Chapter

Mycelium Differentiation and Development of *Streptomyces* in Liquid Nonsporulating Cultures: Programmed Cell Death, Differentiation, and Lysis Condition Secondary Metabolite Production

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Abstract

Streptomycetes are mycelium-forming sporulating bacteria that produce two thirds of clinically relevant secondary metabolites. Secondary metabolite production is activated at specific developmental stages of *Streptomyces* life cycle. Despite this, *Streptomyces* differentiation in liquid nonsporulating cultures (flasks and industrial bioreactors) tends to be underestimated and the most important parameters managed are only indirectly related to differentiation: modifications to the culture media, optimization of productive strains by random or directed mutagenesis, analysis of biophysical parameters, etc. In this chapter, we review the relationship between differentiation and antibiotic production in liquid cultures. Morphological differentiation in liquid cultures is comparable to that occurring during presporulation stages in solid cultures: an initial compartmentalized mycelium suffers a programmed cell death, and remaining viable segments then differentiate to a second multinucleated antibiotic-producing mycelium. Differentiation is one of the keys to interpreting biophysical fermentation parameters and to rationalizing the optimization of secondary metabolite production in liquid cultures.

Keywords: *Streptomyces*, bioreactor, differentiation, antibiotics, programmed cell death

1. Introduction

Streptomycetes are gram-positive, environmental soil bacteria that play important roles in the mineralization of organic matter. *Streptomyces* is extremely important in biotechnology, given that approximately two thirds of all clinical antibiotics and several other bioactive compounds are synthesized by members of this genus [1].

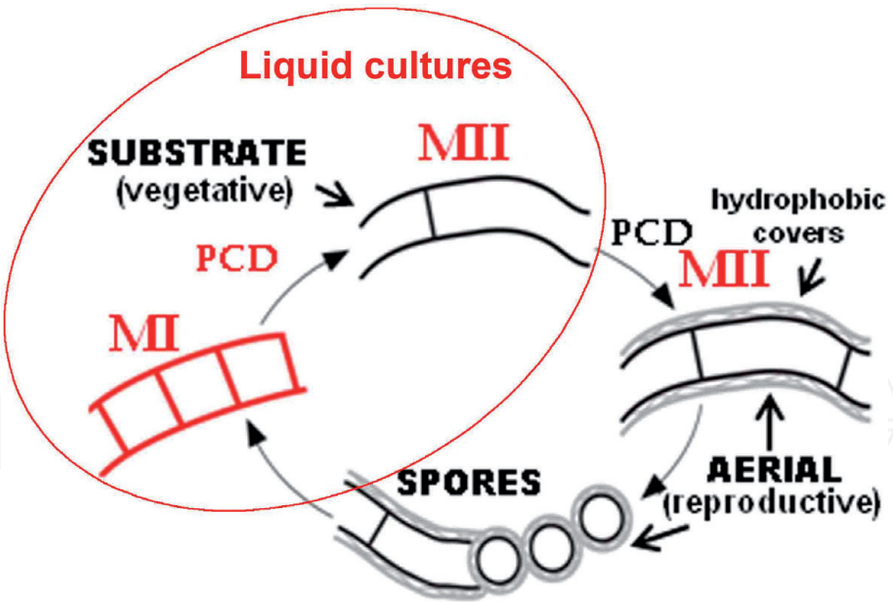

Figure 1.
Streptomyces developmental cycle. New developmental stages are highlighted in red. MI, first compartmentalized mycelium, vegetative; MII, second multinucleated mycelium producing secondary metabolites. The full cycle is developed in solid cultures. Liquid cultures do not develop aerial mycelium or spores.

Streptomycetes have a complex developmental cycle that makes this bacterium a multicellular prokaryotic model. The classical *Streptomyces* developmental cycle defined 50 years ago by Waskman [2] and Wildermuth [3] in laboratory solid cultures focused on sporulation (**Figure 1**): after spore germination, hypha grow inside the culture (substrate mycelium); the substrate mycelium differentiates into a new mycelium that begins to express hydrophobic surfaces and grows into the air (aerial mycelium); the substrate and aerial mycelia are multinucleated with sporadic septa; both mycelia eventually undergo a programmed cell death (PCD), and the remaining viable hyphae differentiate into chains of unigenomic spores. Most streptomycetes do not sporulate in liquid cultures, and it was traditionally thought that there was no differentiation in these conditions. Despite this, most processes for secondary metabolite production are performed in liquid (flask or bioreactor). It was postulated that secondary metabolites would be produced in liquid cultures by the substrate mycelium at the stationary stage [4–8].

The main objective of this work is to review the state of the art of *Streptomyces* differentiation in liquid cultures, especially in lab-scale bioreactors, defining the kind of differentiation present under these conditions, how differentiation, fermentation parameters (dissolved oxygen tension, oxygen uptake rate, oxygen transfer rate, pH, temperature, agitation, culture medium, etc.) and secondary metabolite production are correlated, and describing a general model applicable to improving secondary metabolite production in *Streptomyces* industrial fermentations.

2. *Streptomyces* development in liquid cultures

During the last decade, new knowledge regarding *Streptomyces* development during the early differentiation stages, those occurring between spore germination and substrate mycelium differentiation, was generated (red labels in **Figure 1**): a previously unidentified, young, compartmentalized mycelium (MI) undergoes a PCD; and viable MI segments differentiate into a multinucleated mycelium with sporadic septa (MII). One-micron spaced MI compartments are separated by

cross-membranes without peptidoglycan cell walls, a kind of cell division unprecedented in bacteria [9]. MII corresponds to the substrate mycelium in early development, and to the aerial mycelium once it starts to express hydrophobic surfaces. In liquid cultures, there is no hydrophobic surface formation or sporulation, but there is a compartmentalized MI, which differentiates into a multinucleated MII after PCD. MII produces secondary metabolites in solid and liquid cultures. This was the first time that secondary metabolism was associated with a specific mycelial stage (MII) [10]. Transcriptomic [11] and proteomic [12, 13] analyses performed by our group corroborated that MI is the vegetative mycelium expressing/translating primary metabolism genes/proteins, while MII is the reproductive mycelium expressing/translating secondary metabolism and sporulation genes/proteins.

Microbial multinucleated structures such as the substrate and aerial mycelia are fragile, uncommon, and usually related to transitory reproductive stages [14]. Contrary to what was postulated during the last 50 years, *Streptomyces* is not an exception, and the compartmentalized MI is the predominant mycelium in cultures resembling natural conditions, such as nonamended soils [15]. The MI lifespan is very short in laboratory cultures, which is why it was ignored in most *Streptomyces* works [15].

3. *Streptomyces* differentiation and industrial fermentations

The absence of an understanding of *Streptomyces* differentiation in liquid cultures has long precluded the existence of a general consensus as to how morphological and biophysical parameters correlate with secondary metabolite production. Pharmaceutical companies have addressed the optimization of industrial fermentation empirically for each strain and compound. For example, pellet and clump formation has been described as essential for obtaining good production of retamycin or nikkomycin [16], but in the case of virginiamycin, there is no relationship between morphology and secondary metabolite production [17]; high dissolved oxygen tensions (DOT) have been reported as being necessary for the production of vancomycin [18], but not for the production of erythromycin [19], just to name a few examples.

Only recently has basic knowledge about differentiation in liquid cultures been generated. As introduced above, MII was demonstrated to be the antibiotic-producing mycelium. In a recent work, our group demonstrated that differentiation is one of the keys to interpreting typical fermentation parameters (growth, antibiotic production, dissolved oxygen tension, agitation, and oxygen uptake rates) [20] in bioreactors. Pellet and clump formation greatly influences PCD, usually occurring in the center of the mycelium pellet [10] and MII differentiation from MI living cells at the pellet periphery [10]. We proposed a general consensus to improve secondary metabolite production in *S. coelicolor*: optimization of the differentiation of the antibiotic-producing mycelium (MII) [20]. In the past few years, other groups have contributed to identifying some genetic determinants controlling pellet and clump formation [21, 22].

3.1 Differentiation of *Streptomyces* in bioreactors

There are important differences between liquid cultures in laboratory flasks and bioreactors. Despite the obvious hydrodynamic differences between the two systems, there are also differences in the culture media. In this sense, one of the most important differences between flasks and bioreactors is the use of antifoams. Antifoams are often used in bioreactors to prevent foam formation and its interference with the bioreactor probes [23]. Apparently, antifoams do not affect

development and they are usually added automatically in small amounts when foam is detected by a specific probe, and in some cases, they are added directly to the culture **medium** at concentrations up to 0.1% [23]. Rioseras et al. [20] analyzed for the first time *Streptomyces* differentiation (MI and MII differentiation) in bioreactors and demonstrated an important effect of the antifoam in macroscopic morphology of the cultures (pellet and clump formation), which conditions mycelium differentiation and secondary metabolite production.

3.1.1 Differentiation of S. coelicolor in antifoam-free media

Mycelium differentiation in bioreactors is comparable to differentiation in laboratory flasks [20] (outlined in **Figure 2**): at early time points, hyphae presented the regular discontinuities and gaps previously described for MI hyphae [10]; MI differentiates into a second multinucleated mycelium (MII) after a programmed cell death (reviewed in Yagüe et al. [24]). However, there are important differences between development in flask and bioreactor.

One of the most important differences observed in the bioreactor with respect to laboratory flasks for *S. coelicolor* [20] is the existence of massive fragmentation and disintegration of mycelial pellets at around 50 hours of fermentation. This massive disintegration is observed macroscopically, in the form of the apparent clarification of the culture medium, and correlates with a sudden fall in intracellular protein levels. This kind of mycelial disintegration has been previously described as "massive lysis" in several *Streptomyces* fermentations, such as *S. clavuligerus* (reducing mycelium by more than 30%) [25], *Streptomyces* spp. [26], *Streptomyces albulus* [27], or *S. coelicolor* [28], to name just a few examples. This "massive lysis" differs from the "fragmentation of the mycelial clumps" described in some cases [29], flasks and bioreactors, which basically consists of the fragmentation of large clumps into small clumps, but without the early massive hyphal lysis reported in several bioreactor

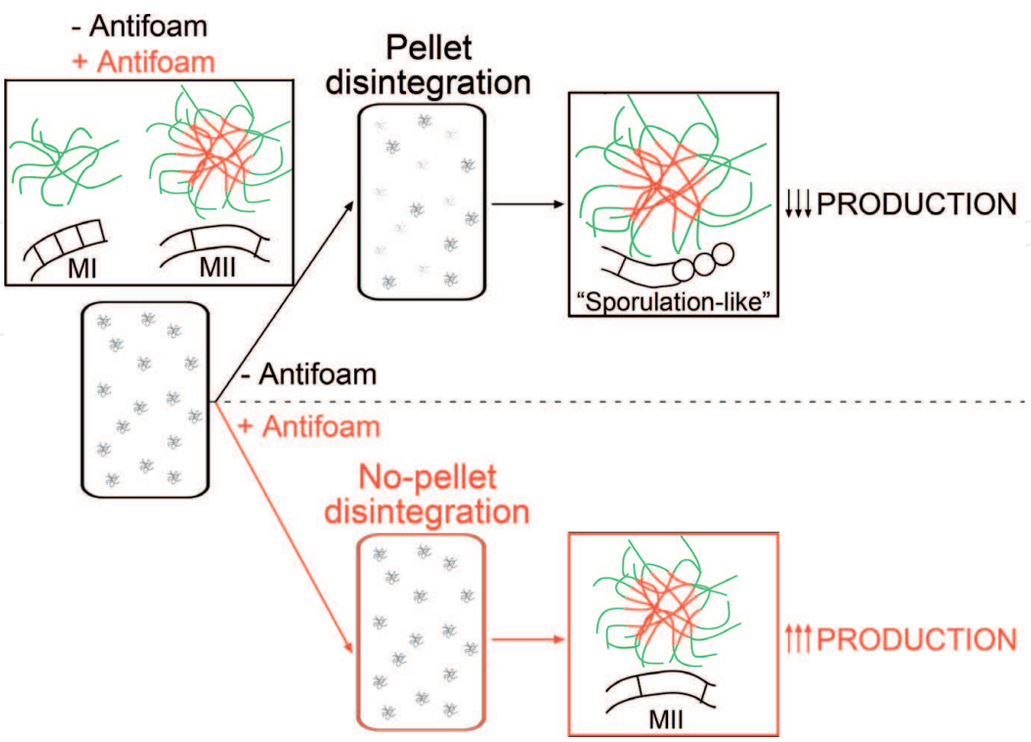

Figure 2.
Scheme illustrating S. coelicolor differentiation in bioreactors in media with or without antifoam. Red corresponds to dying hyphae (PI staining) and green to viable hyphae (SYTO9 staining). The optimal fermentation workflow is highlighted in red.

fermentations [20, 25–28]. The reason why this phenomenon occurred in some streptomycetes and not in others remains unknown. Understanding and controlling mycelial lysis would be essential to optimize fermentations. In *S. coelicolor* growing in laboratory flasks containing the same media used by Rioseras et al. [20] in bioreactors, this massive lysis does not occur [10]. Hence, Rioseras et al. [20] proposed that massive pellet disintegration depends on the hydrodynamics of the bioreactor combined with the tendency of *S. coelicolor* to form large pellets. Further work will be necessary to define if the same is happening in the other streptomycetes suffering the same phenomenon in bioreactor cultures.

MII differentiation and antibiotic production is accelerated in the *S. coelicolor* bioreactor cultures, peaking at 100–140 hours in laboratory flasks vs. 50 hours in the bioreactor [20]. However, antibiotic biosynthesis was halted after pellet disintegration, with maximum undecylprodigiosin and actinorhodin production levels lower in the bioreactor than in laboratory flasks [20].

Biophysical fermentation parameters, such as dissolved oxygen tension (DOT), agitation, and oxygen uptake rates (OURs), correlated well with differentiation [20]: DOT falls from saturation at time zero to the fixed level (50% saturation), due to hyphal growth and respiration; there is a concomitant increase in agitation to maintain oxygen levels at the fixed level; once pellet disintegration starts, biological oxygen consumption and agitation decrease gradually, and dissolved oxygen levels increase suddenly to saturation. OUR values fall during the MI PCD and are not recovered until the MII differentiation. This description of mycelium differentiation and OUR in bioreactors constitutes an elegant example of the importance of understanding *Streptomyces* differentiation, to interpret classical biophysical fermentation parameters in the model strain *S. coelicolor* and conceivably in other industrial relevant streptomycetes. Information concerning oxygen uptake kinetics of *Streptomyces* cultures is scarce despite their industrial importance. OUR values vary widely between strains, from 2.88 mg O_2 g cell^{-1} h^{-1} in *S. lividans* [30] to 320 mg O_2 g cell^{-1} h^{-1} in *S. clavuligerus* [31]. The meaning of these differences is difficult to interpret due to the absence of any indication as to mycelium differentiation/PCD in most of these works. An analysis of hyphae differentiation, development, and PCD would be essential to address these differences in OURs between different *Streptomyces* strains and culture conditions.

3.1.2 Differentiation of S. coelicolor in bioreactors supplemented with antifoam

Rioseras et al. [20] modified growing conditions in bioreactors to prevent the early massive lysis described above. The most obvious difference between bioreactors and laboratory flasks is the impellers used for agitation in the case of the bioreactor, so the first experimental approach to trying to prevent lysis was to reduce agitation to minimum levels (50 rpm); however, the same extension of pellet disintegration was observed [20]. Similar results were observed at different agitation rates (50, 100, 200, or 300 rpm) or by replacing Rushton impellers by a gentle impeller (pitched blade impellers) [20]. The only modification that worked to avoid the massive mycelial lysis observed in bioreactors was the modification of the culture medium's rheology reducing surface tension by means of an antifoam agent (Biospumex 153 K, BASF). This effect of preventing early fragmentation/lysis was only observed at relatively high antifoam concentrations (1%) (outlined in **Figure 2**).

The reason why antifoam prevents pellet disintegration is as yet unknown. However, the antifoam tended to coat the mycelial pellets, and the hydrophobic forces generated may have prevented this phenomenon. Antifoams are often used with *Streptomyces coelicolor* [23] as well as other *Streptomyces* fermentations to prevent foam formation, or even, in some cases to be used as carbon sources [32].

They are usually added automatically in small amounts when foam is detected by a specific probe, and in some cases, they are added directly to the culture medium at concentrations up to 0.1% [23]. However, Rioseras et al. [20] were the first authors who demonstrated that antifoams added to the culture media at relatively high concentrations prevent mycelium lysis, a fact that might be useful for preventing lysis in other industrial streptomycetes.

Biophysical fermentation parameters also correlated well with differentiation in cultures with antifoam: the absence of pellet disintegration prolonged the oxygen consumption phase, generating two peaks of OUR (MI and MII stages) separated by a stage of low oxygen consumption [20]. These two maxima in OUR are very unusual in industrial fermentation, and are another nice illustration of the necessity of understanding *Streptomyces* differentiation in order to interpret fermentation parameters in *Streptomyces* fermentations. As in the case of fermentations without antifoam, oxygen levels did not limit growth.

3.2 Sporulation of *Streptomyces* in bioreactors

Another important difference between bioreactor- and laboratory flask-S. *coelicolor* cultures is the existence of a sporulation-like process, affecting some 5% of hyphae in bioreactors [20]. Two of the most important features of sporulation, division, and separation of nucleoids, and the physical strangulation of hypha forming chains of individual round segments, were observed in bioreactors [20]. Sporulation in *S. coelicolor* liquid cultures is very unusual and has only been reported once before in laboratory flasks suffering nutritional downshifts [33]. The differentiation signals activating sporulation in the bioreactors remain unknown. However, if it is considered that sporulation is triggered by environmental/biological stresses [34], the high growth rates achieved in the bioreactors together with pellet disintegration might approach the development occurring in stressed solid sporulating cultures. In the absence of pellet disintegration, putative differentiation diffusible signals [35, 36] generated by stressed cells suffering from PCD [24] would be hidden in the centers of the pellets [20].

4. *Streptomyces* differentiation and screening for new bioactive compounds

Streptomycetes are important biotechnological bacteria from which two thirds of the bioactive secondary metabolites used clinically (mainly antibiotics, but also antitumourals, immunosuppressors, etc.) were discovered [37]. Drug discovery became challenging once the most common antibiotics were discovered. In fact, during the past 30 years, only three new classes of antibiotics have been brought to the clinic (mutilins, lipopeptides, and oxazolidinones) [38, 39]. At the same time, microbial resistance to existing antibiotics has increased dramatically, rendering some microbial infections extremely hard to treat.

New antibiotics are urgently needed in the clinic. No valid alternatives to screening natural strains have emerged to find new scaffolds and families of antibiotics [40]. New workflows are needed to access the natural secondary metabolites that remain inaccessible in the laboratory [41]. Nonnatural synthetic antibiotics obtained by chemical/combinatorial biosynthesis exist, but most of them are variations of natural molecules [40]. The best way to find structurally novel bioactive compounds is to resume screening from natural streptomycetes. The most obvious approach to look for new bioactive compounds from natural streptomycetes is to study streptomycetes isolated from relatively lowly explored niches such as marine

ecosystems [42], symbiotic streptomycetes [43], etc., an approach followed by several research groups and biotechnology companies. On the other hand, genomic analyses revealed that Streptomyces genomes encode an average of 30 secondary metabolite pathways [44], but only a fraction of these pathways (around four per strain [43]) is active in laboratory cultures. Consequently, there is a huge amount of potentially bioactive compounds produced by streptomycetes that are never observed in the lab (cryptic pathways) and remain unexplored. There is a consensus in the scientific community about the necessity to activate the expression of these cryptic pathways in order to overcome the present bottleneck in drug discovery.

Several research groups and biotechnology companies face the challenge of activating cryptic pathways to try to mimic the ecological niche of the bacteria by making co-cultures of different microbes [45], looking for elicitor activating pathways (nutrients such as glucose, xylose, and small molecules such as GlcNac and phosphate) [46] or making heterologous expression [47]. As stated above, *Streptomyces* differentiation conditions secondary metabolism [20], and differentiation can be one of the keys to activate cryptic pathways by modulating the differentiation of the antibiotic producer mycelium (MII). The "MII approach" will be useful to activate secondary metabolism production in strains discarded as producers in the classical screening campaigns, strains that did not produce in the lab because they did not reach the MII stage, or because they sporulated in liquid cultures (during sporulation metabolism is stopped), i.e. false negatives [48]. This approach was already useful to enhance flavonoid production in *S. albus* [49], as well as microbial transglutaminase in *S. mobaraensis* [50].

5. Conclusions

Different streptomycetes show different behaviors in liquid cultures: some species form large pellets, such as *S. coelicolor*, others grow more dispersed, as for instance *S. clavuligerus* [25], while some species such as *S. griseus* or *S. venezuelae* sporulate in liquid cultures [51, 52]. As a consequence, the effect of fermentation parameter modifications in different species cannot be easily predicted. The "MII approach" described here, i.e. optimization of antibiotic-producing mycelium differentiation, prevention of sporulation, might be applied to rationalizing the biological effects of classical biophysical fermentation parameters, and to facilitating the optimization of secondary metabolite production in industrial streptomycetes. In addition, preventing early massive pellet fragmentation/lysis by adding antifoam directly to the culture medium at relatively high concentrations is novel and may be useful for preventing lysis in other industrial streptomycetes. The "MII approach" also has applications in the screening for new secondary metabolites, allowing the activation of cryptic pathways in streptomycetes that did not reach the MII stage in the lab.

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Conflict of interest

There is no conflict of interest in this work.

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