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Streptomyces as a Source of Natural Products and Industrial Enzymes

Arina Koroleva

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Dedicated with love and gratitude to my family and close ones

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ABSTRACT

Nature has gifted bacteria of the genus *Streptomyces* with immense metabolic potential, especially in their ability to produce a diverse array of natural products and enzymes. Many classes of natural products with antibiotic, antifungal, and anticancer activities were discovered in the 20th century and continue to be widely used in modern medicine. However, our demand for novel drugs continues to grow, while the rate of new molecule discovery steadily declines. Furthermore, *Streptomyces* remains a valuable source of industrially relevant enzymes with applications from the food industry to sustainable biofuel production.

My doctoral research aimed to harness the full biosynthetic potential encoded in *Streptomyces* genomes by applying diverse, but complementary strategies to activate natural products biosynthetic pathways and improve enzyme production. Microbial interactions with yeasts served as a potent natural trigger to induce the biosynthesis of multiple antifungal polyene compounds and yeast cell wall-degrading, carbohydrate-active enzymes. Through the combined use of microscopy, transcriptomics, and enzymatic activity assays, the study showed that *Streptomyces* deploys natural products and extracellular enzymes to degrade the cell walls and membranes of co-existing microorganisms, suggesting a form of facultative predatory behavior.

In addition, we developed single-cell mutant selection (SCMS) for targeted activation of silent biosynthetic gene clusters. SCMS, which integrates traditional random mutagenesis with reporter-guided selection and ultra-high-throughput screening, was particularly efficient in enhancing product yields. This was demonstrated by a 22.8-fold increase in cholesterol oxidase production without the need to optimize growth conditions, resulting in higher yields than through classical heterologous expression in *Streptomyces* or *Escherichia coli* model hosts. The method was also shown to be useful in increasing yields of natural products by an order of magnitude, demonstrating broad utility for pharmaceutical and biotechnological applications, particularly in the development of industrially applicable microbes.

KEYWORDS: Streptomyces, gene cluster activation, natural products, industrial enzymes, microbial interactions

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TIIVISTELMÄ

Luonto on varustanut *Streptomyces*-suvun bakteerit huomattavalla biosynteettisellä potentiaalilla, minkä ansiosta Streptomykeetit kykenevät tuottamaan laajan kirjon erilaisia luonnonyhdisteitä ja entsyymejä. Jo 1900-luvulla löydettiin useita Streptomykeettien tuottamia luonnonyhdisteitä, joita käytetään yhä laajasti nykylääketieteessä muun muassa antibiootteina, sienilääkkeinä ja syöpälääkkeinä. Kuitenkin tarve uusille lääkkeille kasvaa jatkuvasti, mutta samalla uusien yhdisteiden löytäminen on hidastunut huomattavasti. Lisäksi Streptomykeetit ovat merkittävä lähde teollisesti tärkeille entsyymeille, joita hyödynnetään muun muassa elintarviketeollisuudessa ja kestävän biopolttoaineen tuotannossa.

Väitöskirjatutkimukseni tavoitteena oli hyödyntää Streptomykeettien genomien koko biosynteettinen potentiaali soveltamalla monipuolisia mutta toisiaan täydentäviä strategioita luonnonyhdisteiden biosynteesireittien aktivoimiseksi ja entsyymituotannon tehostamiseksi. Mikrobien väliset vuorovaikutukset toimivat tehokkaasti luonnollisina biosynteesireittien aktivoijina. Erityisesti Streptomykeettien vuorovaikutukset hiivojen kanssa aktivoivat useiden sienilääkkeinä toimivien polyeeniyhdisteiden sekä hiilihydraatteja muokkaavien entsyymien tuottoa. Yhdistimme tutkimuksessamme mikroskopian, transkriptomiikan ja entsyymien aktiivisuusmittausten menetelmiä, jotka yhdessä osoittivat Streptomykeettien käyttävän sekä luonnonyhdisteitä että solun ulkopuolisia entsyymejä kilpailevien mikrobien soluseinien ja -kalvojen hajottamiseen. Tämä osoittaa Streptomykeettien kykenevän saalistamaan muita mikrobeja fakultatiivisesti.

Lisäksi kehitimme yksittäissolutason mutanttivalintamenetelmän hiljaisten biosynteettisten geeniklusterien kohdennettua aktivointia varten. Menetelmä yhdistää perinteisen satunnaismutageneesin, reportteriohjatun valinnan ja erittäin korkean seulontakapasiteetin, minkä johdosta tämä menetelmä osoittautui erityisen tehokkaaksi tuotantomäärien kasvattamisessa. Menetelmää käyttämällä onnistuttiin lisäämään kolesterolioksidaasin tuotantoa 22,8-kertaisesti ilman kasvatusolosuhteiden optimointia, mikä johti korkeampiin tuottoihin kuin mitä on aiemmin saavutettu klassisella heterologisella ilmentämisellä. Menetelmä osoittautui hyödylliseksi myös luonnonyhdisteiden tuotannon tehostamisessa, mikä korostaa sen laajaa sovellettavuutta teollisten tuotantokantojen kehittämisessä lääke- ja bioteknologian aloilla.

AVAINSANAT: Streptomykeetit, geeniklusterien aktivointi, luonnonyhdisteet, teolliset entsyymit, mikrobien vuorovaikutukset

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Abbreviations

A	adenylation
AA	auxiliary activities
ABC	ATP-binding cassette
ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ACP	acyl carrier protein
ARO	aromatase
AT	acyltransferase
BGC	biosynthetic gene cluster
CAZymes	carbohydrate-active enzymes
C	condensation
CBMs	carbohydrate-binding modules
CCR	carbon catabolite repression
CEs	carbohydrate esterases
ChoD	cholesterol oxidase
CYC	cyclase
DH	dehydratase
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNS	dinitrosalicylic acid
ER	enoyl reductase
FACS	fluorescence-activated cell sorting
FAD	flavin adenine dinucleotide
G/C	guanine/ cytosine
GFP	green fluorescent protein
GHs	glycoside hydrolases
GlcNAc	N-acetylglucosamine
GTs	glycosyltransferases
HGT	horizontal gene transfer
HiTES	high-throughput elicitor screening
KS	ketosynthase
KR	ketoreductase

LB	Luria-Bertani medium
MACB	mycolic acid-containing bacteria
MIC	minimum inhibitory concentration
NAD	nicotinamide adenine dinucleotide
NMR	nuclear magnetic resonance
NRPS	non-ribosomal peptide synthase
OM	outer membrane
OSMAC	one strain many compounds
PC	phosphatidylcholine
PCP	peptidyl carrier protein
PE	phosphatidylethanolamine
PKS	polyketide synthase
PLs	polysaccharide lyases
PTS	phosphotransferase system
PTM	post-translational modifications
RGMS	reporter-guided mutant selection
RiPP	post-translationally modified peptide
RNAP	RNA polymerase
SARP	*Streptomyces* antibiotic regulatory proteins
SCMS	single-cell mutant selection
Sec	secretory
SEM	scanning electron microscopy
TAT	twin-arginine translocation
TCS	two-component regulatory system
TE	thioesterase
TetR	Tet-repressor protein
TIRs	terminal inverted repeats
TSB	tryptic soy broth
TY	tryptone yeast extract medium

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Yamada K, **Koroleva A**, Laughlin M, Oksanen N, Akhgari A, Safronova V, Yakovleva E, Kolodyaznaya V, Buldakova T, Metsä-Ketelä M. Characterization and overproduction of cell-associated cholesterol oxidase ChoD from *Streptomyces lavendulae* YAKB-15. *Scientific Reports*, 2019; 9: 1-8. doi: 10.1038/s41598-019-48132-1

II Akhgari A*, Baral B*, **Koroleva A**, Siitonen V, Fewer D, Melancon C, Rahkila J, Metsä-Ketelä M. Single cell mutant selection for metabolic engineering of actinomycetes. *Metabolic Engineering*, 2022, 73: 124-133. doi:10.1016/j.ymben.2022.07.002

III **Koroleva A***, Yamada K*, Tirkkonen H, Siitonen V, Laughlin M, Matroodi S, Akhgari A, Mazurier G, Niemi J, Metsä-Ketelä M. *Streptomyces* are facultative predators that feed on yeast cells. Manuscript, 2025

*Equal contribution

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1 Introduction

The microbial world is characterized by remarkable abundance and diversity, with estimates suggesting that there are nearly 10^{11}–10^{12} microbial species in total.[1] Among them, bacteria represent the second largest biomass component on Earth after plants[2] and populate diverse ecological niches in environments, including waters, soils, humans, and animal bodies. They can also be found in extreme environments[3,4] and can even survive under extraterrestrial conditions.[5] Various bacteria and their metabolites find applications in many fields due to their unique metabolic capabilities and adaptability. Wild types and engineered bacteria are used as cell factories in the production of pharmaceuticals, biofuels, and food products, as well as in agriculture and bioremediation.[6]

A key habitat where bacterial diversity thrives is soil. Soil is a complex ecosystem that contains various microenvironments where factors such as soil organic matter concentration, pH, texture, water, and oxygen content vary significantly. These variations shape the diversity and composition of bacterial species.[7] Bacteria, in turn, influence the soil ecosystem by maintaining its health and fertility.[8] These microorganisms possess great metabolic capabilities and play crucial roles in cycling of soil nutrients such as carbon, nitrogen, and phosphorus. In particular, microbes produce numerous extracellular enzymes and significantly participate in the decomposition of plant materials, such as cellulose, other polysaccharides and lignin, contributing to carbon turnover.[9]

There have been numerous efforts to analyze the microbial taxa present in the soil.[10–12] For example, a study of the most abundant and widespread bacterial groups revealed that only 2% of bacterial taxa dominate the soil communities worldwide.[13] Moreover, at the species level, less than 1% of bacterial species are common, whereas the rest are considered rare and undescribed.[14] Among the most prevalent microbial groups in soil are members of the phylum Actinomycetota (Actinobacteria), which includes the large genus *Streptomyces*.[13] This genus is of particular interest to humans for its near-exclusive production of naturally derived antimicrobial compounds used in medicine today. In addition to natural products, *Streptomyces* are an important source of industrial enzymes for biocatalysis.

1.1 *Streptomyces* as soil bacteria

Streptomyces are filamentous Gram-positive bacteria that play important ecological and medical roles. They are primarily considered soil-dwelling organisms, but also inhabit marine environments and have been isolated from deserts and Arctic soils.[15,16] In fact, the characteristic earthy smell of soil comes from the volatile compound geosmin, the production of which is widespread among *Streptomyces*.[17] In the soil environment, this genus exists both as free-living organisms and in strong association with other bacteria, plants, insects, and animals.[18] To successfully compete for space and nutrition, *Streptomyces* adopt several survival strategies that let them thrive in the soil.

The multicellular structure and complex life cycle distinguish *Streptomyces* from other bacteria. Classical development includes three sequential morphological stages: formation of spores, vegetative mycelium, and aerial mycelium **(Figure 1)**. The morphological differentiation is tightly connected with nutrient availability. In a nutrient-favorable environment, *Streptomyces* spores germinate and develop hyphae, which spread through the substrate and form a branched vegetative mycelium.[19,20] The development of an extensive mycelial network is necessary for the exploration of the surrounding area since *Streptomyces* are non-motile. At this stage, *Streptomyces* secretes numerous hydrolytic enzymes to degrade a variety of complex organic polymers that are mainly of plant and animal origin.[21] For example, chitin, a linear polysaccharide made of N-acetylglucosamine (GlcNAc), is a component of both the fungal cell wall and insect exoskeleton. It can be efficiently decomposed by chitinolytic enzymes (chitinases).[22] It has also been shown that *Streptomyces* are able to break down plant components such as starch and lignocellulose, a complex polymer composed of cellulose, hemicellulose, and lignin.[23] Consumption of nutrients leads to aerial mycelium growth, which extends above the substrate surface to the air. Aerial hyphae then differentiate into spores, and the cycle repeats.[19,20] Spore formation ensures dispersal in nature and survival under stress. At the same time as aerial mycelium develops, *Streptomyces* switches to the production of biologically active secondary metabolites.

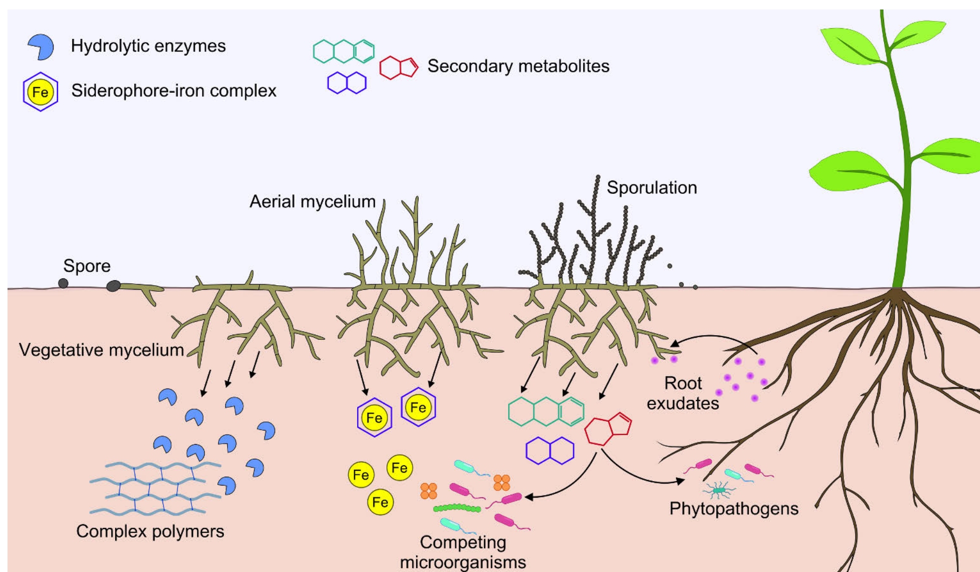


Figure 1 **Classic *Streptomyces* life cycle.** Illustration of the *Streptomyces* life cycle, highlighting spore germination, mycelial growth, and sporulation. The cycle includes the release of hydrolytic enzymes and natural products, competition with other microorganisms, and symbiotic interactions with plants. (Koroleva, A., 2025, CorelDraw)

These specialized compounds are natural products that are not involved in growth and development, but they play important roles in the survival of the producing organism.[24] They serve as the instrument in competition against other microbes, and the arsenal of molecules is highly diverse. Antimicrobial compounds produced by *Streptomyces* inhibit growth and kill competing microorganisms, including other *Streptomyces*, freeing up space.[25] Some metabolites facilitate the environmental distribution of *Streptomyces*. For example, volatile organic compounds, such as the terpenoids geosmin and 2-methylisoborneol, are released during sporulation. These compounds attract springtail arthropods, which feed on the bacteria, but also assist in spore dispersal.[26] Siderophores assist in nutrient acquisition and specifically chelate iron from the environment, reducing its availability for other microbes.[27] The siderophore coelichelin has been shown to sequester iron from the surrounding environment, which influences the growth of competing *Bacillus* and makes it sensitive to phage infection **(Figure 1)**.[28]

In soil, *Streptomyces* are part of the rhizosphere microbiome and form a mutualistic relationship with plants.[18,29] The rhizosphere is the area in close proximity to the plant roots and rich in plant exudates, which are rich in sugars, amino and organic acids, and enzymes, which are utilized by the microbiome.[30] As plant-associated microorganisms, *Streptomyces* utilize nutrients that they acquire from the plants, but at the same time, secrete metabolites that promote plant growth and protect them from phytopathogens. In line with this, a recent study demonstrated that

Streptomyces produce pteridic acids H and F, polyketides that activate photosynthesis and regulate multiple stress-response genes in Arabidopsis plant under abiotic stress conditions.[31] Additionally, *Streptomyces* species also form mutualistic associations with insects, such as termites, leaf-cutting ants, honeybees, and wasps, and provide antimicrobial protection.[32] Specifically, antifungal compounds, candicidins and natamycin A, produced by insect-associated *Streptomyces*, protect their hosts against pathogenic fungi.[33,34] Recent studies have suggested that *Streptomyces* can also have deleterious effects on insects. For example, *Streptomyces* produce the volatile compound 2-methylisoborneol to attract fruit flies, while the co-produced anthracycline antibiotic cosmomycin D is lethal to their larvae.[35] In rare cases, streptomycetes can act as pathogens themselves. Several species, such as *Streptomyces scabiei* and *Streptomyces turgidiscabies,* cause potato common scab by producing the phytotoxic compound thaxtomin A.[36]

These examples highlight the heterogeneity of *Streptomyces* relationships with other organisms in the environment, from mutualistic to antagonistic. Moreover, they reflect the diversity and specificity of the secondary metabolites, which are habitat-specific and most likely a result of the ecological adaptation of bacteria to their surroundings.[37]

1.2 Natural products and enzymes from *Streptomyces*

1.2.1 Bioactive compounds from *Streptomyces*

Streptomyces attracted massive attention as a great source of antibiotics after the discovery of streptomycin in 1943 by Albert Schatz and Selman Waksman. This aminoglycoside antimicrobial, produced by a strain of *Streptomyces griseus*, showed activity against *Mycobacterium tuberculosis*, therefore, it was efficient in the treatment of tuberculosis.[38] This finding marked the beginning of the large drug discovery programs when researchers and pharmaceutical companies screened thousands of soil isolates, including other Actinobacteria, against pathogens to identify natural products with significant therapeutic effects on infectious diseases and minimal toxicity.[39] This period between the 1950s and 1980s was marked by the discovery of various classes of antibiotics, including already mentioned aminoglycosides (streptomycin, kanamycin), tetracyclines (tetracycline, oxytetracycline), chloramphenicol, macrolides (erythromycin), and glycopeptides (vancomycin) **(Figure 2)**. These antibiotics, except vancomycin, are characterized by broad-spectrum activity, meaning they are effective against both Gram-positive and Gram-negative bacteria.[38] Many are still in clinical use; for example, vancomycin, discovered in 1953, remains crucial for treating bacterial infections, especially methicillin-resistant strains of Gram-positive *Staphylococcus aureus* (MRSA).

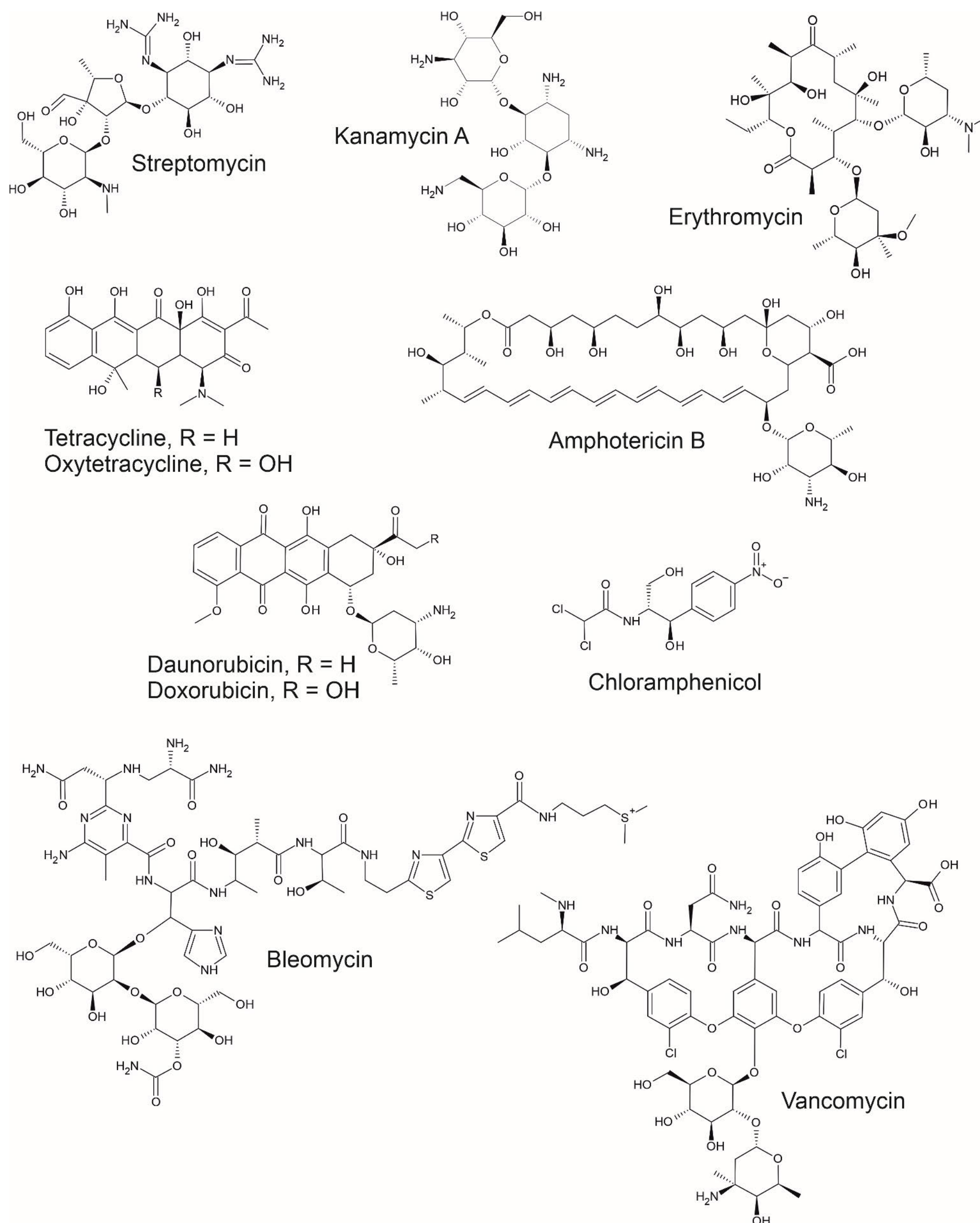


Figure 2 Structural diversity of *Streptomyces* natural products. (Koroleva, A., 2025, ChemSketch)

The bioactivity of these molecules is not limited to antibacterial. Actinomycetes also produce antifungal compounds from the polyene macrolide group, such as nystatin and amphotericin B, active against pathogenic fungi.[40] Many microbial natural products have also been shown to harbor significant anticancer activity. As

examples, anthracyclines such as doxorubicin and daunorubicin, and glycopeptide, bleomycin, are in use as chemotherapy drugs **(Figure 2)**.[41]

Despite the great success of these compounds, the rate of novel molecule discoveries significantly declined in the second half of the 20th century. Pharmaceutical companies increasingly encountered problems with the rediscovery of known compounds.[42] Notably, the last major class of antibiotics discovered was the lipopeptide daptomycin in the 1980s.[43] Today, the antibiotic development pipeline suffers from a lack of investments, which is linked to high risks, as the costs of screening and development often significantly exceed potential revenues.[44] Another issue is the rapid emergence of antimicrobial resistance caused by the misuse of antibiotics in medicine and industry, as well as improper waste disposal by the pharmaceutical industry. Of particular concern are multidrug-resistant pathogens that have developed resistance to several antibiotics.[40] Furthermore, the increase in the incidence of cancer necessitated the discovery of improved antiproliferative agents. These needs have reinvigorated interest in finding new natural products but have required the development of advanced techniques in biotechnology (see below) to harness the full genetic potential of actinomycetes for drug discovery.

1.2.2 Industrial enzymes from *Streptomyces*

1.2.2.1 Carbohydrate-active enzymes

Streptomyces possess a remarkable ability to produce industrially relevant enzymes, which can find application in biocatalysis across various industries. Particularly relevant products are the large array of carbohydrate-active enzymes (CAZymes) obtained from *Streptomyces*. CAZymes act on various structurally diverse carbohydrate substrates, involved in their breakdown, modification, and biosynthesis.[45] According to the CAZy database, these enzymes are categorized based on their activity into several classes, including glycoside hydrolases (GHs), glycosyltransferases (GTs), polysaccharide lyases (PLs), carbohydrate esterases (CEs), and auxiliary activities (AA) enzymes. Additionally, the database allocates carbohydrate-binding modules (CBMs) in a separate class. CBMs are accessory protein domains that do not have catalytic activity but are found associated with CAZymes to enhance enzyme-substrate interactions.[46] Among these, the largest class comprises GH enzymes that are involved in the hydrolysis of glycoside bonds. These bonds link two or more carbohydrates (di-, oligo-, and polysaccharides) and can also occur between a carbohydrate and a non-carbohydrate molecule (glycoconjugates).[47] Therefore, GHs play a key role in the degradation of complex carbohydrates and carbohydrate-containing molecules. As of today, the CAZy database reports more than 190 GH families, and is constantly updated with newly found families.[48] The principle of family formation is based on protein sequences, which group together

enzymes that are similar in structure and catalytic mechanism, but often distinct in substrate specificity.

The *Streptomyces* genus produces a variety of GHs, such as cellulases, amylases, hemicellulases like xylanases, and chitinases.[49] These enzymes catalyze the breakdown of corresponding polysaccharides: cellulose, starch, hemicellulose, and chitin, respectively. The enzymatic breakdown of these polymers is primarily achieved by the combined action of GH enzymes from different families. For example, the decomposition of linear polymer cellulose, made of β-1,4-linked glucose units, is carried out by a mixture of cellulases.[50] For example, *Streptomyces* species have been shown to produce the following cellulases: endoglucanases (GH5, GH9, GH12), which cleave internal β-1,4-glucosidic bonds within the cellulose chain, and exoglucanases (also known as cellobiohydrolases, GH6, GH48), which act on the reducing or nonreducing end of the chain.[51] Finally, β-glucosidases (GH1, GH3) perform the final step of the degradation process and release glucose monomer **(Figure 3a)**.

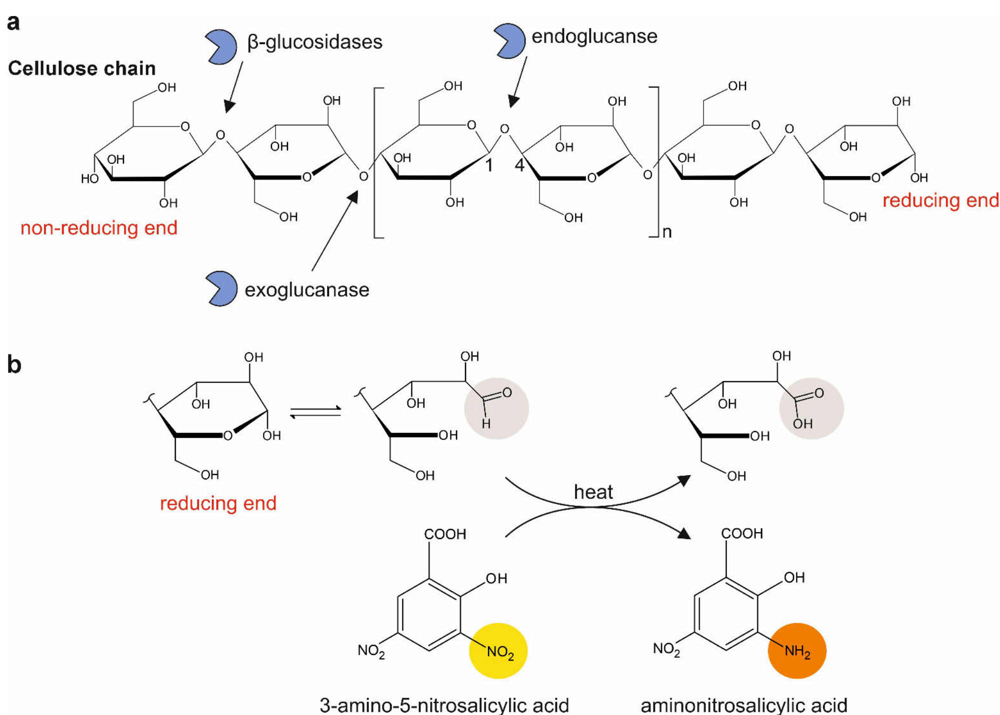


Figure 3 Degradation of polysaccharides using cellulose as an example. (a) Schematic of a cellulose polymer chain with designated non-reducing and reducing ends. (b) Colorimetric detection of GHs enzymatic activity using the DNS assay (Koroleva, A., 2025, CorelDraw)

Understanding the difference between reducing and non-reducing ends of sugars is important in measuring GH activity, particularly when assessing enzymatic degradation of carbohydrates. Reducing sugars have a free anomeric carbon (C1 or C2), which, in its open sugar conformation, has a reactive aldehyde group acting as a reducing agent **(Figure 3b)**.[52] Conversely, non-reducing sugars have an anomeric carbon that is involved in a glycosidic bond, which prevents it from reacting. The ability of reducing sugars to donate electrons (reduce) to oxidizing agents is the principle used in several colorimetric assays.[53] In a quantitative DNS assay and the Nelson-Somogyi assay, reducing sugars reduce 3-amino-5-nitrosalicylic acid or copper(II) ions, respectively, resulting in the production of colored compounds. For example, in the DNS assay, the reduction results in the formation of aminonitrosalicylic acid with a characteristic red-brown color, which can be measured spectrophotometrically at 540 nm **(Figure 3b)**.[54] These methods are inexpensive but less sensitive than other analytical techniques. In addition to reducing sugar-based methods, other approaches include chromogenic and fluorogenic substrate assays, agar plate assays, HPLC, and mass spectrometry.[55]

Enzymes derived from *Streptomyces* are valuable in many industrial bioprocesses. Celluloses and amylases are widely used in the textile, paper and pulp, and detergent industries.[49,56] Amylase is also a principal enzyme for the food and beverage industries, specifically for the production of syrups, juice clarification, and starch solubilization.[57] Chitinases can serve as a non-toxic alternative to insecticides and fungicides in agriculture, protecting crops from fungal pathogens.[58] *Streptomyces* chitinases from the GH18 and GH19 families both showed inhibitory effects on fungal cells.[59,60] In effect, *Streptomyces* cultures producing chitinases have been directly used as biological control agents. The company Novozymes uses *Streptomyces lydicus* WYEC108 strain as a main ingredient in a biological fungicide that can be applied to soil to protect plants against various fungal diseases. After colonizing plant roots, bacteria produce chitinases to disrupt the structural integrity of pathogens, such as *Fusarium* species.[61] The production of renewable energy is another promising field that demands efficient enzymes. Biofuels obtained from plant biomass, such as lignocellulose, are a sustainable alternative to fossil fuels. The conversion of lignocellulose to biofuel involves multiple processing steps, one of which is enzymatic hydrolysis. Enzymes such as cellulases, xylanases, and mannanases are used to break down carbohydrates into fermentable sugars for bioethanol production.[62,63]

1.2.2.2 Cholesterol oxidase in sterol degradation

Additionally, *Streptomyces* degrades sterols such as cholesterol via oxidative enzymes like cholesterol oxidase. Cholesterol oxidase (ChoD, 3β-hydroxysterol oxidase) is a bifunctional, flavin adenine dinucleotide (FAD)-dependent enzyme that

performs the initial step in sterol catabolism.[64] The first step is initiated by the oxidation of the 3β-hydroxyl group of cholesterol to the 3-keto group using oxygen as an electron acceptor **(Figure 4a)**. This results in the production of cholest-5-en-3-one and hydrogen peroxide, H_2O_2. The second step involves isomerization of the Δ5 double bond in the steroid ring to the Δ4 position occurs to produce cholest-4-en-3-one.[65]

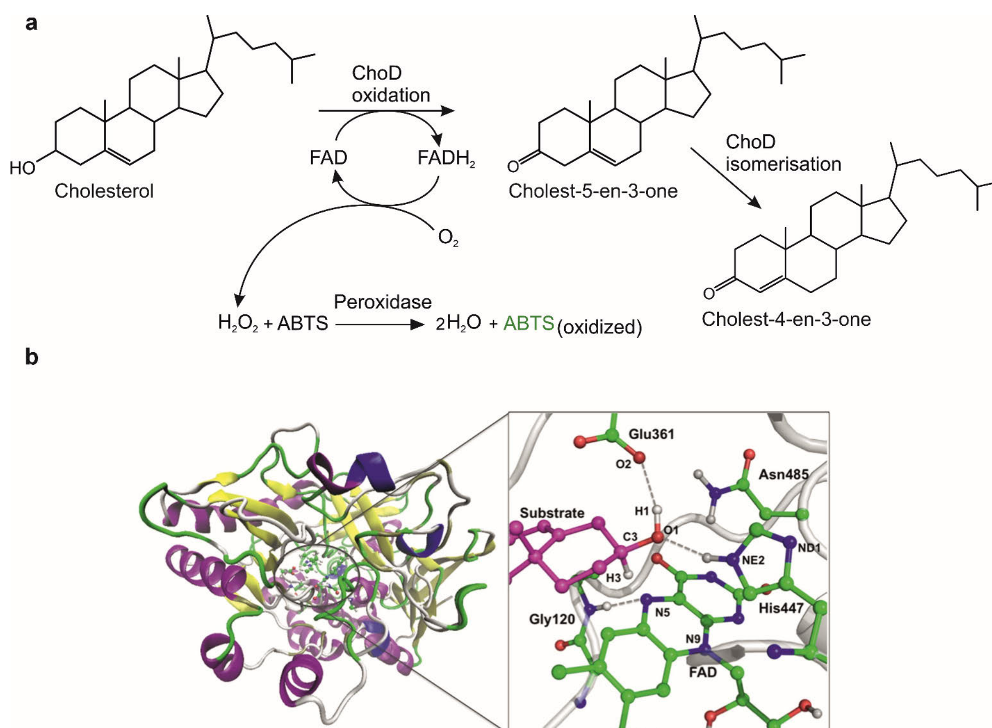


Figure 4 Cholesterol oxidase activity on the cholesterol as substrate. (a) Sequential oxidation and isomerization of cholesterol. The enzymatic reaction produces hydrogen peroxide, which is subsequently used to assess enzyme activity. Source: modified from Article I. (b) Overview of crystal structure of the ChoD and key catalytic residues. Source: modified from Figure 1[66], licensed under CC BY 4.0.

In addition to cholesterol, the ChoD enzyme also acts on various 3β-hydroxysteroids, including plant sterols such as β-sitosterol and stigmasterol, as well as the fungal sterol ergosterol. The production of hydrogen peroxide can be quantified using a coupled reaction with horseradish peroxidase and a chromogenic substrate, such as ABTS, which changes color upon oxidation. The color change can be measured spectrophotometrically to determine the activity of ChoD and the concentration of cholesterol in a sample.[67]

Structural studies showed that depending on FAD binding, ChoD enzymes are classified into class I (noncovalently bound FAD) and class II (covalently bound FAD). Enzymes from two classes share catalytic activity but are different in structure and kinetic characteristics.[68] Class I enzyme has been predominantly identified in actinomycetes (*Streptomyces* spp., *Mycobacterium* spp., *Rhodococcus* spp.) and belongs to the glucose-methanol-choline (GMC) oxidoreductase family **(Figure 4b).**[69] This family possesses a characteristic Rossmann fold (a distinctive βαβ arrangement, creating a three-layered sandwich) with a consensus sequence for FAD binding. This structure is common to many enzymes that bind nucleotide cofactors such as NAD+ or FAD.[70]

The ChoD enzyme is widely used in clinical diagnostics to monitor cholesterol levels in blood samples and assess the risk of cardiovascular diseases.[69] Therefore, enzyme is commercially produced by various companies (Merck, Sekisui Diagnostics, and Creative Enzymes) as both native and recombinant proteins in hosts such as *Escherichia coli*, *Streptomyces sp.*, *Pseudomonas sp.*, and *Nocardia sp.* for industrial and research applications. Additionally, the enzyme can find useful applications in agriculture due to its insecticidal activity and in biocatalysis for the transformation of sterols and biosynthesis of steroid-based pharmaceuticals.[69]

1.2.2.3 Recombinant proteins

The natural enzyme-producing capabilities and the suitability for large-scale industrial cultivation make *Streptomyces* an attractive candidate for the production of recombinant proteins. The development of DNA technology and biotechnology boosted the protein market, and today, recombinant proteins are used as biopharmaceuticals, diagnostic reagents, industrial enzymes, and sustainable food sources.[71] Production of these molecules is carried out via various expression hosts, including both prokaryotic and eukaryotic systems: bacteria (e.g., *E. coli*), yeasts (e.g., *Saccharomyces cerevisiae, Komagataella phaffii* (previously *Pichia pastoris*)), mammalian cells, and insects and plants.[72] The choice of host significantly relies on the biological characteristics of the target protein, complexity of post-translational modification (PTM), growth rate and expression yield, and cost-efficiency.[73] Among bacteria, *E. coli* is the first-choice host for heterologous protein production due to its rapid growth, ease of genetic manipulation, and typically high production yield. However, *E. coli* exhibits several disadvantages that limit its use, particularly the lack of an effective secretion system, protein folding issues, and accumulation of proteins in insoluble inclusion bodies. This is due to the presence of an outer membrane (OM) layer in Gram-negative bacteria, which makes protein translocation difficult.[74]

In contrast, *Streptomyces* offers several advantages over other systems when used as a heterologous host. As Gram-positive bacteria, they lack the OM and exploit several protein translocation systems to directly export target proteins through the

cytoplasmic membrane to the extracellular space during cultivation.[75] Two translocation systems are well characterized: i) the secretory (Sec) system, responsible for the transfer of unfolded polypeptides during or after synthesis; ii) the twin-arginine translocation (TAT) system translocates proteins that are already folded.[76] These characteristics facilitate protein secretion and eliminate the need for cell disruption, simplifying downstream processing. Additionally, due to the absence of OM, *Streptomyces* do not produce lipopolysaccharides (LPS), also known as endotoxins, which are the common contaminant during recombinant protein production in *E. coli*. A widely used heterologous host for protein production among *Streptomyces* is *Streptomyces lividans*, a model species within the genus. Compared to other species, *S. lividans* possesses a limited restriction–modification system, which facilitates the introduction of foreign methylated DNA.[77] It has been successfully employed for decades to express a variety of prokaryotic and eukaryotic proteins. Successful examples would be the recombinant production of fibrinolytic protein streptokinase from the Gram-positive *Streptococcus equisimilis*[78], cellulase from the Gram-negative *Rhodothermus marinus* [79], as well as human interleukin-6 and transforming growth factor α (TGFα)[80].

1.3 *Streptomyces* genome and BGCs

The remarkable ability to produce a vast array of enzymes and secondary metabolites is embedded in the genome of *Streptomyces*. The first complete genome sequence was acquired for the model organism *Streptomyces coelicolor* A3(2) and published in 2002, followed by the sequencing of the industrially important *Streptomyces avermitilis* ATCC31267 strain.[81,82] The continued advancement and increasing accessibility of sequencing technologies have since led to numerous genome publications, providing more details on the distinct features of the *Streptomyces* genome and metabolic potential of the genus.

Streptomyces have notably large single linear chromosomes with an average size of 8.4 Mbp and a high G/C nucleotide content of approximately 70%.[83] Notably, there is considerable variation in genome size across species, ranging from 4.8 Mbp to 13.6 Mbp.[84] This variation is largely attributed to the chromosome structure, which consists of a conserved central region and more variable chromosomal arms. The central part is highly conserved throughout the *Streptomyces* genus and contains core genes for basic cellular processes (e.g., cell division, genetic information processing, survival). In contrast, the genetic content of chromosome arms varies considerably at the species level. These regions are enriched in genes responsible for the biosynthesis of specialized metabolites and hydrolytic enzymes.[81,85] For instance, a comparative analysis of 213 *Streptomyces* genomes revealed that the number of genes encoding CAZymes ranges from 73 to 383, depending on the ecological niche of the species.[86]

This variability arises from the genetic instability of the chromosome arms, which are prone to horizontal gene transfers (HGT), recombination, insertions/deletions, or duplications.[68,70] As a result, this enables *Streptomyces* to acquire adaptive traits in response to environmental changes. Additionally, the telomeric ends of *Streptomyces* chromosomes feature terminal inverted repeats (TIRs), which can range in size from less than 100 bp to nearly 3 Mbp, further contributing to genome size diversity.[88]

In *Streptomyces*, the genome contains, on average, 7,000 protein-coding genes — a notably large number compared to other bacteria.[65] A significant portion of these genes encode regulatory elements, which take up to 12% of the genome.[81] Notably, the number of sigma factor genes ranges from 27 to 80, while transcription factor genes vary between 401 and 1,018.[90] Secretory proteins with hydrolytic activity (such as chitinase, protease, cellulase) are also highly prevalent in the genome.[81] The pangenome analysis of 205 *Streptomyces* species revealed 44,504 genes putatively encoding CAZyme proteins, from which glycoside hydrolases (GHs) were the most abundant.[90] Complex polysaccharides, broken down to simple sugars like monosaccharides and disaccharides, are taken up by specific transporters. In the *Streptomyces* genomes, genes encoding various transporter systems are abundant and account for 1,000 on average. Of these, approximately 27% encode carbon source transporters.[91] For example, ATP-binding cassette (ABC) transporters facilitate the uptake of sugars such as maltose, cellobiose, and xylobiose, while the phosphotransferase system (PTS) is responsible for transporting GlcNAc.[92–94]

Genome sequencing has also revealed a great number of genes dedicated to the biosynthesis of secondary metabolites. Secondary metabolites of *Streptomyces*, including antimicrobial compounds and other valuable natural products, are synthesized by specific regions of the genome termed biosynthetic gene clusters (BGCs).[81] This region is represented by a set of genes, which are clustered together and encode the production of specific secondary metabolites and their derivatives. David Hopwood and his colleagues significantly contributed to establishing the concept of BGCs in the 1980s. Their research focused on the actinorhodin pathway, which produces the blue-coloured polyketide antibiotic, and they attempted to identify all the key genes involved in its biosynthesis **(Figure 5)**.[95,96] By using various mutants defective at different biosynthetic steps and a gene complementation approach, they were able to identify the organization of these genes. The key finding was that the genes are arranged within a discrete region in the chromosome of *S. coelicolor*, the *act* gene cluster. As confirmation, the whole region was heterologously expressed in another *Streptomyces* host, resulting in the appearance of the blue-pigmented actinorhodin antibiotic in the host.[97]

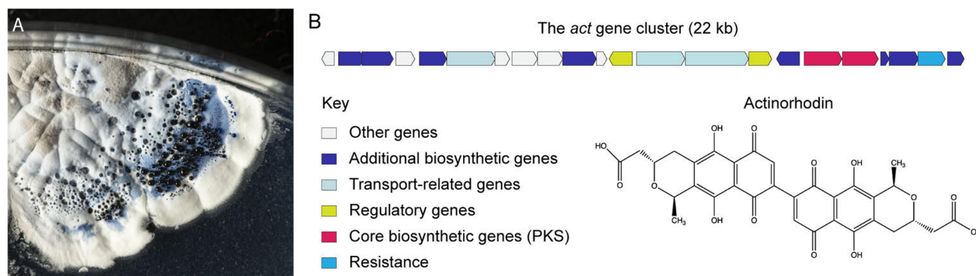


Figure 5 Actinorhodin BGC (a) *Streptomyces coelicolor* producing the characteristic, blue-pigmented antibiotic actinorhodin. (b) Schematic representation of the actinorhodin BGC, highlighting key biosynthetic genes and the chemical structure of actinorhodin. Reprinted with permission from Schlimpert, S., Elliot, M.A., Journal of Bacteriology, 2023; 205: e00153-23. Copyright © 2013 American Society for Microbiology.

The contents of all BGCs can be generalized to contain genes for different functionalities **(Figure 5b)**. Core biosynthetic genes are responsible for assembling the carbon skeleton of the molecules. The core biosynthetic genes are complemented with additional genes that encode tailoring enzymes to further modify the molecule structures (e.g., glycosylation, methylation), thus enhancing chemical diversity. BGCs also typically contain genes for self-resistance, whereas regulatory genes control the expression of the metabolic pathways.[98] The specific set of biosynthetic genes is typically highly conserved and determines the type of biosynthetic cluster and the natural product that is synthesized.[99]

1.3.1 Genetic and biochemical diversity of BGCs

Prior to genome sequencing, *S. coelicolor* was studied for several decades and was known to produce four natural products, including actinorhodin. However, the genome sequence revealed over 20 BGCs for the production of additional compounds.[81] More recently, large-scale genome sequencing efforts have shown that actinobacteria commonly contain around 30 BGCs per genome, although the exact number varies significantly between species, from 8 to 83 BGCs per each genome.[89,100]

The *Streptomyces* BGCs demonstrate remarkable structural diversity and can be categorized into groups based on the biosynthetic logic and type of cluster. The most widespread BGCs are represented by polyketide synthase (PKS), non-ribosomal peptide synthase (NRPS), PKS-NRPS hybrids, ribosomally synthesized and post-translationally modified peptide (RiPP), terpene, and siderophore gene clusters.[84] Together, the products of PKS and NRPS gene clusters—polyketides, nonribosomal peptides, and their hybrids—account for approximately 50% of all antibiotics currently in use.[101] Additionally, PKSs are classified into several subclasses based on

the biosynthetic mechanisms: type I PKSs, type II PKSs, and type III PKSs **(Figure 6)**. Most of the medically important polyketides are primarily generated through type I and II PKSs. [102]

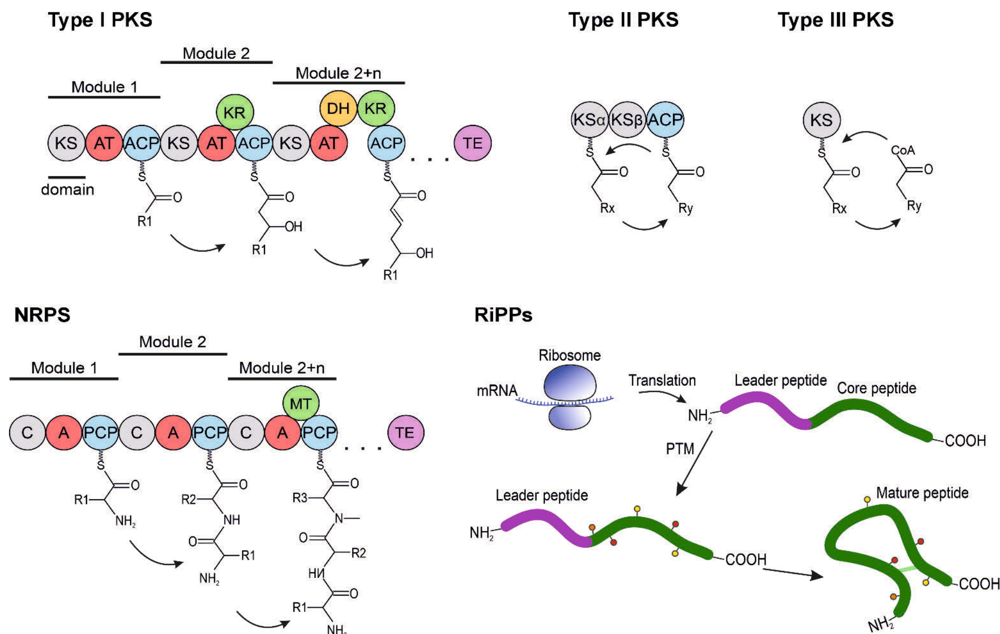


Figure 6 Biosynthesis of natural products via major enzymatic pathways. Schematic representation of polyketide natural product biosynthesis via type I, II, and III polyketide synthase (PKS) pathways, and peptide natural product biosynthesis via nonribosomal peptide synthetase (NRPS) and ribosomally synthesized and post-translationally modified peptides (RiPPs) pathways. (Koroleva, A., 2025, CorelDraw)

In *Streptomyces*, type I PKSs function as large modular enzymes that form an assembly line that synthesizes the backbones of polyketide compounds **(Figure 6)**. PKSs synthesize polyketide natural products from small acyl-coenzyme A units, such as acetyl-CoA or malonyl-CoA. Each PKS consists of modules with linearly arranged domains, where each domain performs a distinct catalytic activity during the assembly process. [103] Key domains present in each module are acyltransferase (AT), acyl carrier protein (ACP), and ketosynthase (KS). Together, these domains perform the two-carbon elongation process. The AT domain selects the appropriate extender unit, while KS interacts with ACP to facilitate C–C bond formation through a decarboxylative Claisen condensation reaction. In addition, each module may include optional domains such as ketoreductase (KR), dehydratase (DH), and enoylreductase (ER), which subsequently reduce the ketone group to a saturated C–C bond. Additional modifications are made after the release of the polyketide chain from PKS

through post-PKS mechanisms, for example, alkylation, glycosylation, and oxygenation.[104,105] The completed polyketide chains are released from the PKS enzymes by a terminal thioesterase (TE) domain, resulting in either linear (hydrolysis) or cyclic (cyclization) products.[103] Examples of type I PKS products include the antibiotic rifamycin and the antifungal compound amphotericin B.[101]

Type II PKS, unlike type I PKS, operates as a complex of several distinct enzymes, each containing a specific functional domain. For carbon chain extension, type II PKS requires the presence of two KSs, referred to as KSα and KSβ, along with ACP, which together form a major complex called "minimal PKS" that iteratively assembles polyketides of various lengths. The highly reactive intermediate polyketides, which are typically between 16 and 24 carbons in length, are bound to the ACP that protects the molecules from non-enzymatic cyclization. Additional enzymatic components, such as KR, cyclases (CYC), and aromatases (ARO), contribute to the formation of the aromatic core of the compound, while tailoring enzymes like oxygenases, glycosyl- and methyltransferases further modify the structure. Typical type II PKS-derived polyketides are aromatic natural products, such as anticancer doxorubicin and antibacterial tetracyclines.[102,105,106]

Type III PKSs are also involved in the biosynthesis of aromatic polyketides but feature relatively simple biosynthetic machinery. These enzymes lack an ACP domain; instead, type III PKSs consist of a multifunctional KS homodimer that directly accepts free acyl-CoAs as extender units and performs iterative elongation followed by cyclization. Their broad substrate flexibility and varying numbers of condensation reactions allow for the production of molecules with diverse structures. The cluster may include additional genes for various tailoring reactions. In *Streptomyces*, type III PKS is represented by the Gcs synthase, which produces the spore germination inhibitor germicidin.

Nonribosomal peptides are synthesized from amino acids (both proteinogenic and nonproteinogenic), which are linked together by NRPS enzymes, independently of the ribosomes **(Figure 6)**. NRPSs have a modular structure similar to type I PKSs and require a minimum of three catalytic domains: adenylation (A), a peptidyl carrier protein (PCP), and a condensation (C) domain. Similar to type I PKS, these domains together form an assembly line that synthesizes the growing peptide chain. The A domain activates the amino acid to load it on the PCP domain, which is structurally related to ACPs from PKSs. The C domain is responsible for forming the peptide bond between amino acids loaded on adjacent PCP domains. Additionally, modifications of the peptide chain can be introduced through reactions such as epimerization, heterocyclization, methylation, hydroxylation, and halogenation.[105,107,108] The completed polypeptide chains are also released through the action of the TE domain.[103] A well-known product of NRPS is the antibiotic

daptomycin, while an example of a hybrid PKS and NRPS gene cluster is the anticancer bleomycin compound.[101]

Another important type of peptide natural products produced by *Streptomyces* is RiPPs **(Figure 6)**. In contrast to NRPSs, RiPPs are synthesized by ribosomes using only proteinogenic amino acids. Initially, a precursor peptide is formed, which typically consists of a leader and a core segment. After translation, the peptide undergoes extensive post-translational modifications (PTM) that occur in the core segment, while the leader peptide guides the modification process. The PTMs are essential, as these modifications significantly increase the structural diversity and biological activity of the final products. Modifications include a variety of cyclization reactions, dehydration, incorporation of non-proteinogenic amino acids, and proteolytic cleavage of the leader peptide, leading to the formation of more than 20 diverse RiPPs subclasses such as lanthipeptides, lasso peptides, and thiopeptides.[109–111]

1.3.2 Regulatory networks

The production of many secondary metabolites simultaneously is an energy-intensive process, especially as the majority of metabolites are exported outside of the cells. Therefore, production needs to be tightly regulated in a manner where expression of BGC starts only when needed, in response to internal and external triggers. The transition from an environmental trigger to BGC gene expression occurs through transcriptional regulation and can be represented as a cascade of interacting elements. The key elements here are regulatory proteins - transcriptional regulators. They have DNA-binding properties and specifically bind to the promoter region of BGC thereby activating or repressing it.[112]

Pathway-specific, also termed cluster-situated, regulators are located within the specific BGCs and directly control gene expression. For example, ActII-ORF4 and OtcR regulators activate the expression of biosynthetic pathways of actinorhodin and oxytetracycline, respectively.[113,114] Regulators are divided into various families depending on their sequence and domain structure similarities. Both ActII-ORF4 and OtcR belong to the SARP family (*Streptomyces* antibiotic regulatory proteins). This group is highly distributed among *Streptomyces* and includes many activators.[115] In contrast, the TetR family mainly consists of transcriptional repressors.[116] Additionally, despite being pathway-specific, some regulators were shown to coordinate the expression of multiple BGCs. As seen in the case of *Streptomyces albus* S4, where FscRI, the pathway-specific activator of the candicidin cluster, also cross-regulates the production of antimycin.[117]

Control of pathway-specific regulators is achieved through higher-order regulation by global regulators. Global regulators are not linked to a specific BGC. Instead, they control multiple BGCs and have broader (pleiotropic) effects on *Streptomyces*,

influencing both morphological development and metabolite production. One such regulator is AdpA, a global transcriptional regulator shown to control the expression of over 500 genes directly.[118] AdpA can function as both an activator and a repressor, depending on the target gene. For example, it activates streptomycin biosynthesis and represses oviedomycin biosynthesis by controlling the expression of corresponding pathway-specific regulators, *strR* and *ovmZ/ovmW*, respectively.[119,120]

Global regulators react in response to specific environmental signals such as nutrient limitations, pH, temperature, and other changing parameters of the environment. In *Streptomyces*, there are multiple global nutrient-sensing regulators. For example, DasR responds to N-acetylglucosamine, a carbon and nitrogen source; GlnR regulates nitrogen metabolism; and the PhoR–PhoP system responds to phosphate availability.[121] PhoR–PhoP is a two-component regulatory system (TCS), where PhoR functions as a sensor kinase that detects environmental signals, while PhoP acts as the response regulator, modulating gene expression accordingly.[122]

1.3.3 Self-resistance to natural products

While producing a plethora of specialized metabolites, *Streptomyces* have also evolved self-resistance mechanisms as protection from the toxic effects of their own metabolites. Genes encoding such resistance are embedded within BGCs of specific metabolites. A common mechanism involves efflux pumps, often ABC transporters, which actively export metabolites to the extracellular environment. For example, oxytetracycline and doxorubicin BGCs encode such ABC transporters OtrC and DrrAB, respectively.[123,124] Alternative resistance mechanisms utilize sequestration proteins, such as BlmA; it binds the anticancer bleomycin and neutralizes its effects. The bleomycin BGC also encodes additional self-resistance elements, including several transporters and the BlmB protein that acetylates the bleomycin metal-binding domain, which is crucial for its DNA-damaging activity.[125]

1.4 Techniques for activation of silent BGCs

The abundance of BGCs in *Streptomyces* genomes has tremendous potential for drug discovery, as each uncharacterized cluster may produce a medically relevant natural product. To date, extensive studies of about 5000 Actinobacteria genomes have identified 80,947 BGCs, of which fewer than 5% have been linked to BGCs with characterized functions.[126] However, despite their high number, these clusters are often not expressed when the bacterial strain is cultured in the laboratory. Due to this reason, they are termed “silent”, and their products are often unknown. Therefore, one of the biggest challenges and goals in drug discovery has become the activation of these clusters. Unleashing the full potential of *Streptomyces* is an attractive goal, since

microbial natural products have a proven track record as a prolific source of antibiotics. For this reason, to date, there are a plethora of available approaches for the activation of BGCs in *Streptomyces*. Some techniques were developed long before DNA sequencing technologies emerged, whereas others became available after genome sequencing.

1.4.1 Culture-based methods

This group of methods emerged from the understanding that *Streptomyces* inhabit a complex environment where bacteria are constantly exposed to various abiotic and biotic factors. Abiotic factors include nutrient availability and fluctuation in pH and temperature, while biotic factors encompass interactions with other living microorganisms and exchange of chemical signals. This environmental context directly and indirectly influences natural product biosynthesis; therefore, compound production can be induced by mimicking these conditions. Even minor changes in culture conditions cause so-called pleiotropic effects, which cause global alterations in microbial metabolism and affect the expression of many genes at different levels.

1.4.1.1 OSMAC

One of the simplest means to activate BGCs is the OSMAC approach, which stands for One Strain Many Compounds. This approach works on the idea that a single microbial strain can produce a variety of compounds under changing cultivation conditions **(Figure 7a,b)**. The term OSMAC was first introduced in 2002.[127] However, the concept of changing cultivation conditions has a long history of application in the industry for improving the yields of antibiotics.[128] Like in the natural habitat, changes in pH, temperature, nutrient composition, and oxygen level in a flask or bioreactor can trigger the expression of BGCs. Often, changing just one parameter is enough to boost metabolite production. For instance, screening dozens of culture extracts of thermotolerant Actinobacteria cultivated at 45°C resulted in new secondary metabolite peaks appearing in the chromatogram, whereas no production was observed at 30°C, a typical temperature used for laboratory cultivation of *Streptomyces*.[129] Additional variables for consideration are the type of culture vessel and the solid/liquid state of cultivation.[130]

Media typically consist of various sources of carbon, nitrogen, phosphorus, and salts. The type, concentration, and ratio of nutrients greatly affect *Streptomyces* growth, morphological differentiation, and metabolism.[131] For example, glucose is a primary source of carbon and energy for heterotrophs like streptomycetes. However, when present in high concentration in the culture medium, it represses secondary metabolite synthesis through a process known as carbon catabolite repression (CCR). This global regulatory mechanism controls the order of carbohydrate usage by bacteria, ensuring the utilization of readily available sugars such as glucose first. This

limits the expression of genes involved in the assimilation of alternative carbon sources and secondary metabolism.[132,133] The control mechanism is complex and involves a cascade of interactions between global and pathway-specific transcription factors. Over 30 cases of secondary metabolite repression by various carbon sources have been reported.[132] The repression can be relieved by switching to another carbon source. In the case of *S. lividans*, glucose represses the synthesis of actinorhodin through control of the AfsR2 regulatory protein. The change from glucose to glycerol relieves repression and activates the *act* cluster.[134] Overall, the OSMAC approach has resulted in the activation of structurally diverse secondary metabolites.[135]

1.4.1.2 Chemical elicitors

The OSMAC approach can be extended by using specific chemical elicitors – external and internal signaling molecules that regulate the expression of silent BGCs. The nature of these small molecules is highly diverse. One of the first characterized bacterial signals is γ-butyrolactone, which controls the biosynthesis of streptomycin and is also known as an autoregulator produced by *Streptomyces*.[136] Autoregulators are widely distributed among *Streptomyces* and, along with γ-butyrolactones, they include furanes, γ-butenolides, diketopiperazines, and PI (pimaricin inducer[137]) factor. Regulation occurs through the binding of the signaling molecules directly to transcriptional regulators.[138] Examples of external elicitors are plant and animal stress hormones.[139,140] For example, the animal stress hormone epinephrine (adrenaline), specifically its catechol chemical constituent moiety, elicits the production of siderophore and specialized metabolites from the angucyclines class of natural products in *Streptomyces* sp. MBT84.[140] Organic solvents (ethanol and DMSO), metal ions (nickel and cobalt), and the rare earth element (scandium) were reported to cause metabolic changes when added to the culture, too. [141–143]

Interestingly, antibiotics themselves can act as elicitors. Typically, the biological activity of antibiotics is defined by the minimum inhibitory concentration (MIC). It is the lowest concentration of an antibiotic that inhibits microbial growth *in vitro*. On the other hand, the concentration of antibiotics in nature is often much lower and can be as much as 1% of the MIC.[144] These small concentrations are called subinhibitory and act as environmental signals and trigger biological responses from bacteria, including phenotypic changes and secondary metabolite production.[145] As seen in the case of *S. coelicolor*, the addition of a low concentration of angucycline antibiotic jadomycin B (JdB) to the liquid culture led to several events. In a concentration-dependent manner, JdB triggered the synthesis of the red pigment undecylprodigiosin and morphological differentiation in *S. coelicolor*. The regulation occurred at the transcriptional level through the binding of JdB to the ScbR2 regulator that represses antibiotic production and morphogenesis.[146]

The identification and testing of individual elicitors, which can account for

thousands of candidate molecules, is a challenging task. To expedite this process, various high-throughput screening methods have been developed. The screening can be untargeted when microbial strain is screened against large elicitor libraries in a high-throughput manner and analyzed based on any phenotypic and metabolic changes.[147] Alternatively, when the genetic information about the target BGC is available, the use of a reporter gene can facilitate the identification of elicitors that activate specific clusters. One such approach, HiTES (High-Throughput Elicitor Screening), has been tested with the model strain *S. albus* J1074. As a result of targeted screening against ~500 elicitors, several inducers, two of which, etoposide and ivermectin, were shown to trigger activation of the *sur* cluster (NRPS type). These two compounds also induced the production of 14 novel metabolites.[148] In cases when genetic manipulations are challenging, HiTES has recently been adapted by coupling it with various detection systems, including bioactivity screening, imaging mass spectrometry, and fluorescence-based DNA cleavage assay.[149–151]

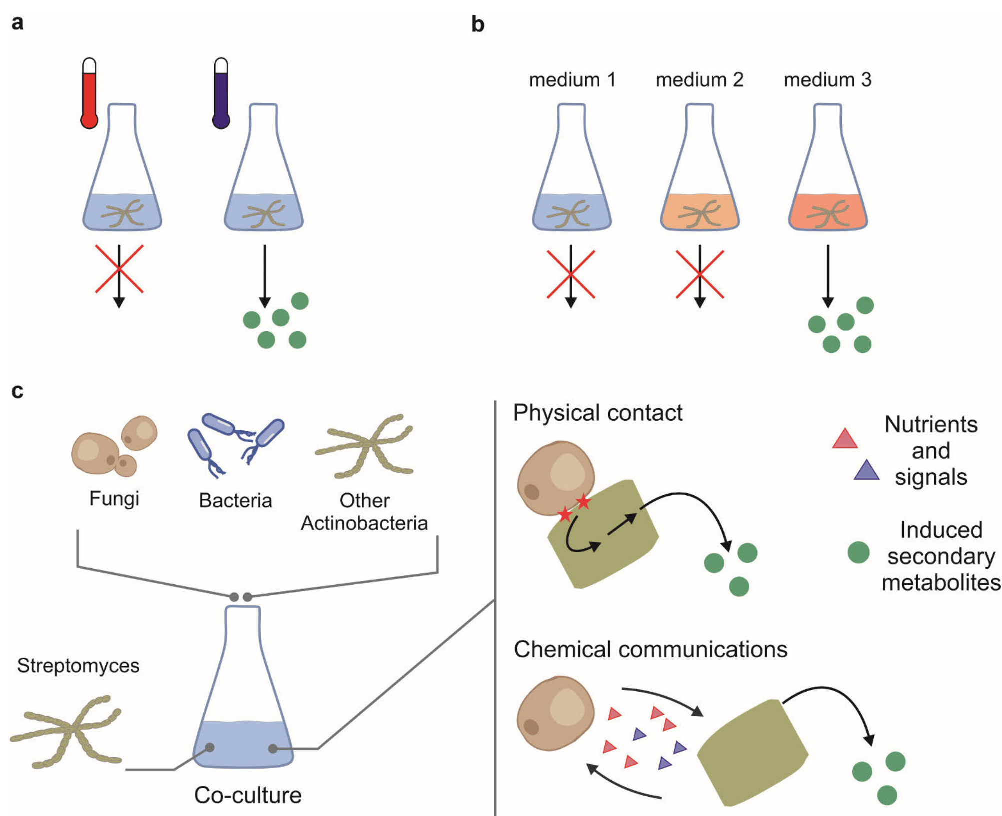


Figure 7 General overview of culture-based methods for BGC activation. **(a)** Variation of culture parameters, specifically temperature; **(b)** Media composition variations; **(c)** Co-culture with various microorganisms and different mechanisms of interaction. (Koroleva, A., 2025, CorelDraw)

1.4.1.3 Co-culture

Co-cultivation also represents a conventional approach for the activation of biosynthetic pathways that often remain silent in monoculture. This method involves cultivating the target microbe with one or more microorganisms in close proximity, imitating the interactions occurring naturally **(Figure 7c)**. The approach is also rather simple, although it requires some consideration in the choice of microorganisms for co-culture, their ratio, and cultivation conditions. *Streptomyces* bacteria have been extensively co-cultured with other bacteria and fungi, which has unlocked their metabolic potential. For these purposes, solid and liquid media have been used, as well as special culture systems, including one with a dialysis membrane.[152]

The biosynthesis of secondary metabolites in *Streptomyces* can be triggered by a diverse range of bacteria, including other actinomycetes, human pathogens, and model organisms like *E. coli*.[153] The exact mechanism behind this activation is often unclear, but it can be generally mediated by physical contact, signaling molecule exchange, and/or nutrient competition **(Figure 7c)**. For instance, mycolic acid-containing bacteria (MACB), such as *Tsukamurella pulmonis* from the Actinobacteria phylum, can stimulate the synthesis of various secondary metabolites in several species of *Streptomyces* via direct physical contact.[154] This interaction has resulted in the production of a novel cytotoxic alkaloid arcyriaflavin E from *Streptomyces cinnamoneus* NBRC 13823 and a polyketide antibiotic alchivemycin A from *Streptomyces endus* S-522.[154,155] The latter is the product of a hybrid PKS-NRPS cluster and has potential as an antimicrobial and antitumor compound.[156] Contact-dependent mechanism was confirmed when dead MACB cells, which remain intact after inactivation and retain a lipid layer with mycolic acid, lost the ability to adhere to *Streptomyces* mycelia and, hence, failed to elicit the metabolic response.[157] In contrast, another study showed that BGC activation can occur without physical contact between the two species. For example, the activation of the actinorhodin cluster in *Streptomyces coelicolor* was observed during competition for iron acquisition with *Myxococcus xanthus, highlighting a mechanism based on resource competition.*[158]

The use of fungi-*Streptomyces* co-cultures has also been successful. These organisms share the same niche; therefore, they often mutually stimulate the production of secondary metabolites.[159] Although more emphasis has been placed on the activation of gene clusters in the fungal partner[160] there are reports on the activation of bacterial clusters via cultivation with *Aspergillus* fungi and *Saccharomyces* yeasts. For example, the co-culture of *Streptomyces* sp. with a co-isolated *Aspergillus* sp. activated the production of a rare fungistatic secondary metabolite heronapyrrole B in the bacterial partner.[161] The study also revealed that the fungus partner produced a chemical cue, cyclo-(L-Phe-trans-4-hydroxy-L-Pro),

that stimulated the bacteria to produce nitric oxide that acts as a transcriptional regulator for the silent BGC responsible for heronapyrrole B biosynthesis.

1.4.2 Genetic-based methods

These methods switch from searching for external triggers and cues and focus on internal changes that lead to BGCs activation.

1.4.2.1 Manipulation of BGC regulatory elements

One promising strategy for cluster activation is to manipulate the regulatory system that controls *Streptomyces* secondary metabolism. Regulation can be governed at the global level, which can be manipulated using overexpression or inactivation of global regulators. For example, the knock-out of the global transcriptional regulator, *adpA*, in *Streptomyces ansochromogenes* resulted in the activation of a silent BGC for oviedomycin production. The inactivation of *adpA* led to the depression of cluster-specific regulators, *ovmZ* and *ovmW*.[120] Alternatively, overexpression of the activators, such as *afsQ1,* has induced the production of cryptic metabolites.[162] This strategy is quite similar to the methods mentioned in the previous section. This method is quite unpredictable because global regulators simultaneously influence the expression of hundreds of genes.

Manipulation by pathway-specific regulators offers a more targeted approach for activation **(Figure 8)**. A silent large PKS gene cluster, nearly 150 kilobases in size, from *Streptomyces ambofaciens* ATCC23877 was activated through constitutive overexpression of a positive regulator situated within the cluster. This activation resulted in the production of four previously unidentified glycosylated macrolides, stambomycins A, B, C, and D.[163]

The promoter is another regulatory element that can be manipulated, as it is essential for transcription. Inactive native promoters can be replaced with more active variants, such as constitutive promoters *ermEp* and its derivatives or engineered synthetic promoters, *kasOp∗* or *SP44*. Inducible promoters, like cumate-inducible promoters, are also available for controlled gene expression.[164] Numerous promoters are now available for various applications.[165] For instance, inserting a strong *kasOp∗* promoter upstream of the first biosynthetic gene or regulatory element in a target BGC led to the production of previously uncharacterized metabolites, such as a polyketide with a unique core structure from *Streptomyces viridochromogenes*.[166]

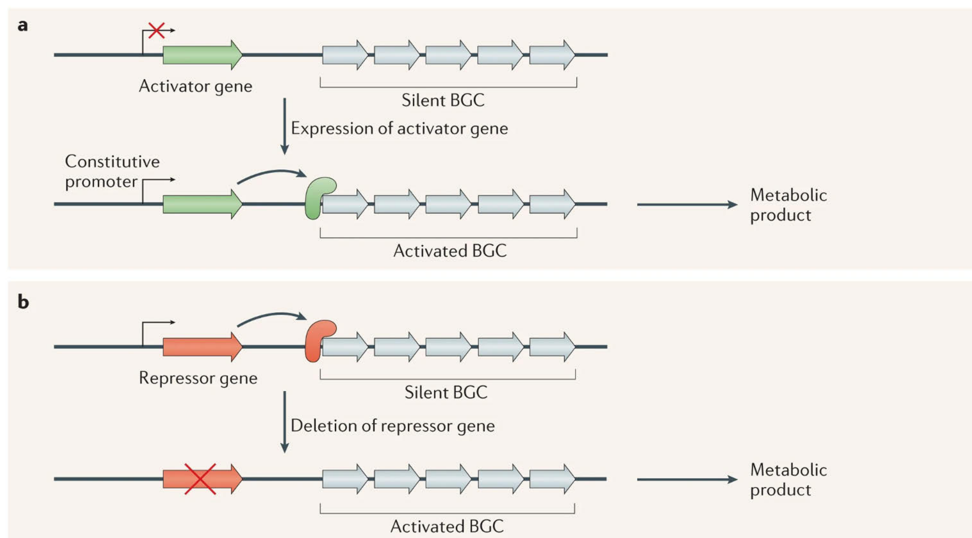


Figure 8 Examples of BGC activation via manipulation of regulatory elements. (a) Overexpression of pathway-specific regulator (activator gene) **(b)** Inactivation of pathway-specific regulator (repressor gene). Source: modified from Figure 4[167], licensed under CC BY 4.0.

1.4.2.2 Mutagenesis

1.4.2.2.1 Ribosome engineering

Mutagenesis can be an effective tool for altering bacterial secondary metabolism. Random genome-wide mutations can, for example, inactivate genes involved in antibiotic biosynthesis, thereby helping to elucidate their function.[168] Mutations in genes encoding RNA polymerase (RNAP) and ribosomal proteins have been shown to enhance the transcription and translation machinery, respectively, and trigger the production of previously unexpressed compounds.[169] This concept forms the foundation of ribosomal engineering. Bacterial strains exposed to streptomycin can develop drug-resistant mutants with specific mutations in the *rpsL* gene of the ribosomal protein S12.[170] Similarly, treating bacteria with rifampicin leads to the selection of resistant mutants with mutations in the *rpoB* gene of the β-subunit of RNAP.[171] *Streptomyces* strains with these types of mutations have been associated with activated antibiotic biosynthesis.

This method has been further expanded by using antibiotics such as erythromycin, gentamicin, lincomycin, and tetracycline.[172] Systematic antibiotic treatment of 1068 actinomycetes showed that 43% of *Streptomyces* species switched from non-producers to producers after acquiring resistance mutations, leading to the discovery of novel cyclic depsipeptides named piperidamycins A–H from *Streptomyces mauvecolor* mutants.[173] Despite its success, this method still relies on

labour-intensive mutant screening and can yield false positive results. Recently, a new strategy was developed where, instead of generating spontaneous mutations, exogenous mutated *rpsL* and *rpoB* genes were constitutively overexpressed in three wild-type isolates of *Streptomyces*. This led to the activation of metabolic pathways in the strains and the discovery of two polyketide antibiotics, piloquinone and homopiloquinone.[174]

1.4.2.2.2 Reporter-guided mutant selection (RGMS)

Random mutagenesis may also be coupled to reporter-guided screening, which has allowed for targeted activation of BGCs.[175] In a typical RGMS workflow, the first step involves identifying the promoter region of the silent BGC of interest. This promoter sequence is coupled with a reporter gene in a plasmid, that is typically integrated into the genome. This links transcription of the BGC to the reporter gene, which produces an easily detectable signal and "reports" when the promoter becomes active. There are several established systems, including the use of: i) green or red fluorescent protein signal (*gfp* or *rfp* gene)[176]; ii) antibiotic resistance[177]; iii) production of a coloured product (*xylE* gene produces the enzyme catechol 2,3-dioxygenase that cleaves colourless substrate and produces yellow substance)[178]. The second step is random mutagenesis for the generation of a library of mutants. Mutations can be introduced via UV, chemical or transposon mutagenesis. The library is then screened based on the signal of the reporter system and positive mutants are analysed for the presence of new activated metabolites. RGMS showed efficiency in cluster activation of aminoglycoside polyketides, gaudimycin D/E in *Streptomyces* sp. PGA64 (kanamycin resistance and *xylE* gene as reporters).[175]

1.4.2.3 Heterologous expression of BGCs

All previously described culture and genetic-based strategies aim to activate the BGCs in the native *Streptomyces* producer strains. However, sometimes native producers are genetically intractable or cannot be cultured under laboratory conditions, which makes BGC activation challenging. The heterologous expression of BGCs is another approach to activate production from BGCs. With the availability of genome sequences, the development of genetic manipulation tools, and the use of optimized host microbes for gene expression, it is now possible to transfer BGCs from native, non-producing *Streptomyces* strains into heterologous hosts, where metabolite production can be initiated.[179]

In the case of *Streptomyces*, several engineered variant strains of the species, such as *S. coelicolor*, *S. albus*, *S. avermitilis,* and *S. lividans*, have been widely used for the expression of BGCs from Actinobacteria. These producer strains were optimized through strategies such as deletion of native BGCs to remove metabolite backgrounds

or introduction of additional site-specific integration loci for better cloning of foreign DNA.[180,181] In addition, they can be easily transformed and genetically manipulated.

Several techniques have been developed for the retrieval of the entire target BGC directly from the native producer.[182,183] The classical approach is to generate and screen a random gene library (sequencing-independent), in which fragmented genomic DNA is cloned in a vector such as a cosmid (>40 kb) or a bacterial artificial chromosome (BAC, 100 kb).[184,185] The library is then screened for the target BGC. It is an overall time-consuming method and requires extensive screening. A more straightforward alternative is direct cloning of the BGC using techniques such as homologous recombination (transformation-associated recombination, TAR), which was successfully used to activate the silent taromycin A BGC.[186] Additionally, phage recombination (Red/ET recombineering) and site-specific recombination-based methods are also used for capturing BGCs.[187,188] The major limitation of these methods is that the BGC often remains silent in a heterologous host. For instance, in the case of the taromycin A BGC, activation was achieved only after the knockout of a negative regulator encoded within the cluster.[186]

The heterologous expression can be expanded by the use of synthetic biology tools for pathway refactoring, which is an alternative approach to activate silent BGCs in a heterologous host. Assembly of entire gene clusters from synthetic DNA allows control over the selection of biosynthetic genes, promoters, and regulatory elements to be included in the refactored pathway. The method can be used to bypass native regulatory mechanisms through modification of the regulatory elements of BGC, such as promoter replacement or introduction of additional constitutive promoters.[186]

The majority of heterologous expression studies have been carried out in *Streptomyces*, but other hosts have also been explored. The well-established *E. coli* host is not suitable for the expression of complex PKS and NRPS gene clusters, as these genes encode large multidomain proteins that are not typically produced in a soluble form in *E. coli*. This leads to several challenges, including a limited supply of metabolic substrates, differences in codon usage, poor protein solubility, and overall low production yields. Despite these challenges, there have been successful examples of the expression of BGCs in *E coli*. For example, the type II PKS-derived oviedomycin was produced in *E. coli* B24 strain with a yield of 120 mg/L. Efficient production required extensive metabolic engineering of the *E. coli* host strain, inducing the enhancement of protein solubility through co-expression of chaperones and tRNAs, optimization of precursor availability, and the expression of efflux pumps to alleviate product toxicity.[189]

1.4.3 Advantages and Limitations

This literature review summarizes the diverse methods developed and applied for the activation of silent BGCs in *Streptomyces*. Cultivation-based methods aim to mimic specific environmental signals by altering cultivation conditions or employing co-cultivation. Methods in this category are technically simple and cost-effective. Their implementation does not require prior knowledge of natural product biosynthesis; thus, genetic manipulations are generally unnecessary, with rare exceptions.

However, several significant limitations exist. In nature, a microbial population can experience multiple stimuli simultaneously, making it challenging to determine the right combinations and concentrations in laboratory settings, which can be labour-intensive and time-consuming. Furthermore, the regulatory cascades controlling compound production are complex, making it difficult to predict the effects of changing conditions in advance. For instance, co-cultures can result in the simultaneous activation of multiple co-culture-derived metabolites, making it difficult to link a specific metabolite to the producing strain.[190] Additionally, untargeted methods often result in the rediscovery of known pathways/compounds and their derivatives, which is the key issue in classical microbial drug discovery. In such instances, efficient screening of compounds in extracts and a dereplication process are necessary. Dereplication typically employs analytical techniques such as tandem mass spectrometry combined with natural product database searches to identify previously known molecules rapidly.[191,192] Therefore, to date, cultivation-based methods are often coupled with transcriptomics[193] and metabolomics[194] to facilitate the compounds discovery process.

On the other hand, genetic-based methods aim to activate biosynthetic pathways through genetic modification of a bacterial genome. These methods vary from simple, non-targeted techniques such as random mutagenesis and ribosome engineering to more precise manipulation of BGC. Untargeted genome manipulations face similar problems to culture-based methods, as broad changes in the genome often lead to unpredictable metabolic changes. Recently, conventional random mutagenesis has been combined with reporter-guided screening to enable targeted activation of specific BGCs. Mutagenesis allows for the introduction of multiple changes at both the global and pathway-specific levels, while the reporter system significantly increases throughput and identifies mutants with activated BGCs. Targeted, BGC-specific approaches such as manipulating regulatory elements offer the key advantage of directly activating the desired cluster, thereby bypassing complex regulatory cascades in *Streptomyces*. This results in greater predictability compared to culture-based or untargeted genetic methods.

However, while successful, targeted genetic-based methods have limitations, as some native isolates of *Streptomyces spp*. may be difficult targets to manipulate

genetically, making application impossible. In such situations, BGC activation may instead be achieved through heterologous expression in genetically tractable *Streptomyces* hosts. Nonetheless, this strategy presents its own challenges, particularly when expressing large or complex BGCs. Even after a successful cluster transfer, the introduced BGC may require extensive engineering to initiate biosynthesis or to achieve acceptable product yields.

Overall, no single approach is universally effective for the activation of biosynthetic pathways, as the process is complex and influenced by many factors, including gene cluster architecture, regulatory mechanisms, the genetic background of the producing strain, and environmental conditions. Therefore, the strengths and limitations of each strategy should be carefully evaluated in the context of the specific biological system being studied. It is also important to note that, although not discussed in detail in this review, these methods are widely used to enhance the yield of natural products and enzymes.[141,152,182,195]

2 Aims

This work aims to develop novel methods to harness the full genetic potential of *Streptomyces* for the production of valuable microbial natural products and industrial enzymes.

More specifically, the aims are to utilize two complementary approaches:

I. To employ naturally occurring events such as interactions with other microorganisms to activate metabolic pathways (Articles I and III)

II. To develop a novel metabolic engineering tool that can be used to activate BGCs and increase yields of enzymes and natural products (Article II)

3 Materials and Methods

3.1 Microorganisms used in the study

All *Streptomyces* strains used in this study were obtained from an in-house strain collection. *Streptomyces lavendulae* YAKB-15 was the primary microbe used throughout the study. In Article I, *Streptomyces albus* J1074 and *Streptomyces lividans* TK24 were employed as heterologous expression hosts. In Article III, the following isolates of *Streptomyces* species were employed: *Streptomyces albus* J1074, *Streptomyces lividans* TK24, *Streptomyces kanamyceticus* DSM 40500, *Streptomyces galilaeus* ATCC 31615, *Streptomyces showdoensis* ATCC 15227, *Streptomyces candidus* NRRL 3601, *Streptomyces platensis* NRRL 8035, and *Streptomyces peucetius* ATCC 27952. For co-culture experiments, *Saccharomyces cerevisiae* BY25610 yeast strain was used. [196] *Escherichia coli* TOP10 was used for general cloning and production of recombinant proteins. *E. coli* ET12567/pUZ8002 served as the donor strain for conjugative plasmid transfer into *Streptomyces*.[197]

3.2 Media and culture conditions

E. coli bacteria were routinely cultivated in Luria-Bertani (LB) broth and on LB agar plates. For protein expression, cultures were grown in 2×tryptone yeast extract (TY) medium (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl). Incubations were conducted at 200–250 rpm, at 30–37°C for general cultivation; room temperature was used for protein production. When required, the following antibiotics were added to the media: ampicillin (100 µg/mL), apramycin (50 µg/mL), kanamycin (50 µg/mL), and chloramphenicol (25 µg/mL), as described in Articles I, II, and III.

Streptomyces bacteria were cultivated on both solid and liquid media at 30 °C with shaking at 200–250 rpm for liquid cultures. Solid agar media included: P medium (1 g/L peptone, 4.55 g/L glucose, 0.4 g/L $MgSO_4 \cdot 7H_2O$, 0.4 g/L K_2HPO_4, 22 g/L agar, and 100 g/L potato juice), ISP4 (BD DifcoTM), and MS (mannitol-soy flour) medium made according to standard protocol.[197] Liquid media included: Y medium (9.1 g/L anhydrous glucose, 2 g/L NH_4NO_3, 2 g/L $CaCO_3$, and 26 g/L common bakery yeast), YE medium (same as Y but with 26 g/L of yeast extract), TSB medium (17 g/L tryptone, 3 g/L soy, 5 g/L NaCl, 2.5 g/L glucose), GYM

medium (4 g/L glucose, 4 g/L yeast extract, 10 g/L malt extract), SC medium (1.7 g/L yeast nitrogen base without amino acids, 5 g/L $(NH_4)_2SO_4$, 20 g/L dextrose, 2 g/L casamino acids, 1g/L uracil). When required, following antibiotics were added: thiostrepton (50 µg/mL), apramycin (50 µg/mL), and kanamycin (50 µg/mL).

3.3 Plasmids and constructs

In Articles I and III, the pBADHisBΔ plasmid was employed for the recombinant production of cholesterol oxidase and hydrolytic enzymes. The pBADHisBΔ constructs with corresponding genes were introduced into *E. coli* via the standard heat shock transformation protocol.[198] In Article I, pIJE486 and a modified pSET152 plasmid were employed to overexpress the *choD* gene. The *choD* gene was PCR-amplified from genomic DNA using Phusion High-Fidelity DNA Polymerase (ThermoFisher Scientific). Constructs were introduced into *Streptomyces* hosts by protoplast transformation and conjugation as described in the protocols.[197] In Article II, pSET152 plasmid was used to generate the reporter vector pS-GK, which was subsequently used in Article III as a reporter system to monitor gene expression during microscopy.

3.4 Protein production and purification

In Articles I and III, genes for protein production were codon optimized for *E. coli* and synthesized as synthetic DNA fragments. These DNA fragments were cloned into the pBADHisBΔ plasmid to express proteins with an N-terminal His-tag. The constructs were introduced into *E. coli* TOP10, and protein expression was induced with 0.02% (w/v) L-arabinose during cultivation and incubated overnight. Recombinant proteins were purified using polyhistidine-tag affinity chromatography with TALON Superflow resin (GE Healthcare), followed by buffer exchange and desalting using a PD-10 column (GE Healthcare). The purified recombinant enzymes were analyzed by SDS-PAGE using a precast gel (Bolt™ 4-12% Bis-Tris Plus Gel) and further evaluated using reducing sugar assays to confirm activity.

3.5 Enzyme activity assays

Cholesterol oxidase activity was assessed via a spectrophotometric assay detecting H_2O_2 produced during cholesterol oxidation. After cultivation, the cells were harvested, and proteins were extracted from the cell fraction using 50 mM phosphate buffer (pH 7.0) containing 0.15% Tween 80. The enzymatic assay was performed in a 96-well plate format and included 120 µL of 50 mM phosphate buffer with 0.05% Triton X-100 (pH 7), 10 µL of 9.1 mM ABTS (2,2'-azino-bis(3-

ethylbenzothiazoline-6-sulfonic acid), 2.5 µL of cholesterol (1 mg/mL in ethanol), and 1.5 µL of horseradish peroxidase (150 U/mL, type II, Sigma-Aldrich). The reaction was initiated by adding 20 µL of the culture extract. Absorbance was measured at 405 nm. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 µmol of H_2O_2 per minute at pH 7.0 and 27°C.

Activity of certain GHs CAZymes was measured using the 3,5-dinitrosalicylic acid (DNS) assay.[54] Streptomyces bacteria were cultivated for three days in Y medium and TSB medium, then centrifuged and filtered (0.45 µm) to obtain cell-free supernatants, which were concentrated 5-fold using Amicon® Ultra-15 centrifugal filters (Millipore, 10,000 MWCO) at +4°C. Enzymatic activity of purified proteins and concentrated supernatants was tested with laminarin (Sigma-Aldrich), colloidal chitin, mannan (Sigma-Aldrich), and a 25% (w/v) yeast cell suspension in water as substrates. For each reaction, 50 µL of enzyme sample was combined with 450 µL of substrate (10 mg/mL in 50 mM phosphate buffer, pH 7) in a tube and incubated overnight at 37 °C in the heat cabinet without shaking. Reactions were terminated by adding 750 µL of DNS reagent, followed by heating at 95 °C for 10 minutes. After centrifugation, the absorbance of samples was measured at 540 nm. Reactions without enzyme served as negative controls, and reducing sugars were quantified using a glucose standard curve.

Colloidal chitin was prepared by slowly adding chitin powder (2 g) to 80 mL concentrated HCl at below 5 °C with vigorous stirring, then heating to 37 °C until the viscosity decreased. The mixture was filtered through glass wool, and the filtrate was poured into 0.8 L of cold deionized water to reprecipitate chitin. After stirring (30 min) and overnight storage at below 5 °C, the precipitate was collected by centrifugation, washed to neutral pH, and resuspended in 50 mL deionized water.

3.6 Extraction and HPLC analysis of metabolites

In Article III, intracellular compounds were extracted from *Streptomyces* cells harvested after cultivation using methanol. Extracts were analyzed by high-performance liquid chromatography (HPLC) using a Shimadzu Nexera X3 system equipped with a photodiode array detector and a C18 reversed-phase column (2.6 µM, 100 Å, 4.6 × 100 mm Kinetex column (Phenomenex)). The mobile phase consisted of: solvent A - 0.1 % (vol/vol%) formic acid, 15 % acetonitrile, 85 % H_2O; solvent B - 100 % acetonitrile. The gradient program was as follows: gradient: 0-2 min, 0 % B; 2-20 min, 0-60 % B; 20-24 min, 100 % B; 24-29 min, 0 % B. The flow rate was set at 0.5 mL/min. The reaction products were detected by their retention time and absorbance spectra according to comparison to standards. Elution chromatograms were followed as absorbance at 430 nm wavelength.

3.7 Language tool

The language and style of this thesis were reviewed with the assistance of Copilot tool to ensure clarity and consistency.

4 Results and Discussion

My doctoral studies focused on *Streptomyces lavendulae* YAKB-15, which produces the industrially important cholesterol oxidase (ChoD) enzyme. As discussed in section 1.2.2.2, the ChoD catalyzes the oxidation of cholesterol to cholest-4-en-3-one and production of hydrogen peroxide H_2O_2 **(Figure 4a)**. Interestingly, our initial observation indicated that *S. lavendulae* YAKB-15 requires specific conditions for enzyme production - the presence of whole yeast cells, either live or autoclaved, in the culture medium. This requirement poses a significant challenge for industrial-scale production and raises important questions about the nature of *Streptomyces*-yeast interactions.

This thesis consists of both fundamental and applied research aspects. In **Article I**, we focused on the identification and characterization of *S. lavendulae* ChoD enzyme, followed by enzyme overproduction using new vector constructs with various promoters and other *Streptomyce*s species as hosts. In **Article II**, we engineered the native strain of *S. lavendulae* YAKB-15 to overproduce ChoD in large quantities. Finally, **Article III** focused on *Streptomyces*-yeast interactions and how these interactions influence the biology of *S. lavendulae* YAKB-15.

4.1 Characterization and enhanced production of cholesterol oxidase via heterologous expression

In **Article I**, we characterized and overproduced the cholesterol oxidase ChoD enzyme from *S. lavendulae* YAKB-15. For this purpose, we sequenced and assembled the genome, which led to the identification of the *choD* gene, using protein BLAST analysis. The putative ChoD protein had 82% identity to a known cholesterol oxidase from *Streptomyces* sp. SA-COO (UniProt P12676). Subsequently, the *choD* coding region was amplified by PCR from the genomic DNA and used for heterologous expression in two model *Streptomyces* hosts.

Notably, we found that *S. lavendulae* YAKB-15 predominantly produced cell-associated ChoD, with only minor activity detected in the culture supernatant. Subsequent *in silico* analysis of ChoD showed the presence of a TAT signal peptide at the N-terminal region. This suggests that the intracellularly synthesized protein

was exported across the cell membrane via the TAT translocation pathway, and then remains associated with the cell wall.[199]

4.1.1 Heterologous expression of *choD*

For ChoD enzyme overproduction, I utilized various *Streptomyce*s hosts, plasmids, and promoters. The first engineered strain, *S. lividans* TK24, expressed the *choD* coding region under the control of the natural constitutive *ermE* promoter using the multicopy pIJE486 plasmid. The second strain, *S. albus* J1074, expressed the *choD* gene under the control of the strong synthetic promoter SP44 in the integrative pSET152 plasmid. The constructs were introduced to *S. lividans* TK24 and *S. albus* J1074 by protoplast transformation and conjugation, respectively.

Next, I evaluated the production of the ChoD enzyme by the native host and the two overexpression strains in Y and YE production media. Both media contain glucose, ammonium nitrate (NH_4NO_3), and calcium carbonate ($CaCO_3$) as their primary components. The Y medium also contains baker's yeast as whole cells that are inactivated by autoclaving. In YE medium, we replaced yeast cells with an equivalent amount of yeast extract, a common media component in industrial fermentation. Notably, yeast extract is manufactured from yeast cell biomass through cell disruption and autolysis to extract the water-soluble content of yeast cells and remove the insoluble cell wall.[200,201]

As a result, the *S. lavendulae* YAKB-15 strain exhibited peak enzyme activity, 1.25 U/mL, after 40 hours of cultivation in the Y medium, followed by a rapid decline. No enzyme activity was detected in the YE medium, indicating that the presence of whole yeast cells is essential for ChoD production **(Figure 9).**

In *S. albus* J1074/pS_ChoD, ChoD activity was also dependent on the type of cultivation media. In Y medium, the enzyme activity in the engineered *S. albus* J1074 was 3-fold lower (0.4 U/mL) compared to the native strain. Notably, in the YE medium, the engineered *S. albus* J1074 was the only strain that produced ChoD, with a maximum activity of 0.78 U/mL **(Figure 9b).** This activity level was comparable to the level obtained with the native produce *S. lavendulae* YAKB-15 in Y medium. Similarly, heterologous production of ChoD in *S. albus J1074* also resulted in enzyme activity primarily associated with the cell fraction and not the supernatant. Importantly, this result demonstrated that *choD* expression can be achieved without whole yeast cells. *S. lividans* TK24, despite being a widely used host for heterologous protein expression, produced only a small amount of active ChoD enzyme under both conditions tested **(Figure 9)**.

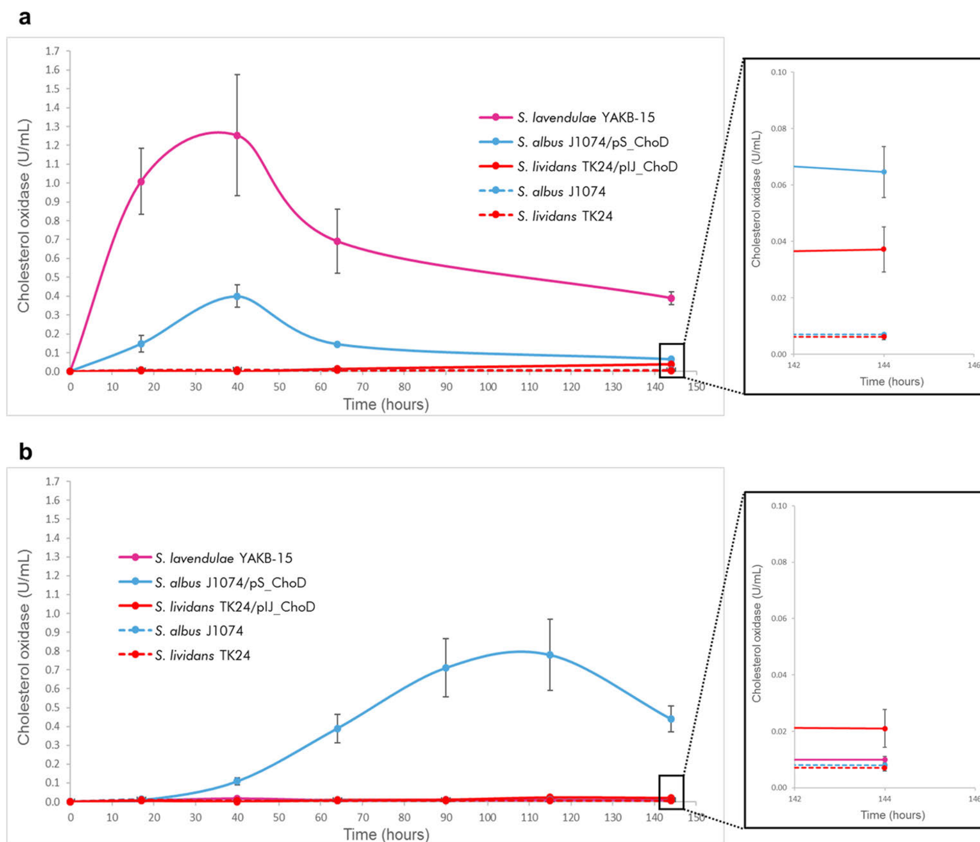


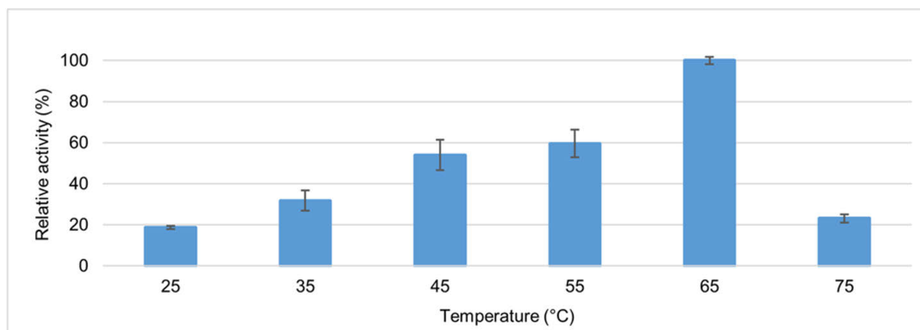
Figure 9 Time course analysis of ChoD activity in *S. lavendulae* YAKB-15 and two engineered strains of *S. albus* and S. *lividans* for expression of *S. lavendulae* ChoD. (a) Production in Y medium and **(b)** YE medium. Error bars represent standard deviation from three biological replicates. Source: Article I.

The ChoD activity of the native producer *S. lavendulae YAKB-15* was not observed when yeast extract was used as the nutrient source. Notably, unlike whole yeast cells, yeast extract lacks cell wall components, suggesting that these structural elements may serve as critical induction signals.[201] This hypothesis is supported by other studies showing that fungal cell wall components, specifically chitin, can stimulate enzymatic activity and the production of secondary metabolites in *Streptomyces* and cyanobacteria.[202,203] Moreover, the requirement for whole yeast cells in the medium further suggests that ChoD may play a role in signaling during interactions between *Streptomyces* and fungi. For example, a previous study identified the cholesterol oxidase *pimE* gene within the polyene pimaricin BGC in *Streptomyces natalensis*. The *pimE* inactivation has confirmed that cholesterol oxidase is essential for the production of this antifungal compound, indicating a signaling role for the enzyme.[204]

4.1.2 Characteristics of the recombinant ChoD enzyme

Next, I studied the effect of temperature and pH on the activity of the recombinant ChoD produced by the heterologous host *S. albus* J1074/pS_ChoD. Within the tested temperature range of 25–75°C, the optimal temperature for enzyme activity was found to be 65°C. Enzyme activity was also measured across a pH range of 4 to 9, with the highest activity observed at pH 5 **(Figure 10)**.

a



b

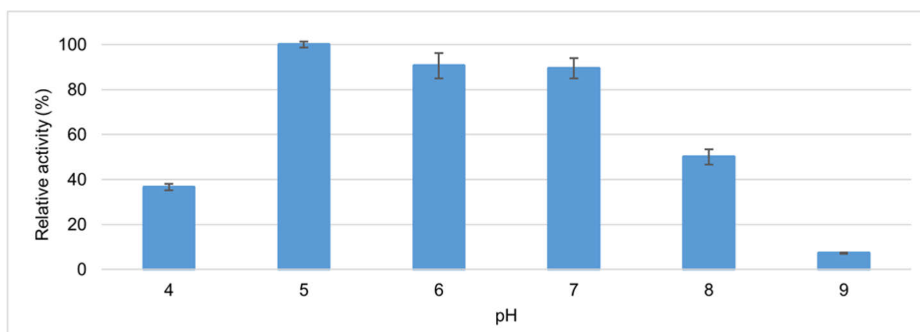


Figure 10 The impact of temperature and pH on the activity of the recombinant ChoD enzyme produced by *S. albus* J1074/pS_ChoD. Error bars represent standard deviation from three technical replicates. Source: Article I.

To further study enzyme kinetics, we expressed the codon-optimized *choD* in *E. coli* and purified the recombinant enzyme as an N-terminal His-tagged protein. The key kinetic parameters for the standard assay at pH 7 and temperature 30°C using cholesterol as substrate were calculated, resulting with a turnover number (kcat) of 10.35 s^{-1} and a Km of 15.91 µM. The Km value of ChoD can vary depending on the specific strain and experimental conditions. This result for *S. lavendulae* ChoD falls within the range of previously reported values, such as a Km of 3 µM for *Streptomyces sp*. SA-COO and 152 µM for *S. aegyptia* NEAE 102.[205,206]

4.2 Single-cell mutant selection for improved production of ChoD and mutaxanthenes

As discussed in introuction, mutagenesis and media optimization are conventional strategies for activating and enhancing the production of natural products with certain limitations. For example, in the case of mutagenesis, overall screening throughput is often limited due to the need to scan thousands of mutants.[207] In **Article II,** we developed a broadly applicable method that combines traditional random mutagenesis with reporter-guided selection and ultra-high-throughput screening. By using fluorescence-activated cell sorting (FACS), we were able to sort millions of *Streptomyces* mutants at a single-cell level directly from liquid cultures. Prior to this study, the use of FACS has been limited to *Streptomyces* due to the challenges associated with mycelial growth. The issue was solved by fragmenting the mycelium by sonication, followed by filtration and rigorous gating in FACS to identify single cells.

The detailed workflow of the method is described in **Article II**. Briefly, the first step in the process is the construction of a reporter plasmid. My colleagues constructed a *pSET152*-based vector, pS-GK, that featured a double-reporter system: *kan* gene for kanamycin resistance selection, and an *sfGFP* gene for selection based on fluorescence intensity via FACS **(Figure 11a).** The plasmid pS-GK was designed to harbor a promoter probe of a target gene or BGC upstream of the reporter genes. In essence, the plasmid links the transcription of the targeted operon in the BGC to the survival of the strain under kanamycin selection and the intensity of the GFP signal. After the introduction of the construct into a *Streptomyces* strain, genome-wide mutations were introduced via chemical mutagenesis (ethyl methanesulfonate) or UV irradiation, generating a mutant library. The objective of the step was to randomly generate a mutant where the targeted BGC had become active due to alterations in the complex regulatory networks controlling secondary metabolism in *Streptomyces*. We selected mutants from the library based on their ability to grow in the presence of increasing concentrations of kanamycin, indicating that increased transcription from the promoter plasmid. The ability to survive under the higher concentration of kanamycin correlated with stronger expression of the resistant gene. Enriched by kanamycin, positive mutants were subsequently screened in FACS to select the *Streptomyces* strain with the highest fluorescent signal. Additionally, FACS enabled single-cell sorting of *Streptomyces* mycelia, significantly increasing throughput to up to 20 million cells per library.

4.2.1 ChoD overproduction

We applied SCMS to the *S. lavendulae* YAKB-15 strain to enhance ChoD production. To achieve this, the promoter region of the *choD* operon was inserted

into the pS-GK reported construct and integrated into the *S. lavendulae* YAKB-15 genome **(Figure 11b, c).** After the random chemical mutagenesis, we grew mutants in Y medium (with whole yeast cells) with different concentrations of kanamycin to enrich the mutant library. The result showed that some *Streptomyces* mutants could tolerate up to 400 µg/ml of the antibiotic, indicating an increase in *kan* gene expression from the target promoter.

Further, we used FACS to select individual mutants exhibiting the highest sfGFP fluorescence, hence high ChoD expression. I was involved in the cultivation of selected mutants in Y medium and the analysis of ChoD activity. The first round of SCMS led to a 1.8-fold increase in the yield of ChoD. Subsequently, the best mutant from the first round was subjected to the second and third mutagenesis rounds. Overall, after three SCMC rounds, ChoD production increased 5-fold, reaching 20.4 U/g compared to 4.1 U/g in the native *S. lavendulae* YAKB-15 strain **(Figure 11d).**

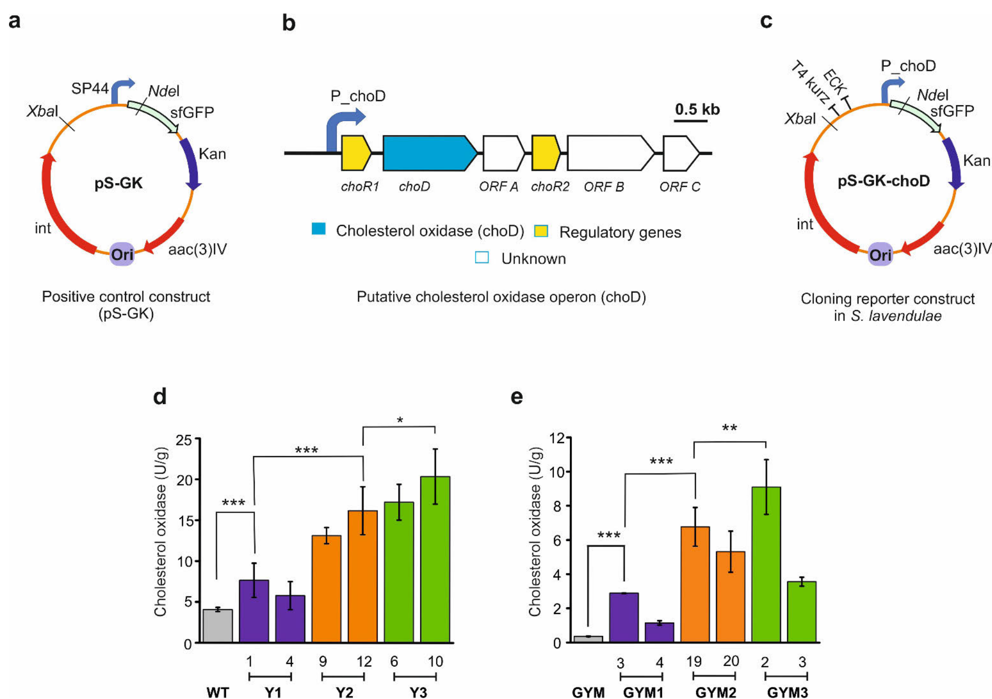


Figure 11 Enhancement of ChoD protein production in *S. lavendulae* YAKB-15. (a) Plasmid map of pS-GK featuring a dual-reporter system. **(b, c)** Structure of the *choD* operon and final constructs for strain improvement. **(d, e)** The ChoD activity analysis of selected mutants in (a) Y medium and (e) GYM medium. Error bars represent standard deviation from three biological replicates. ***p < 0.001, **p < 0.01, *p<0.05. Statistical significance was determined by one-way ANOVA with Tukey's post hoc analysis. by Source: modified from Article II.

Using SCMS, we achieved a substantial improvement in ChoD production in the native *S. lavendulae* YAKB-15 strain cultivated in Y medium. However, the use of yeast cells is not preferable in industrial bioprocessing as this medium component is non-standard. Moreover, refining the medium composition through conventional one-factor-at-a-time or statistical optimization techniques is a complex and time-consuming process that also requires multiple iterative experiments.[208] As an alternative approach, we preselected a relatively simple and inexpensive GYM medium (with yeast extract) and applied SCMS to evolve the *S. lavendulae* YAKB-15 strain to produce ChoD under these conditions. The initial ChoD yield in GYM medium was low (0.4 U/g) **(Figure 11e)**. To improve this, *S. lavendulae* YAKB-15 harboring the *choD* promoter-reporter construct underwent three rounds of SCMS in GYM medium. The results demonstrated continuous improvement in ChoD yield throughout the rounds, with a remarkable 22.8-fold increase in the best mutant **(Figure 10e).** These findings demonstrate that the SCMS is a highly effective strategy for increasing enzyme production in the native host. Moreover, this method presents a viable alternative to traditional medium optimization approaches.

4.2.2 Mutaxanthene cluster activation

In addition, my colleague successfully employed the SCMS approach for activating a previously silent mutaxanthene biosynthetic pathway in *Amycolatopsis orientalis* NRRL F3213 **(Article II, Figures 3 and 4)**. Genome mining revealed a type II PKS gene cluster responsible for mutaxanthene production. Subsequently, the promoter region of the cluster-situated transcriptional regulator was cloned into the *pS-GK* reporter construct. The strain was then subjected to UV mutagenesis, and after one round of antibiotic and FACS enrichment, several mutants produced visible pigmented compounds. These compounds were confirmed by NMR as mutaxanthenes A, B, and D. The following second round of SCMS demonstrated a significant increase in product yields. Specifically, the average mutaxanthenes titer rose from 11 mg/L to 55 mg/L, which is a 9-fold increase in production. The best mutant yielded 99 mg/L. These results highlight the value of the method for activation of BGC, making it a promising tool in drug discovery. In addition, the improvement of compound yield helps to obtain sufficient material for further structure elucidation and bioactivity assays.

4.3 *Streptomyces*-yeast interactions for activation of metabolic pathways and enzyme production

As mentioned in 4.1, *S. lavendulae* YAKB-15 produces the ChoD enzyme only in the presence of whole yeast cells in Y medium. This raised the question of whether the production of ChoD was important for more general interactions between *Streptomyces* and fungi. The hypothesis became more relevant during co-cultivation experiments, where we observed an unusual phenomenon in which yeast cells appeared to physically adhere to the *Streptomyces* mycelium. More intriguingly, the yeast cells eventually disappeared upon extended cultivation, indicating that *Streptomyces* might utilize the yeast cells as nutrients **(Figure 12)**. We became interested in the molecular basis of these interactions **(Article III)** and decided to investigate their effect on the production of natural products and extracellular enzymes by *Streptomyces*.

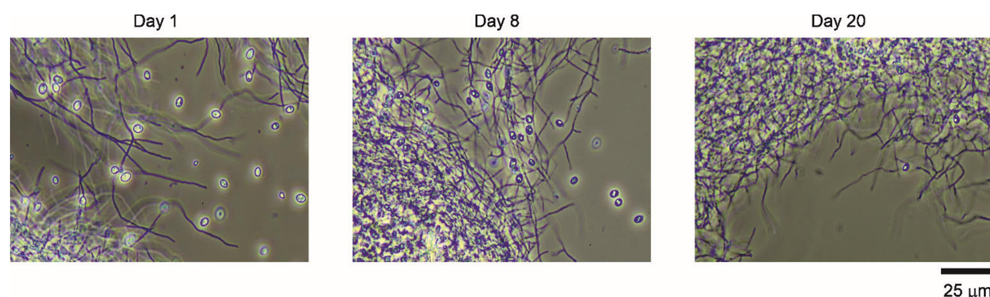


Figure 12 The initial observation of attachment and digestion of yeast cells by Streptomyces. The *S. lavendulae* YAKB-15 was cultured in Y medium with autoclaved yeast and monitored over 20 days using optical phase contrast microscopy. Source: Article III.

4.3.1 Physical contact triggers *Streptomyces* to prey on yeast

The interaction between *S. lavendulae* YAKB-15 and *Saccharomyces cerevisiae* was studied in co-cultures using various microscopy techniques. We first used time-lapse fluorescence microscopy to capture the onset of the interaction. For this purpose, we used the *S. lavendulae* YAKB-15 strain in which the *sfGFP* gene was linked to the promoter of the *choD* gene, allowing us to monitor *choD* expression through green fluorescence. The yeast strain used in the co-cultures was *S. cerevisiae* BY25610, which constitutively expressed the red fluorescent protein mCherry as a fluorescent marker. Continuous co-cultivation revealed that *choD* expression was activated when *S. lavendulae* YAKB-15 mycelia physically interacted with yeast cells, as indicated by the appearance of green fluorescence at the contact sites **(Figure 13a)**. Conversely, the yeast cells that interacted with *Streptomyces* showed a gradual loss

of red fluorescence and exhibited slowed growth. These findings demonstrate that physical contact between *S. lavendulae* mycelia and yeast cells elicits the production of the ChoD enzyme.

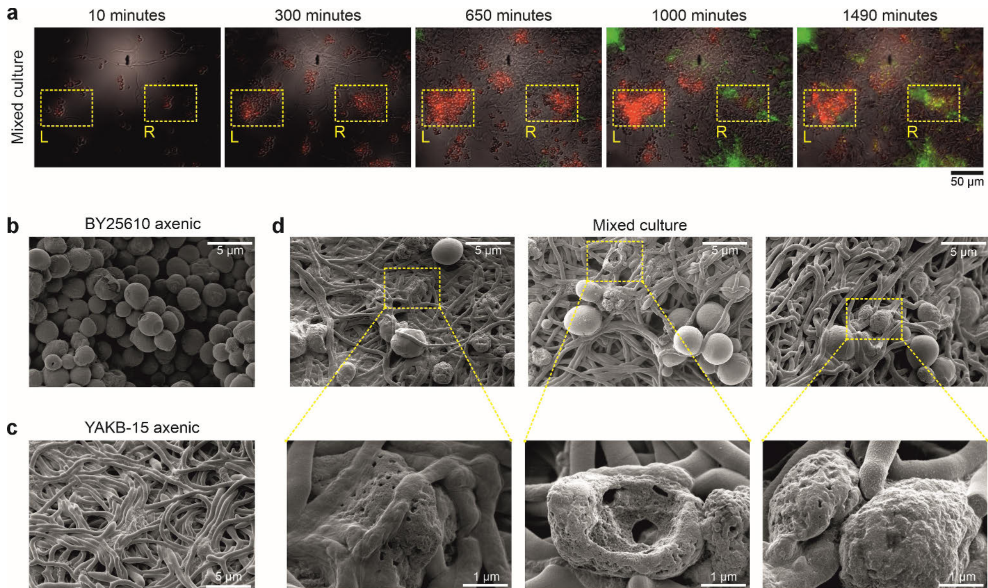


Figure 13 Microscopic analysis of *Streptomyces* and live yeast interactions. (a) Time-lapse fluorescence microscopy of *S. lavendulae* (*choD*-GFP) co-cultured with *S. cerevisiae* BY25610 (mCherry). Two distinct yeast cell populations are marked with yellow boxes: the left population (L), not in contact with *S. lavendulae*, displayed increased RFP intensity over time; the right population (R), which interacted with *S. lavendulae*, showed consistently low RFP intensity. (b, c, d) Scanning electron microscopy images of (b) *S. cerevisiae* BY25610 monoculture and (c) *S. lavendulae* YAKB-15 monoculture; (d) mixed cultures showing structural damage to yeast cells. Source: modified from Article III.

Next, we employed scanning electron microscopy (SEM) to examine possible changes in yeast cell morphology. After three days of cultivation with *S. lavendulae* YAKB-15, *S. cerevisiae* BY25610 exhibited significant structural alterations **(Figure 13d)**. Yeast cells transformed from their typical round shape to a shrunken and deformed morphology. In addition, cell surfaces appeared irregular and rough, with visible pores and cavities in some cells, indicating cell wall and membrane digestion. These types of damage were not observed in yeast monocultures **(Figure 13b)**. The observed destructive effect on live yeast cells suggests a predatory behavior of *S. lavendulae* YAKB-15. Predatory bacteria, such as *Bdellovibrio* and *Myxococcus*, obtain nutrients by killing other organisms, referred to as prey. Various mechanisms of attack have been described, including direct invasion, attachment to prey cells, and coordinated group attacks. In addition, the production of specialized

metabolites that kill the prey, along with digestive enzymes that degrade the cell wall and internal contents, has been shown.[209–211] *Streptomyces* and other members of the phylum Actinobacteria have not traditionally been considered predatory; however, increasing evidence suggests they may possess facultative predatory capabilities.[212]

4.3.2 Transcriptomics and metabolomics reveal large-scale changes in *Streptomyces* upon contact with yeast

Comparative transcriptome analysis revealed that physical contact with yeasts activated the expression of broad sets of genes in *Streptomyces*. We observed significant upregulation of 185 CAZyme genes, particularly those encoding enzymes of CAZyme GH families: α-mannosidases, β-glucanases, and chitinases. These enzymes participate in the degradation of the yeast cell wall **(Figure 14a)**. Furthermore, genes associated with the breakdown of yeast cellular membranes were also upregulated, including *choD*; the gene *cyp125A13* encoding a putative P450 sterol monooxygenase involved in sterol degradation; and two genes *pld1* and *pld2,* encoding putative phospholipases-degrading enzymes **(Figure 14b)**. Finally, the transcriptomics data indicated that several BGCs were activated in *S. lavendulae* YAKB-15 during co-cultures with yeast, including a polyene-type BGC that remained silent under monoculture conditions. Other induced BGCs were associated with a putative diisonitrile antibiotic SF2768 with antifungal activity, a foxicin-type siderophore, and a stenothricin-type antibiotic **(Figure 14c and d)**.

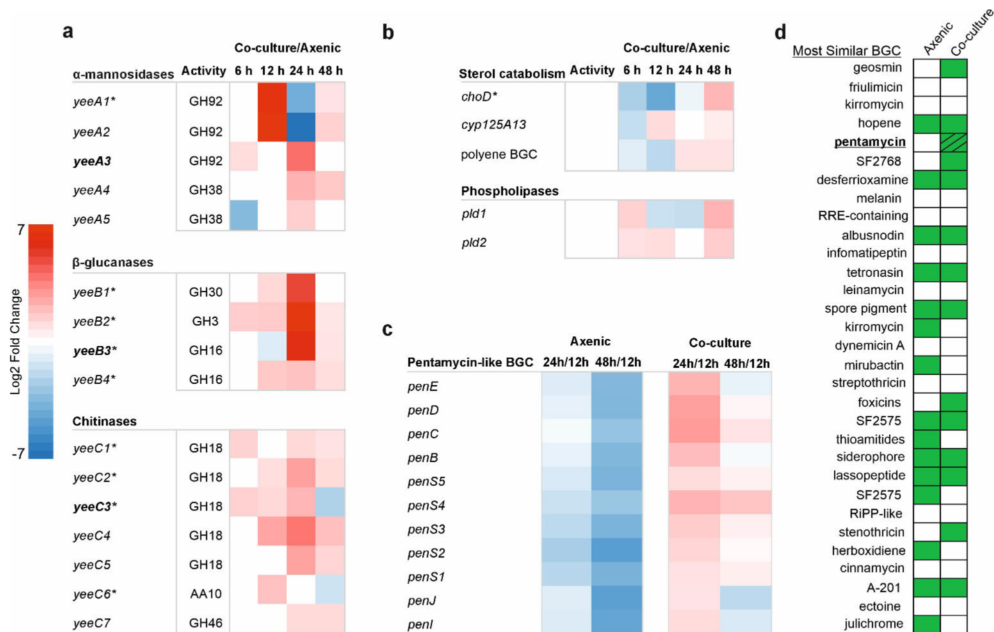


Figure 14 Transcriptomic changes in *S. lavendulae* YAKB-15 upon contact with yeasts. (a, b) Comparative transcriptomic analysis highlights differentially expressed genes encoding enzymes for degrading yeast (a) cell wall and (b) membrane components. Bold text indicates heterologously expressed proteins**. (c)** Transcriptomic analysis of polyene biosynthetic genes over time (12, 24, 48 hours). In *S. lavendulae* monoculture, polyene production remains inactive, in contrast, co-culturing with yeast triggers polyene biosynthesis. **(d)** A comparison of expressed BGCs in *S. lavendulae* after 24 hours under axenic conditions and during co-culture with yeast. Active clusters are shown in green. Source: modified from Article III.

Comparative metabolome analyses of co-culture and monoculture carried out by a co-worker showed that *S. lavendulae* YAKB-15 consumed the phospholipids and sterols from the yeast cell membrane **(Article III, Figure 3)**. Specifically, the main phospholipids of yeast cell membranes, such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE), disappeared in co-culture samples after 4 days of incubation. The same applied to sterols, specifically ergosterol, which were both consumed and modified by *Streptomyces*.

4.3.3 Enzyme assays confirm the ability of hydrolytic enzymes from *Streptomyces* to digest the yeast cell wall

In *S. cerevisiae* yeasts, the cell wall maintains cell shape and integrity while also protecting the cell from environmental damage and stress. It is composed of carbohydrate polymers and proteins, primarily β-glucans, mannoproteins, and chitin. Glucan is the major polymer, accounting for up to 60% of the dry weight of the cell

wall. It consists of differently linked glucose units that create a highly branched network of β-1,3-glucans and β-1,6-glucans. The mannan part of the mannoprotein complex is a mannose polymer composed of α-1,6-linked mannan with α-1,2- and α-1,3-mannan branches. Chitin, a linear polymer of N-acetylglucosamine (GlcNAc), constitutes about 1–2% of the cell wall **(Figure 15a).**[213–215]

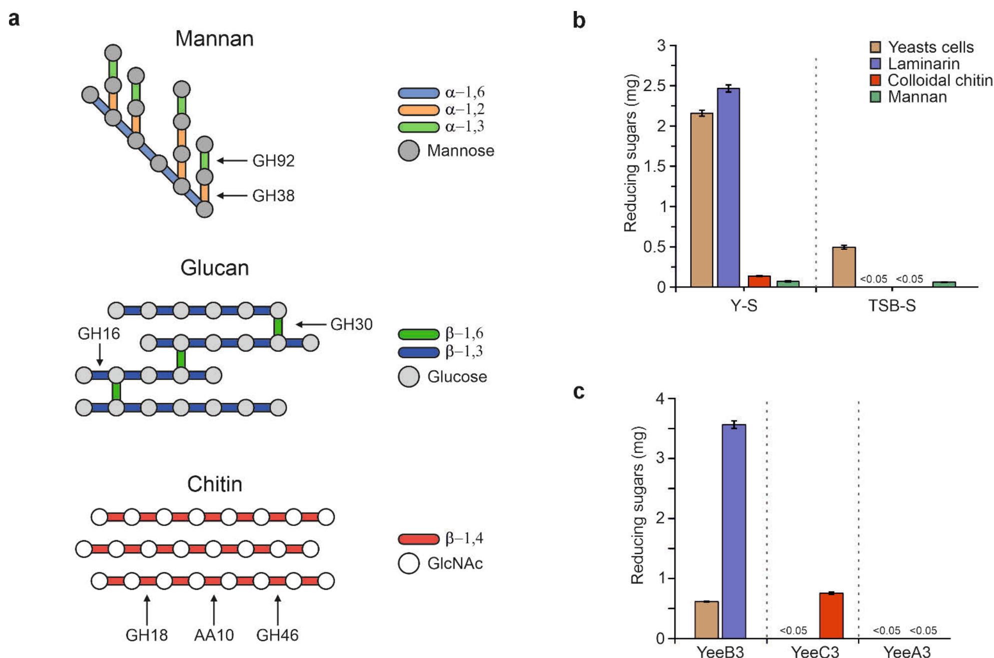


Figure 15 Enzymatic abilities of *S. lavendulae* to degrade yeast cell wall components. (a) Schematic representation of the structural components of the yeast cell wall: mannan, glucan, and chitin. **(b)** Enzymatic activity of *S. lavendulae* supernatant from yeast-free medium (TSB-S) and with whole autoclaved yeast cells (Y-S) cultures. Activity was measured with supplementation of autoclaved yeast cells or the cell wall components as substrates. **(c)** Recombinant glucanase (YeeB3), chitinase (YeeC3), and mannosidase (YeeA3) activity was measured with supplementation of autoclaved yeast cells or the cell wall components as substrates. Source: modified from Article III.

The ability of *S. lavendulae* YAKB-15 to degrade yeast cell wall components was confirmed by the analysis of CAZymes activity using the colorimetric 3,5-dinitrosalicylic acid (DNS) assay. Using an oxidizing agent, such as DNS, the method detects the presence of reducing sugars released during the enzymatic hydrolysis of complex carbohydrate polymers. Reducing sugars react with DNS, which undergoes a color change upon reduction. This change can be measured spectrophotometrically.[54] For analysis, I cultured *S. lavendulae* YAKB-15 in the presence (Y medium, Y-S) and absence (TSB medium, TSB-S) of autoclaved yeast cells. The resulting supernatants were used as the source of CAZymes, while

laminarin, mannan, colloidal chitin, and autoclaved yeast cells served as substrates. Laminarin is a structural analog of β-1,3-glucans, and colloidal chitin was produced by partial hydrolysis of shrimp shells to obtain water-soluble chitin. With dead yeast cells as substrate, the supernatant from the co-cultures exhibited higher total hydrolytic activity compared to the monoculture supernatant, with a 4.4-fold increase in reducing sugar content **(Figure 15b)**. Furthermore, the same co-culture supernatant displayed particularly high glucanase activity in reaction with laminarin, whereas chitinase and mannosidase activities were lower.

In addition, the putative *S. lavendulae* glucanase, chitinase, and mannosidase enzymes encoded by the genes *yeeB3*, *yeeC3*, and *yeeA3*, respectively, were expressed in *E. coli* and purified as N-terminal His-tagged proteins. The resulting recombinant enzymes were also subjected to the enzymatic assays. Results demonstrated that the recombinant glucanase and chitinase enzymes successfully degrade laminarin and chitin into sugar units with reducing ends **(Figure 15c)**. In contrast, the recombinant mannosidase showed no detectable activity against the tested mannan substrate or whole yeast cells. This lack of activity may be attributed to substrate specificity, as the mannosidase selected for recombinant expression was predicted to belong to the CAZy GH92 family that predominantly targets α-1,2-, α-1,3- and α-1,4-glycosidic cleaving activity. These specific linkages may not be present in the substrate I selected.

4.3.4 Isolation of antifungal compounds produced upon interactions with yeast

Transcriptomics and metabolomics experiments revealed the production of polyene antifungal compounds by *S. lavendulae* YAKB-15 grown in Y medium with whole yeast cells **(Figure 16b)**. Following large-scale cultivation, these compounds were purified and structurally identified as pentamycin and filipin III using NMR spectroscopy. Genome mining indicated the existence of a type I PKS gene cluster, designated *pen*, that is responsible for the biosynthesis of these polyene antifungal compounds **(Figure 16a)**. The cluster comprises five core biosynthetic genes, *penS1*-*penS5*, responsible for assembling the core polyene structure, and three tailoring genes *penC*, *penD*, and *penJ*, which encode cytochrome P450 monooxygenases that introduce hydroxyl groups into the structure. Notably, during extraction, we found that the compounds were also associated with the cell fraction. Analysis of the *pen* cluster revealed the absence of transporter genes, which may explain their retainment in the cell extracts.

Pentamycin polyene antibiotic is used against vaginal candidiasis (*Candida* spp. fungal infection) and trichomoniasis (parasitic infection).[216] Polyene antibiotics are known to target fungal membranes by interacting with sterols within the lipid bilayer. Several mechanisms of action have been described, including the formation of the

pores in the membrane and the extraction of ergosterol, both of which disrupt membrane integrity.[217] More recently, polyene mandimycin has been shown to bind directly to various membrane phospholipids, suggesting an additional mode of action.[218]

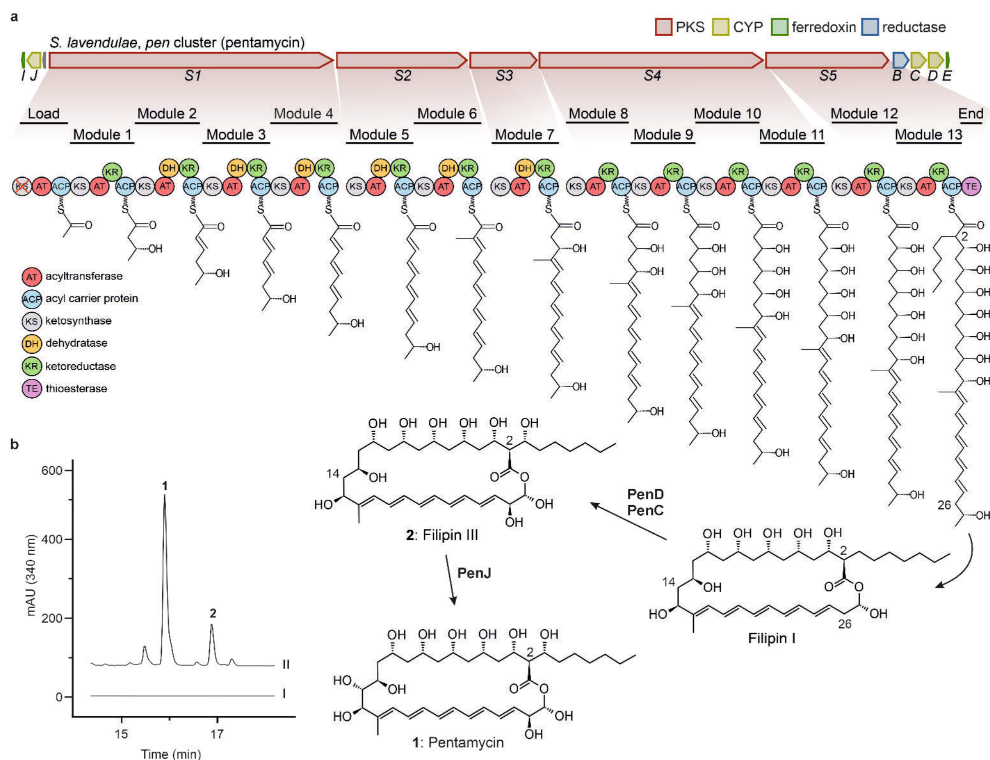


Figure 16 Pentamycin biosynthesis in *S. lavendulae* YAKB15. (a) Pentamycin BGC and domain organization for filipin I biosynthesis. The tailoring enzymes PenC, PenD and PenJ convert filipin I to filipin III (2) and pentamycin (1). **(b)** HPLC analysis of *S. lavendulae* culture extracts reveals polyenes 1 and 2 exclusively in co-cultures with yeast (II), while they are absent in axenic cultures (I). Chromatograms were recorded as absorbance at 340 nm. Source: modified from Article III.

4.3.5 Yeast predation is a common phenomenon in *Streptomyces*

As the next step, we expanded the interaction studies by testing eight additional species of *Streptomycetes* from our microbial collection in co-culture with yeast. I co-cultured each species with autoclaved yeast to assess their ability to degrade yeast cells. Microscopic examination revealed that seven out of eight cultivated species completely digested the yeast cells. Notably, *S. candidus* NRRL 3601 was able to complete degradation within only two days of contact time **(Figure 17a)**.

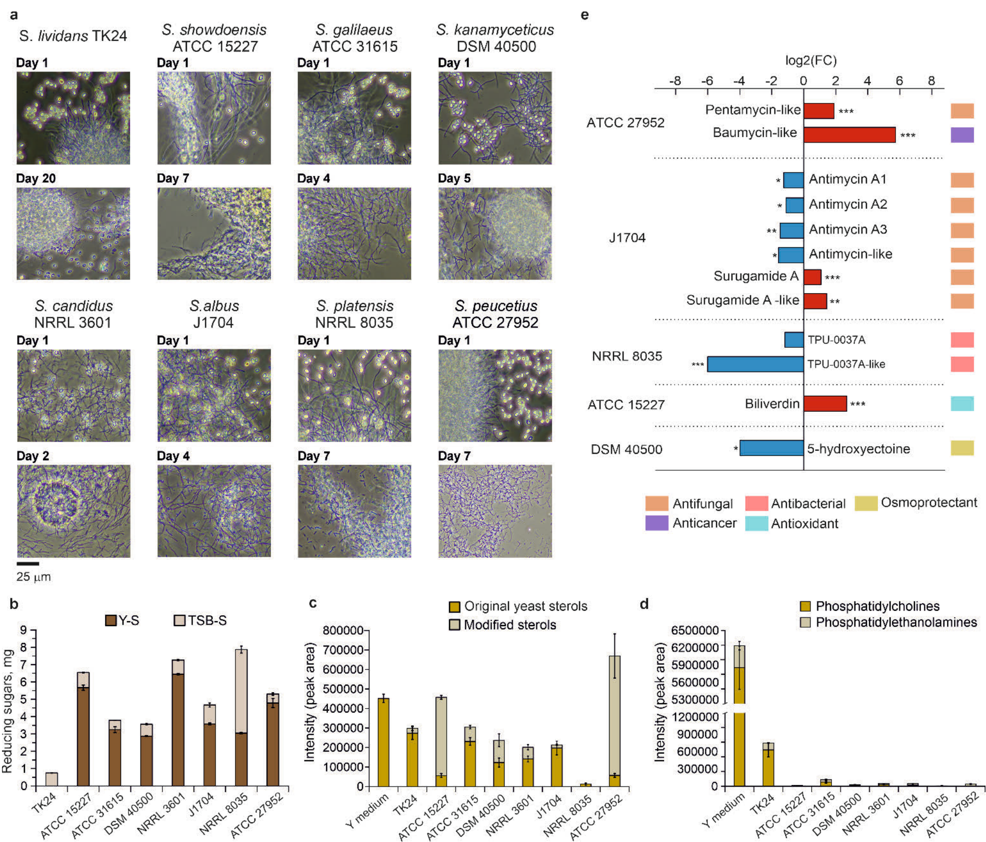


Figure 17 Widespread predation abilities among species of *Streptomyces*. (a) Microscopic analysis of different species of *Streptomycetes* in association with yeast *Saccharomyces cerevisiae* cells. **(b)** Hydrolytic glucanase activity of *Streptomyces* culture supernatants from cultivations on yeast-free medium (TSB-S) and with whole autoclaved yeast cells (Y-S) cultures. Enzymatic activity was determined in the presence of autoclaved yeast cells. **(c, d)** Metabolomic analysis of yeast sterol, phosphatidylcholines, and phosphatidylethanolamines degradation. Error bars represent the standard deviation from three biological replicates. **(e)** Comparative metabolomic analysis of secondary metabolites showing changes in production levels in *Streptomyces* strains during co-culture with yeast. Error bars represent the standard deviation from three biological replicates. ***, $p < 0.01$; **, $p < 0.05$; *, $p < 0.1$. Source: Article III.

S. lividans TK24 stood out among the other tested species of *Streptomycetes*, as it did not consume the yeast even after 20 days of incubation. Furthermore, I assessed the hydrolytic glucanase enzyme activity in cultures of the tested species of *Streptomyces*. Most species, except *S. lividans* and *S. platensis*, exhibited enhanced hydrolytic enzyme activity when cultivated in the Y medium containing autoclaved (dead) yeast cells **(Figure 17b)**.

Metabolomic analysis conducted by my coworker revealed that all eight species of *Streptomycetes* were capable of degrading sterols and phospholipids, with notable variability in degradation efficiency **(Figures 17c, d)**. Additionally, exposure to yeast triggered metabolic shifts across species, including increased production of antifungal, anticancer, and antioxidant compounds.

We conducted a closer investigation of *S. peucetius* ATCC 27952, which, according to metabolomics analysis, produced a pentamycin-like compound. Structure elucidation, performed by my colleague, identified the compound 14-hydroxyisochainin, which is highly similar to the pentamycin compound from *S. lavandulae* YAKB-15 **(Figure 18b)**. Using the available genome sequence of *S. peucetius* ATCC 27952 (CP022438.1), we identified a type I PKS gene cluster, which we named *iso*, responsible for the biosynthesis of 14-hydroxyisochainin. The *iso* cluster had an identical domain organization to the *pen* cluster; however, it lacked one gene (gene D) encoding a cytochrome P450 monooxygenase of the *S. lavendulae pen* cluster **(Figure 18a)**. As a result, the 14-hydroxyisochainin compound lacks the hydroxyl group at the C1 position.

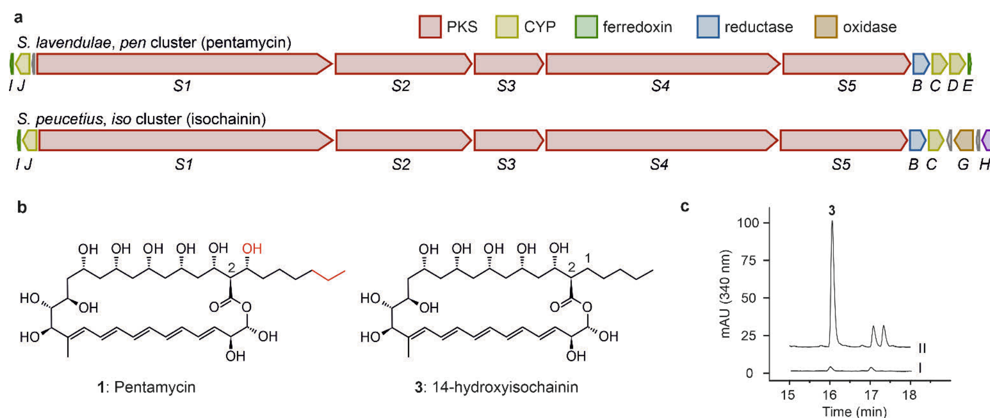


Figure 18 Polyene compounds from *S. lavendulae* YAKB-15 and *S. peucetius* ATCC 27952. **(a)** Comparison of BGCs for pentamycin (*pen*) and 14-hydroxyisochainin (*iso*) biosynthesis. **(b)** Structural differences between pentamycin (1) and 14-hydroxyisochainin (3). **(c)** HPLC analysis of *S. peucetius* culture extracts from (I) axenic cultivation and (II) cultivation in a medium supplemented with autoclaved (dead) yeast cells. Chromatograms were recorded as absorbance at 340 nm.

5 Conclusions

My thesis demonstrated the use of a multifaceted approach to unlock the full genetic potential of *Streptomyces* for the production of high-value products. I employed microbial interactions, heterologous expression, and a newly developed single-cell mutant selection (SCMS) method to activate biosynthetic pathways for both natural products and enzymes, significantly enhancing their production yields.

The study began as a simple investigation into using baker's yeast (*Saccharomyces cerevisiae*) as a medium component to induce the production of cholesterol oxidase (ChoD), an industrially important enzyme. This initial work led to broader insights into microbial interactions. We discovered that physical interaction with yeast cells strongly induced ChoD production in *S. lavendulae* YAKB-15. In addition to ChoD, the co-culture conditions triggered the production of a broad range of industrially relevant carbohydrate-active enzymes (CAZymes), highlighting the role of microbial communication in enzyme expression and the potential biotechnological application of *Streptomyces* as a source of industrial enzymes.

To move beyond the limitations of yeast-dependent conditions, my work contributed to the development and implementation of the SCMS metabolic engineering tool. Using SCMS, we were able to activate ChoD production in preselected yeast-free media, resulting in a remarkable 22-fold increase in enzyme activity and yield. Notably, the use of other *Streptomyces* species as host allowed recombinant ChoD expression without yeast cell supplementation. However, however, the production level remains about a 3-fold lower than in the *S. lavendulae* native host. These results indicate how different strategies can lead to varying outcomes.

In parallel, our investigation of *Streptomyces*-yeast interactions reveals that yeast cells also act as powerful triggers for natural product biosynthesis in *Streptomyces*. Co-cultivation led to the production of several secondary metabolites in *S. lavendulae* YAKB-15, particularly antifungal polyenes pentamycin and filipin III. These compounds were not detectable under standard monoculture conditions, indicating the role of microbial interaction in activating silent BGCs. To further explore natural product activation, SCMS was applied to *Amycolatopsis orientalis*,

enabling the activation of a previously silent BGC responsible for producing mutaxanthenes. This led to up to a 10-fold increase in yield, without the need for medium optimization experiments. Overall, the SCMS technique presents a powerful tool for pharmaceutical and biotechnological industries, enabling rapid development of high-yielding microbial strains and increasing yields of highly valuable drugs.

Beyond these results, our work highlights a new role for the genus *Streptomyces* in an ecological context. Microscopic analysis revealed that yeast cells underwent significant morphological changes during co-cultivation, and the induced metabolites appeared to target and disrupt yeast cells. These findings suggest that *Streptomyces* can exhibit facultative predatory behavior and use natural products and enzymes to attack and extract nutrients from associated and competing organisms. Importantly, this response was consistent across a set of *Streptomyces* species, indicating a conserved predatory strategy.

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Turku, September 2025
Arina Koroleva

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