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Decoding Multicellularity in *Streptomyces*: Single-Cell Technologies Reveal the Link between Phenotypic Heterogeneity and Secondary Metabolite Production

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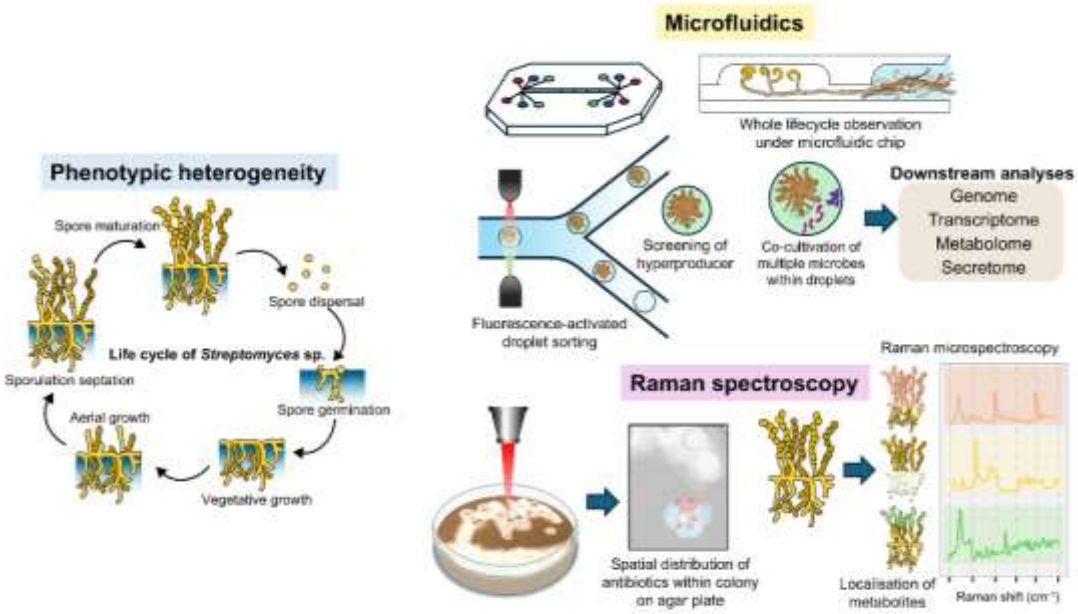
Abstract: *Streptomyces* species are among the most prolific natural sources of antibiotics and other bioactive secondary metabolites, supporting major therapeutic advances, including streptomycin and ivermectin. In addition to their ecological role as saprophytic decomposers, they remain central to modern medicine and agriculture, with nearly two-thirds of clinically used antibiotics originating directly or indirectly from this genus. Recent progress in genome sequencing, CRISPR-Cas9 editing, and synthetic biology has expanded our ability to modify *Streptomyces* genomes, activate silent biosynthetic gene clusters, remove competing pathways, and increase metabolite output. Yet, industrial use of these bacteria continues to be difficult due to their complex multicellular life cycle and pronounced phenotypic heterogeneity. Even genetically identical cells display differences in germination, growth, and metabolic activity, leading to inconsistent fermentation performance and variable yields. More recent studies have shown that this heterogeneity is not simply a technical barrier but a beneficial ecological strategy that improves survival in changing environments. These findings underscore the need to understand the spatial and temporal diversity that shapes *Streptomyces* biology. New single-cell technologies, including microfluidics and Raman spectroscopy, now allow direct observation of developmental processes, spatial mapping of metabolite production, and high-resolution screening of superior producer strains. This review summarises current advances in engineering *Streptomyces* and examines how single-cell approaches are transforming our understanding of their variability. By combining genetic tools with precise phenotypic analysis, researchers are moving toward more consistent and scalable production systems for valuable natural products.



Graphical abstract. Advancements in single-cell technologies, such as microfluidics and Raman spectroscopy, in decoding *Streptoymyces* heterogeneity.

Keywords: *Streptomyces*; secondary metabolites; heterogeneity; microfluidics; Raman spectroscopy; SDG 3 Good health and well-being

1. Introduction

Streptomyces bacteria are among nature's most prolific producers of clinically important antibiotics and other bioactive secondary metabolites [1, 2]. For decades, *Streptomyces* has been central to microbiology and therapeutic discovery, contributing to the Nobel Prize-winning drugs such as streptomycin and ivermectin [3, 4]. These microorganisms are critical to ecological processes as saprophytes, mineralising complex polymers in soil and aquatic sediments, which facilitates the recycling of organic matter. *Streptomyces* also serve as the primary source of natural products used in medicine and agriculture [5]. It is estimated that approximately two-thirds of antibiotics are derived directly or indirectly from *Streptomyces* species [6, 7], along with a diverse range of other bioactive compounds, including anticancer, antifungal, antiparasitic, and immunosuppressant agents [8-12].

The urgent global challenge of antimicrobial resistance sharpens the relevance of *Streptomyces* research [13, 14]. Multidrug-resistant pathogens threaten public health worldwide [15-20], and microbial natural products remain one of the richest sources of new chemical scaffolds for antibiotic discovery [21, 22]. Renewed efforts to discover, activate and optimise biosynthetic gene clusters are therefore strategic priorities for replenishing the antibiotic pipeline and countering resistant infections [23]. These imperatives motivate both fundamental

studies of *Streptomyces* biology and the development of high-throughput, discovery-oriented platforms that can accelerate lead identification and strain improvement.

Recent decades have seen dramatic advances in the genetic manipulation of *Streptomyces*. With the advent of high-throughput genome sequencing, CRISPR-Cas9 based genome editing, and synthetic biology, it is now possible to make precise modifications to the bacterial genome [24-27]. These technologies allow for the deletion of competing metabolic pathways, the activation of silent biosynthetic gene clusters (BGCs), and the optimisation of BGC expression [28-30]. These innovations have unlocked previously cryptic biosynthetic pathways and led to notable successes in enhancing the production of valuable secondary metabolites, providing new opportunities for drug discovery and industrial biotechnology [31, 32]. However, despite these powerful tools, the industrial-scale exploitation of *Streptomyces* remains challenging due to its inherent biological complexity.

One of the central challenges in using *Streptomyces* for large-scale production is its complex, multicellular life cycle [4]. Spore germination gives rise to a branching substrate mycelium that, under nutrient limitation, differentiates to form aerial hyphae and spores; this developmental program is tightly coupled to secondary metabolism, with antibiotic production frequently coinciding with sporulation [33-35]. However, a key feature of this lifecycle is the profound phenotypic heterogeneity observed even within genetically identical populations. Variations in germination timing, growth rates, and metabolic activity among individual cells result in unpredictable batch-to-batch variations and suboptimal yields in industrial fermentations [36, 37].

Nevertheless, recent studies have shifted this perspective, revealing that such heterogeneity serves as a biologically meaningful, adaptive bet-hedging strategy, enhancing the survival of *Streptomyces* populations in the face of fluctuating environmental conditions [34, 38]. This variability occurs across various spatial scales within the colony, from metabolic differences between individual cells within a hyphal filament to larger-scale variations between distinct regions of the mycelial network [39]. While such variability has posed challenges in achieving consistent secondary metabolite production, it also provides critical insights into the complex regulatory networks governing *Streptomyces* biology.

This review aims to illustrate the significant efforts by researchers to engineer *Streptomyces* for the production of secondary metabolites of interest. In doing so, these efforts have uncovered the remarkable heterogeneity within *Streptomyces* populations, which had remained underexplored due to technological constraints. We will highlight how innovations in genetic engineering, combined with advancements in single-cell technologies such as microfluidics and Raman spectroscopy, are unravelling this complexity. These technologies enable researchers to directly observe the life cycles of *Streptomyces* [40], spatially map metabolite production [41, 42], and identify high-producing strains with unprecedented resolution [43-45].

2. Key Genetic Engineering Strategies to Enhance Secondary Metabolite Production in *Streptomyces*

Genetic engineering strategies in *Streptomyces* have long combined classical regulatory manipulation with advanced synthetic biology approaches [46]. These strategies include targeting regulatory genes, manipulating the genetic makeup of the BGC itself, employing heterologous expression, and leveraging advanced genome-editing technologies such as CRISPR-Cas9. Early landmark studies established that pathway-specific activators such as ActII-ORF4 and RedD function as master regulators for the actinorhodin [47] and undecylprodigiosin [48] gene clusters. Increasing their copy number or driving their overexpression reliably activated transcription of the corresponding biosynthetic genes, demonstrating that targeting native *Streptomyces* antibiotic regulatory protein (SARP)-family regulators is an effective way to boost metabolite production [49, 50].

The rapid advancement of whole-genome sequencing technologies has further strengthened these strategies. With thousands of complete or nearly complete *Streptomyces* genomes now publicly available, researchers can comprehensively map regulatory architectures, identify cryptic or silent BGCs, and compare promoter elements and regulatory motifs across species [51-54]. This genomic richness provides a rational foundation for targeted engineering by revealing conserved regulatory logic and cluster organisation. Building on these foundations, promoter engineering has introduced more predictable and tunable control over BGC expression [55]. Strong constitutive promoters such as *kasO*p* variants [56], together with synthetic promoter libraries [57, 58], have enabled rational refactoring of operons and BGCs, leading to substantial and reproducible increases in secondary metabolite yields across multiple pathways [59].

More recent approaches have broadened the engineering toolbox and accelerated the pace of discovery, particularly through the application of clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein (Cas) system for genome editing in *Streptomyces* [24, 60]. The development of CRISPR-based systems, especially the pCRISPomyces platforms, enabled rapid and efficient genome editing in a wide range of *Streptomyces* species [61]. These tools support multiplex deletions, promoter insertions, and even modular replacement within large polyketide synthase and nonribosomal peptide synthetase clusters, greatly expanding what can be achieved in BGC refactoring [61]. Alongside these targeted methods, ribosome engineering remains a valuable strategy for activating silent gene clusters and boosting metabolite production by selecting mutants in ribosomal proteins or RNA polymerase subunits [62], without prior knowledge of specific regulators.

Finally, improvements at the host and systems level make pathway engineering more predictable [63, 64]. Engineered chassis strains such as *S. lividans* and *S. albus* provide reduced background metabolism and fewer interfering pathways, facilitating heterologous expression [65, 66]. Genome-scale metabolic models (GEMs) and algorithms now guide rational interventions to boost precursor supply and reroute central carbon flux [67]. GEMs is a metabolic engineering approach which predicts the flow of metabolic intermediates and identifies the key

genetic targets for modification, illustrating how modelling and experimentation are combined to raise yields [68-70]. Together, these advances have made the activation of silent BGCs and the scale-up of valuable secondary metabolites far more reliable and efficient.

While genetic engineering strategies have undeniably transformed the potential of *Streptomyces* for the industrial production of secondary metabolites, several challenges remain. The inherent complexity of the *Streptomyces* genome, with its vast regulatory networks and multi-layered biosynthetic pathways, often leads to unpredictable outcomes when manipulating these systems [71]. Moreover, while CRISPR-Cas9 and other advanced tools offer unprecedented precision, issues like off-target effects, genetic instability, and host toxicity still pose significant hurdles for their widespread application [72-74].

The success of these strategies often depends on the specific context, such as the nature of the BGC, the host strain, and the production scale [74]. Consequently, achieving optimal outcomes requires a deep understanding of both the genetic underpinnings of *Streptomyces* and the metabolic engineering tools available. Further advancements in tools like CRISPR-Cas9, genome-scale metabolic modelling, and combinatorial biosynthesis, combined with increased knowledge of *Streptomyces* biology, will likely continue to enhance the ability to activate silent BGCs and maximise the production of high-value secondary metabolites.

3. Multifaceted Nature of *Streptomyces* Heterogeneity

Heterogeneity in *Streptomyces* is a major factor that complicates efforts to optimise secondary metabolite production. Although genetic engineering has improved yields in many strains, the substantial phenotypic variation that arises within clonal populations remains insufficiently understood. This variation is strongly tied to the multicellular life cycle of *Streptomyces*, which begins with spore germination, followed by vegetative mycelial growth, and leads to the formation of aerial hyphae and chains of unigenomic spores [34, 75, 76]. These developmental transitions create distinct cellular states within a colony and lead to differences in growth, morphology, and metabolite secretion, even among genetically identical cells.

Because *Streptomyces* grows as an interconnected mycelial network, spatial heterogeneity naturally emerges, whereby cells in different regions of a colony may experience different nutrient levels, stresses, and signalling cues, resulting in varied gene expression and metabolic behaviour. These differences reflect coordinated developmental and ecological responses rather than random noise. As noted by Hoskisson et al. [34], such within-colony variation allows the population to respond to multiple, rapidly changing environmental inputs at the same time. In soil environments where resources and competition are patchy, this flexibility increases overall survival and fitness. A well-studied example of this functional differentiation is the division of labour created by genomic rearrangements. In *Streptomyces coelicolor*, a small subpopulation of cells (around 1–10%) undergoes major deletions or amplifications near the end of their linear chromosome, producing variants that grow poorly but overproduce antibiotics [76]. Meanwhile, the rest of the population retains normal growth and reproductive capacity. When these subpopulations coexist, the community achieves higher

antibiotic output without a net loss in overall fitness. This demonstrates a population-level strategy in which genetically distinct lineages carry out complementary roles for the benefit of the entire population.

Competitive interactions add an ecological layer to the developmental and genetic variation already present in *Streptomyces* colonies. Siderophore production is a good example; by scavenging iron for the producer, siderophores also impose iron limitation on nearby competitors. Zang et al. [77] recently showed that secretion of the siderophore coelichelin can increase *Bacillus subtilis* susceptibility to lytic phage infection, illustrating how a metabolite that mediates nutrient competition can also change competitor physiology in ways that alter community outcomes. These external cues generate heterogeneity even within a single colony. Cells at the boundary with a competitor often activate specific BGCs, secrete defensive or antagonistic metabolites, or change their developmental program, while cells deeper in the colony remain metabolically distinct. Traxler et al. [78] found that siderophore secretion by a neighbouring actinomycete triggered locally increased production of pigmented antibiotics and additional siderophores in *S. coelicolor*; this response was tightly localised to the interface between colonies and varied over time, with at least a dozen distinct acyl-dFO analogues produced sequentially as the colonies interacted. Repeated encounters with competitors can amplify these effects and select for stable specialisation. Over time, subpopulations that invest heavily in defence and metabolite secretion can emerge alongside subpopulations that prioritise growth and reproduction, reinforcing a division of labour that benefits the colony as a whole.

These layers of variation arise from dynamic genomes, complex regulation, and diverse ecological pressures. *Streptomyces* genomes undergo frequent horizontal gene transfer through integrative and conjugative elements (ICEs and AICEs) and contain BGCs positioned near chromosomal ends, where they evolve rapidly [79-81]. Regulatory networks further amplify differences in BGC expression even within clonal populations [82]. Ecologically, different species and strains inhabit soil, marine, and host-associated niches, and this diversity shapes their metabolite profiles [83-85]. Within a single colony, gradients in nutrients and signals lead to localised differences in morphology and metabolism [86-88]. For natural product discovery, these forms of heterogeneity mean that bulk assays often obscure rare high-producing cells that could otherwise yield valuable metabolites [89-91].

In short, the heterogeneity within *Streptomyces* colonies is not a random occurrence but a product of their adaptation to a resource-poor, competitive, and dynamically changing environment. The patchy availability of resources, division of labour among colony members, and the complex regulatory networks governing secondary metabolite production all contribute to this heterogeneity. These factors allow *Streptomyces* to adapt to its environment in a highly flexible and specialised manner, ensuring its survival and success in competitive soil ecosystems. As Hoskisson et al. [34] highlight, understanding the sources of this heterogeneity is crucial for future research, particularly in the fields of antibiotic discovery and the development of new biotechnological applications.

4. Unravelling the Morphological and Functional Heterogeneity in *Streptomyces* Fermentations

In recent years, significant progress has been made in understanding the inherent morphological heterogeneity of *Streptomyces* during industrial fermentations, a key factor influencing secondary metabolite production. The seminal work of van Veluw et al. [92] laid the foundation for understanding the dynamics of pellet formation in liquid cultures. Through a novel flow cytometry approach using the Complex Object Parametric Analyser and Sorter (COPAS), the authors identified two distinct subpopulations of pellets within *Streptomyces* fermentations, which include a uniform population of small mycelia and a more variable population of larger pellets. The functional significance of this morphological divergence was later uncovered through proteomic analysis, revealing that the larger pellets are enriched in stress-related and biosynthetic proteins, positioning them as specialised sites for secondary metabolite production. Conversely, the smaller pellets appear metabolically geared toward growth and replication. This work highlighted the critical role of pellet size in determining both the physiological state and the industrial potential of *Streptomyces* cultures [92].

Building on this foundational work, Zacchetti et al. [93] provided important mechanistic insights into the formation of heterogeneous pellets, demonstrating that aggregation between young germlings was mediated by specific glycans, acting as a key driver of pellet size heterogeneity. In contrast, fragmentation or the shearing off of viable mycelial parts acted as a counterbalancing force, dynamically shaping the final distribution of pellet sizes. Together, these findings establish a model where the interplay between aggregation and fragmentation creates a bimodal distribution of pellet sizes, as observed by van Veluw et al. [92]. These insights are pivotal for the rational design of *Streptomyces* morphology, opening avenues for engineering pellet formation to optimise metabolite yields.

The relationship between pellet morphology and metabolite production has been further elaborated by Wang et al. [94], who demonstrated that the optimal pellet size for secondary metabolite production is metabolite-specific. For example, smaller, denser pellets were found to enhance the production of undecylprodigiosin (Red) in *S. coelicolor*, while a different morphology was required for the production of actinorhodin (Act). Interestingly, Wang et al. [94] also showed that antibiotic treatments could directly manipulate mycelial architecture. Sub-inhibitory concentrations of thiostrepton promoted the formation of larger, more compact pellets, while spectinomycin induced the formation of smaller pellets. These findings suggest that antibiotics, traditionally considered inhibitors of growth, can also serve as chemical tools for morphologically reprogramming *Streptomyces*, providing a novel approach to optimising fermentation conditions and improving secondary metabolite production.

The role of antibiotics as signalling molecules is further exemplified by the study of jadomycin B (JdB), which acts as a ligand for the pseudo-receptor ScbR2 in *S. coelicolor* [95]. This interaction forms a feed-forward loop that regulates both morphological differentiation (via the adpA gene) and antibiotic production (via the redD gene). Notably, varying concentrations of JdB can skew the phenotypic response toward either differentiation or

enhanced antibiotic production, demonstrating that external chemical cues can finely tune the physiological state of *Streptomyces* populations. This discovery underscores the sophisticated regulatory networks that enable *Streptomyces* to adapt to environmental challenges, reinforcing the potential for engineered morphologies to optimise the production of specific metabolites.

Taken together, these studies illustrate the intricate relationship between *Streptomyces* morphology, metabolic specialisation, and secondary metabolite production. The functional division of labour observed in *Streptomyces* pellets opens new opportunities for optimising industrial fermentation processes. By leveraging these insights, researchers can not only better understand the biological underpinnings of *Streptomyces* heterogeneity but also develop more effective strategies for engineering microbial cultures with enhanced yields of valuable bioactive compounds.

5. Microfluidic Technology: A Toolkit for Deconstructing Heterogeneity

Microfluidics uses micrometre-scale channels and compartments to control fluids, cells, nutrients and gases with a precision that is impossible in conventional shake flasks or bioreactors. By reducing culture volumes to picoliters or nanoliters and running many experiments in parallel, droplet and chamber microfluidic platforms enable massively parallel experiments with very low reagent consumption and high temporal control. These platforms were first shown to enable ultrahigh-throughput screening and directed evolution by encapsulating single cells in droplets and screening millions of compartmentalised reactions [96].

Fluorescence-activated droplet sorting (FADS) is the microfluidic analogue of fluorescence-activated cell sorting (FACS). A particular advantage of FADS when compared to conventional sorting system, such as FACS, is that droplets provide a unique tool to link genotype and phenotype through compartmentalisation. Each droplet functions as an independent microreactor, allowing metabolites or reporter signals to accumulate to detectable levels even for slow-growing or low-titer producers [97]. This confinement makes it possible to measure rare phenotypes and to follow the complete life cycle of filamentous microbes inside a closed compartment [98, 99]. Moreover, encapsulated cells can be lysed for biochemical and genetic analyses. These advantages have been exploited for functional profiling of complex communities and for ultrahigh-throughput discovery of bioactive strains and activities. This distinction explains why FADS has become the preferred high-throughput method for screening filamentous producers [100, 101].

Recent studies have extended these approaches to filamentous actinomycetes, showing that droplets can support *Streptomyces* germination, vegetative growth and secondary metabolite production over several days, and can enrich hyperproducers by several hundred fold [102, 103]. The ability to cultivate productive mycelia in droplets and then interrogate and recover productive compartments also enables direct integration with downstream analytics. Lab-on-a-chip mass spectrometry (MS) workflow has been used to detect antibiotics and other secreted metabolites from individual droplets, providing direct chemical confirmation that the

productive phenotype observed by fluorescence corresponds to metabolite production [104]. These analytical integrations close the gap between phenotype and chemistry and set the stage for industry-relevant screening pipelines. Taken together, these features position microfluidics as a platform that both complements and overcomes the main limitations of FACS for *Streptomyces* screening.

5.1. The Challenge of FACS for Streptomyces analysis and sorting

FACS has long been established as an ideal ultra-high-throughput method for strain development in unicellular organisms like yeast and bacteria. Its power lies in the ability to screen and sort millions of individual cells from a liquid culture based on fluorescent markers at incredible speeds. For actinomycetes like *Streptomyces*, which are prolific producers of antibiotics and enzymes, applying FACS for reporter-guided metabolic engineering promises rapid strain improvement. However, a fundamental physical limitation has hindered its application: the filamentous, mycelial growth of *Streptomyces*. The typical mycelial fragments are far too large (260–950 µm) to pass through the standard ~70 µm nozzle of a FACS instrument [105].

Early attempts to circumvent this issue involved creating and sorting protoplasts (cells with their cell walls removed). While technically possible, this approach has not been widely adopted due to the inherent fragility of protoplasts, very low post-sorting survival rates, and the fact that protoplasts do not represent the natural, productive mycelial form of the organism. For instance, Bai et al. [106] demonstrated the different buffers used in the optimisation for maintaining the osmotic pressure and viability of *Streptomyces* protoplasts up to ~50% viability for the development of a quantitative strategy at single-cell resolution for *Streptomyces*. This significant bottleneck limited the use of FACS for direct, high-throughput screening of industrially relevant *Streptomyces* cultures. A recent work has aimed to overcome this barrier by developing novel methodologies that bypass the need for protoplasts. Akhgari et al. [105] demonstrated that the pellet of a 3-days old culture of *Streptomyces* mycelia was subjected to ultrasonication, yielding mycelia fragments that were filtered through a cell-strainer cap prior to FACS.

Despite these efforts, a core limitation persists as FACS-based screening methods involve the screening of spores, protoplasts, or small mycelial fragments, which are not the same as the mature, differentiated mycelium responsible for sustained antibiotic production in industrial fermentations. This discrepancy has led to the development of microfluidic systems capable of encapsulating and analysing the entire filamentous life cycle of *Streptomyces*, offering a more accurate representation of the conditions under which secondary metabolites of interest are produced in industrial-scale fermentations.

5.2. Applications of Microfluidics in Streptomyces and Drug Discovery Research

5.2.1. Unravelling Lifecycle Heterogeneity and Single-Cell Behaviour

Microfluidics also enables the direct observation of *Streptomyces* development from a single spore, a crucial step in understanding lifecycle heterogeneity. Chen et al. [40] designed a chip with microwells and air chambers that mimic the natural water-air interface, crucial for aerial hyphae formation. This allowed them to observe the entire lifecycle of *Streptomyces coelicolor* and *S. griseus* from a single spore to mature mycelium, revealing differentiation processes at a micron scale. This single-cell perspective is vital for understanding heterogeneity in germination and development. Similarly, Koepff et al. [107] used microfluidic cultivation to investigate *Streptomyces lividans* under controlled conditions. Contrary to expectations of high intrinsic heterogeneity, they discovered remarkable uniformity in growth behaviour when environmental conditions were constant. Their key finding was that spores undergo long metabolic adaptation phases (up to > 30 hours) when introduced to new media, rather than employing a "persister" strategy. This suggests that the often-observed batch-to-batch variability in large-scale fermentations is likely a result of environmental gradients and poor controllability, not an intrinsic stochasticity at the single-cell level under stable conditions.

5.2.2. High-Throughput Screening in Industry-Relevant Conditions

Microfluidics has revolutionised high-throughput screening for *Streptomyces* by supporting the entire lifecycle of the organism, from germination to secondary metabolite production. Droplet-based microfluidics solves the limitation of traditional approaches, which are confined to screening spores or protoplasts, which do not represent the mycelial form responsible for antibiotic production in industrial fermentations. As demonstrated by Tu et al. [45], droplets act as picoliter-to-nanoliter bioreactors that can support the *Streptomyces*'s entire life cycle, including germination, vegetative growth, and critically, secondary metabolite production over 5-7 days. The study demonstrated a much better model for the *Streptomyces* fermentation process, allowing for the screening of hyperproducers in their productive mycelial form, achieving enrichment ratios of up to 334.2. For secreted products like antibiotics, Zhang et al. [108] developed the "REPID system" using core–shell microdroplets and a split GFP system to screen for filamentous fungi with high extracellular enzyme secretion. Although the system was demonstrated with fungi, the underlying approach is highly transferable to *Streptomyces*, which shares a similar filamentous growth form. Likewise, Samlali et al. [109] specifically addressed the challenge of sorting filamentous cells after long-term incubation by developing a low-voltage system to sort nanoliter droplets containing fungi based on their secretion of cell-wall-degrading enzymes after several days of growth. Crucially, these studies demonstrated that individual droplets can be interrogated for product formation and then sorted, either with dedicated droplet sorters or by adapting flow-cytometry/FACS workflows, so that droplets containing high-producing mycelia are physically recovered for downstream characterisation.

The integration of advanced detection systems in microfluidic platforms further enhances the specificity and capability of these screening systems. Wink et al. [104] and Mahler

et al. [110] used surfactant-stabilised droplets for long-term incubation and directly coupled them to mass spectrometry (MS). Their "chip-MS" setup allows for the *in situ* tracing of secondary metabolites like streptomycin from single droplets, confirming antibiotic production from fastidious cultures and complex environmental samples. Together, the combination of in-droplet cultivation, sensitive detection, and high-speed sorting creates a realistic, industry-relevant screening pipeline that identifies productive strains in their natural, mycelial state.

5.2.3. Studying Microbial Interactions Using Microfluidics

The study of microbial interactions is a crucial area of microbiological research, as understanding these complex dynamics can unveil new strategies for infection control, antibiotic discovery, and microbial ecology. However, traditional culture-based or bulk analytical methods often fail to capture the spatial and temporal heterogeneity that governs these interactions at the microscale. Building on this need for finer resolution, Terekhov et al. [111] demonstrated that biocompatible MDE droplets can be used for in-droplet co-cultivation of different living species, enabling detailed studies of how pathogens interact with one another, or with their hosts, in highly controlled microenvironments.

By combining droplet-generating machinery with FACS, next-generation sequencing (NGS), and liquid chromatography-mass spectrometry (LC-MS/MS) analysis of droplet-derived secretomes, the MDE–FACS platform offers unprecedented insight into the genotype-phenotype interactions of co-cultured microorganisms [111]. For instance, this system allows researchers to directly observe pathogen interactions within individual droplets, which contain both a "killer" strain (such as an antibiotic-producing *Streptomyces* isolate) and a "target" pathogen (such as *Staphylococcus aureus*). By incorporating fluorescent reporters for each species, droplets can be sorted based on fluorescence signatures that indicate bacterial inhibition, growth, or metabolic activity, enabling rapid identification of inhibitory interactions in real-time [111]. This high-throughput screening system far surpasses traditional agar-based methods in terms of both speed and scalability, offering a more efficient way to identify novel antibiotic-producing strains or understand the intricacies of pathogen dynamics.

Importantly, the MDE platform is compatible with downstream molecular and chemical analyses [111]. The genomes of isolated strains can be sequenced to identify specific biosynthetic gene clusters (BGCs) responsible for the observed inhibition [112], while LC-MS/MS analysis of the droplet secretomes offers direct chemical evidence of the metabolites involved. This integrated workflow, which links genetic profiles of the bacteria to their chemical outputs, has become a transformative tool in natural product research. It allows for the precise identification of the biosynthetic pathways responsible for antimicrobial activity, which is crucial for discovering novel antibiotics or understanding how microbial communities interact within a host or the environment. For example, MDE-enabled screening can be applied to identify probiotic candidates that inhibit gut pathogens, which cause dysbiosis [113, 114], helping to rationalise the development of next-generation probiotics tailored for gastrointestinal health [115-118], improved nutrient absorption, or reduced infection rates [119]. Similarly, in animal

agriculture and aquaculture, the platform can be used to pinpoint beneficial strains that competitively suppress zoonotic pathogens, offering alternatives to antibiotic use [120, 121].

The ability to study these interactions at the level of individual droplets not only accelerates the discovery of therapeutic or probiotic candidates, while deepens our understanding of the ecological and evolutionary pressures shaping pathogen behaviour. As this technology evolves, it holds promise for advancing the study of host-pathogen interactions and microbe-microbe dynamics in both clinical and agricultural contexts that were previously unattainable with traditional microbiological methods.

6. Raman Imaging: A Lens into the Chemical Heterogeneity of *Streptomyces*

Raman imaging has emerged as a transformative tool for dissecting the chemical and metabolic heterogeneity of *Streptomyces*, addressing long-standing limitations in traditional analytical methods. Conventional metabolomic techniques often rely on destructive sampling and yield only pool-averaged measurements, obscuring the spatial and temporal dynamics that govern metabolite production within a biological sample [122, 123]. In contrast, Raman imaging, particularly single-cell Raman microspectroscopy, provides a label-free, non-destructive, and chemically specific means of probing living cells [124]. By detecting the inelastic scattering of light from molecular bonds, it generates a spectroscopic "fingerprint" that reflects the unique chemical state of each cell. This shift from bulk analyses to spatially resolved, single-cell measurements has fundamentally changed how researchers interrogate heterogeneity, enabling direct visualisation of where and when biosynthetic activity occurs within the mycelial network [124].

The power and feasibility of Raman-based analysis in *Streptomyces* were first demonstrated in one of the earliest UV resonance Raman studies, where bulk spectra classified species with >94% accuracy and single-cell spectra achieved 80–93% accuracy despite the microorganism's filamentous hyphal structure [125]. Critically, the study showed that even a single Raman spectrum can capture the complete chemical and physiological state of a *Streptomyces* hyphal cell, confirming that single-cell phenotyping is viable in these multicellular bacteria. It also revealed condition-dependent chemical heterogeneity, whereby young cultures and lipid-rich cells exhibited uniform spectra, while early lipid-producing cells displayed mixed spectral signatures, reflecting intracellular chemical variation. These foundational insights established Raman spectroscopy as a sensitive platform capable of resolving both species-level diversity and fine-scale metabolic heterogeneity.

6.1. Spatial and Temporal Mapping of Metabolite Production

Raman imaging has provided compelling evidence that secondary metabolite production in *Streptomyces* is not uniform but closely tied to morphological development, with metabolites localised in specific regions of the mycelium. One key demonstration of this is the work of Miyaoka et al. [42], who used resonance Raman spectroscopy to study *Streptomyces nodosus* producing the polyene antibiotic amphotericin B (AmB). By leveraging the resonance

Raman effect, which enhances the signal when the laser wavelength matches the molecule's electronic absorption, the researchers were able to detect AmB within the hyphae and map its heterogeneous distribution. This approach also allowed them to monitor the time-dependent accumulation of AmB alongside mycelial growth, providing insights into its production dynamics within the colony. Furthermore, the technique was sensitive enough to detect Raman spectral shifts, enabling the identification of the molecular association state of AmB *in situ* within the cellular environment. Similarly, in a more recent study, Suwa et al. [126] proposed a newer method that directly analyses the secondary metabolite production within *Streptomyces* colonies on agar dishes using multivariate Raman spectral analysis with machine learning algorithms.

Raman imaging has also revealed the dynamic nature of metabolite localisation in *Streptomyces avermitilis*, where the distribution of the anthelmintic agent avermectin varies based on both developmental stage and culture conditions [127]. In solid cultures, avermectin was primarily localised in the later-stage, spore-bearing mycelium and spiral spore chains, but not in the early vegetative mycelium. In contrast, when cultured in liquid, avermectin accumulated within mycelial pellets formed at an earlier morphological stage (MII). This research indicated that morphological differentiation alone is not an accurate predictor of metabolic activity, as the chemical profiles of mycelia at the same stage (MII) varied considerably between solid and liquid cultures [127].

The integration of Raman imaging into *Streptomyces* research marks a paradigm shift from a bulk, averaged perspective to a single-cell resolution, spatially resolved understanding of metabolic heterogeneity. The ability to track metabolite production *in situ*, label-free, and dynamically from a growing colony is unparalleled [126]. By linking specific chemical profiles to morphological features, Raman imaging provides insights into the complex regulatory network that governs the activation of biosynthetic gene clusters. The findings from these studies suggest that heterogeneity is not merely random variation but a structured, ecologically relevant phenomenon. The observed spatial segregation of metabolites like amphotericin B and avermectin points to functional compartmentalisation within the *Streptomyces* colony [127], which could be crucial for optimising production or mitigating self-toxicity.

For industrial applications, the ability to rapidly screen high-producing strains or non-destructively monitor fermentation processes in real time could lead to substantial gains in yield and efficiency [128]. Evidence from studies in other microorganisms has already shown that integrating microfluidic platforms with Raman-based single-cell analysis enables high-throughput trapping, cultivation, and phenotyping at unprecedented resolution [129, 130]. Although there are only limited studies that combine a microfluidic system with Raman spectroscopy, these successes highlight a clear opportunity for *Streptomyces* research. Future work should focus on adapting and optimising such integrated microfluidic-Raman workflows for filamentous bacteria, where controlled microscale environments could stabilise growth, isolate distinct morphological states, and enable systematic interrogation of metabolic heterogeneity. Developing these combinatorial platforms would not only advance natural

product discovery but also create a powerful screening pipeline for strain improvement and industrial process optimisation.

7. Conclusions

Understanding and harnessing *Streptomyces* heterogeneity has become central to improving the discovery and production of bioactive compounds. Microfluidic technologies have shifted the field from a bulk, population-averaged perspective to a high-resolution, single-cell analysis that can track cellular behaviours and secondary metabolite production with unprecedented detail. These advancements are enabling more accurate strain selection, revealing the regulatory networks that govern biosynthetic gene cluster activation, and providing powerful new tools for metabolic engineering. The future of *Streptomyces* research lies in combining these advanced microfluidic techniques with high-resolution genomics, metabolomics, and AI-driven optimisation. By integrating Raman imaging with genome mining, mutagenesis, and other analytics, we can accelerate natural product discovery, refine strain optimisation processes, and push the boundaries of industrial biotechnological applications. As these technologies converge, they will not only deepen our understanding of *Streptomyces* biology but also transform how we approach natural product production, bringing us closer to harnessing the full potential of these remarkable microorganisms.

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