict responses. Experimental runs are then conducted on these unknown data points, with close alignment between experimental and predicted values confirming the machine learning model's accuracy and efficacy (Yarkoni & Westfall, 2017).

2.13 Receptor Tyrosine Kinase

Receptor Tyrosine Kinases (RTKs) play an essential role in controlling various cellular processes such as cell growth, differentiation, migration, and metabolism (Shchemelinin et al., 2006). Structurally, RTKs consist of an extracellular domain that binds ligands, a single-pass transmembrane domain, and an intracellular domain with intrinsic tyrosine kinase activity (Paul & Hristova, 2018). However, dysregulation in RTKs expression, such as overexpression, mutation, or amplification in RTKs, results in their constitutive activation, leading to uncontrolled cell growth and proliferation, a hallmark of multiple types of cancer (Butti et al., 2018; J. Wang et al., 2016). Cancer is a complex and often deadly disease; while treatments such as surgery and radiation can be effective, they are not universally curative and may require integration with other therapeutic approaches (Manda et al., 2009). Focusing on RTKs or their signaling pathways displays a distinct possibility for creating a pharmacological strategy for cancer treatment (Gschwind et al., 2004; Roskoski, 2022). Several receptor tyrosine kinases (RTKs) have been identified as targets for anticancer drug intervention, including the Insulin Growth Factor Receptor (IGFR), Hepatocyte Growth Factor Receptor (HGFR), Fibroblast Growth Factor Receptor (FGFR) family, Vascular Endothelial Growth Factor Receptor (VEGFR) family, Platelet-Derived Growth Factor Receptor (PDGFR) family, and Epidermal Growth Factor Receptor (EGFR) (Zwick et al., 2001). Thus, the identification of secondary metabolites

from the genus Streptomyces that possess the ability to selectively target and inhibit these mutated RTKs potentially leads to novel and targeted anticancer therapies. Therefore, an *insilico* approach including molecular docking, dynamic simulation, and MM/GBSA analysis is an efficacious approach in identifying potential inhibitors from *Streptomyces* that can selectively target the mutated Receptor Tyrosine Kinase (RTKs) (Mohapatra et al., 2023).

2.14 Protein Kinase Inhibitory Assay

The genus *Streptomyces* possesses both eukaryotic signaling systems and prokaryotic two-component regulatory systems, which draw attention to the development of an assay for screening various kinase inhibitors in the eukaryotic signaling pathway. *Streptomyces 85E* strain has been identified as a suitable strain for testing kinase inhibitors using agar diffusion methods due to its growth and development characteristics on solid media and its ability to reveal the cytotoxicity of tested inhibitors (Watersa et al., 2002). The inhibition of hyphae formation has been observed with known eukaryotic kinase inhibitors, such as tyrphostin and genistein, forming "bald" colonies (Shanbhag et al., 2015b).

The *Streptomyces 85E* assay exhibits three distinct phenotypes that can be utilized to deduce the persistence of eukaryotic protein kinase inhibitors (Cheenpracha et al., 2010). These phenotypes encompass the absence of a zone, indicating the drug's inactivity; a clear transparent zone signifying the cytotoxicity towards the cell, which in turn inhibits both growth and sporulation; and a turbid grey zone, characterized by the creation of "bald" colonies that inhibits sporulation and aerial hyphae formation (Batool et al., 2023). However, vegetative mycelia indicate the presence of protein kinase inhibitors in the strain, as shown in Figure 2.4. Additionally, this assay can substantially lower the cost and complexity associated with conventional assays, as it does not require radioisotopes.

Furthermore, the ability to screen for kinase inhibitors using *Streptomyces 85E* can provide valuable insights into regulating eukaryotic signalling pathways, which may have applications in developing new therapeutics for a wide range of diseases.

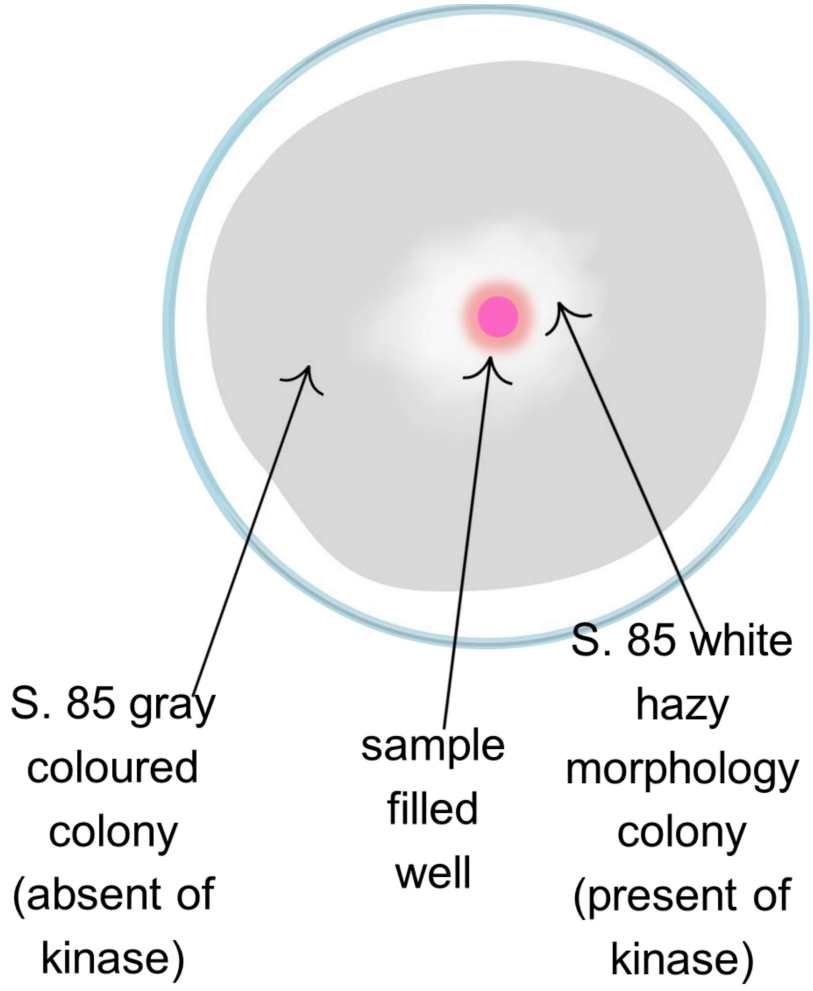

Figure 2.4: Protein Kinase Inhibitory Assay showing the growth pattern of *Streptomyces 85E* strain in the presence and absence of kinase inhibitors.