Bioresource Technology 151 (2014) 191–198

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

# Mycelium differentiation and development of *Streptomyces coelicolor* in lab-scale bioreactors: Programmed cell death, differentiation, and lysis are closely linked to undecylprodigiosin and actinorhodin production

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## HIGHLIGHTS

- Differentiation in bioreactors is comparable to solid sporulating cultures.
- Differentiation is linked to antibiotic production.
- Differentiation is one of the keys to interpreting fermentation parameters.
- General consensus: differentiation of the antibiotic-producing mycelium (MII).
- Antifoams can prevent massive pellet fragmentation/lysis.

## ARTICLE INFO

*Article history:*
Received 26 August 2013
Received in revised form 18 October 2013
Accepted 21 October 2013
Available online 30 October 2013

*Keywords:*
*Streptomyces*
Bioreactor
Differentiation
Antibiotics
Programmed cell death

## ABSTRACT

Streptomycetes are mycelium-forming 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 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 work the relationship between differentiation and antibiotic production in lab-scale bioreactors was defined. *Streptomyces coelicolor* was used as a model strain. Morphological differentiation was comparable to that occurring during pre-sporulation 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 was demonstrated to be one of the keys to interpreting biophysical fermentation parameters and to rationalizing the optimization of secondary metabolite production in bioreactors.



## 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 (Ruiz et al., 2010).

Streptomycetes are mycelial microorganisms with complex developmental cycles that include programmed cell death (PCD) and sporulation (reviewed in Claessen et al. (2006) and Yagüe et al. (2013)). In solid sporulating cultures, a compartmentalized mycelium (MI) initiates development. MI compartments are separated by septa formed by membranes which generally do not display thick cell walls (reviewed in Yagüe et al. (2013)). A fraction of MI cells undergo a highly ordered programmed cell death (PCD) (Yagüe et al., 2013), and remaining viable cells differentiate to a multinucleated mycelium that has only sporadic septa (MII). MII gradually begins to express the chaplin and rodlin proteins that assemble into the rodlet layer that, in turn, provides the surface hydrophobicity necessary to grow into the air (aerial mycelium) (reviewed in Claessen et al. (2006)). At the end of the cycle, hypha septation and sporulation take place. MI fulfills the vegetative role in *Streptomyces* and MII constitutes the reproductive stage that is destined to sporulate and also produces secondary metabolites (Yagüe et al., 2013). In previous works, it was reported that differentiation in non-sporulating liquid cultures (laboratory

*Abbreviations:* PCD, programmed cell death; MI, first compartmentalized mycelium; MII, second multinucleated mycelium; PI, propidium iodide; DOT, dissolved oxygen tension; OTR, oxygen transfer rates; OUR, oxygen uptake rate; PMT, photomultiplier tube.

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http://dx.doi.org/10.1016/j.biortech.2013.10.068

flasks) was similar to that occurring during the pre-sporulation stages in solid cultures (reviewed in Yagüe et al. (2013)): an initial compartmentalized mycelium (MI) undergoes PCD and the remaining viable segments of this mycelium differentiate to a multinucleated mycelium (MII), i.e. the antibiotic-producing mycelium (Yagüe et al., 2013).

Most processes for secondary metabolite production are performed in bioreactors. Nevertheless, *Streptomyces* differentiation under these conditions has barely been studied, mainly due to the fact that most *Streptomyces* strains do not sporulate under these conditions. *Streptomyces* fermentation analysis and optimization has mainly been empirical and focused on the analysis of biophysical parameters, such as mycelial grouping (pellets, clumps), media composition, oxygenation, pH, agitation, temperature and, of course, levels of secondary metabolite production. Several studies have tested the optimal composition of culture media (Wentzel et al., 2012), analyzing the kind of hyphae grouping that is best suited for secondary metabolite production (dispersed hyphae vs. clumps or pellets) (van Veluw et al., 2012; van Wezel et al., 2006), analyzing the effects of bioreactor hydrodynamics on the physiology of *Streptomyces* (reviewed in Olmos et al. (2013)), or optimizing productive strains by random or directed mutagenesis (van Wezel et al., 2006). However, the complex development of *Streptomyces* under these conditions has not been fully understood and, as a direct consequence, there is no general consensus as to how morphology and other biophysical parameters correlate with secondary metabolite production. Fermentation parameters need to be optimized 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 (Pamboukian and Facciotti, 2004), but in the case of virginiamycin, there is no relationship between morphology and secondary metabolite production (Yang et al., 1996); high dissolved oxygen tensions (DOT) have been reported as necessary for the production of vancomycin (Dunstan et al., 2000), but not for the production of erythromycin (Clark et al., 1995), just to name a few examples.

The main objective of this work is to extend understanding of *Streptomyces* differentiation to lab-scale bioreactors, defining the kind of differentiation present under these conditions, how differentiation, fermentation parameters and secondary metabolite production are correlated, and describing a general model applicable to improving secondary metabolite production in *Streptomyces* industrial fermentations. *Streptomyces coelicolor* is one of the best-characterized *Streptomyces* strains (Chater, 2001). It produces various secondary metabolites, including two well-characterized antibiotics: undecylprodigiosin and actinorhodin. In order to facilitate comparisons with differentiation and development in bioreactors and other developmental conditions (solid cultures and laboratory flasks), *S. coelicolor* was used in this work as a model strain.

## 2. Methods

### 2.1. Strains, media, and culture conditions

*S. coelicolor* M145 was the strain used in this work. Cultures were performed in R5A sucrose-free liquid media (Fernandez et al., 1998). This culture medium contains MOPS buffer in sufficient concentration (100 mM) to maintain pH stable during cultivations. Laboratory flasks of 500 ml were filled with 100 ml of culture medium and incubated at 200 rpm and 30 °C. Bioreactor cultivations were performed in a 2-L bioreactor (Bio-Flo 110, New Brunswick Scientific, NJ, USA) equipped with a pH meter (Mettler Toledo, Switzerland), a polarographic dissolved oxygen electrode (InPro 6830, Mettler Toledo, Switzerland), and rushton impellers. As described above, the effect of pitched blade impellers in fermentations was also tested (data not shown). An initial working volume of 1.3 L at 30 °C and aeration of 1 L/min were used. Dissolved oxygen tension was set to a minimum of 3.8 mg/L (50% saturation), using an agitation interval of between 300 and 800 rpm, and pH was set at 6.8 using a computer-controlled peristaltic pump via automatic addition of 2 M KOH and 1 M HCl.

Flasks and bioreactors were inoculated directly with freshly prepared spores at $1 \times 10^7$ ("dense cultures") or $1 \times 10^5$ ("diluted cultures") spores/ml. Where indicated, culture medium was supplemented with antifoam (Biospumex 153K, BASF) to a final concentration of 1%. The effect of the antifoam in preventing early massive fragmentation/lysis was not so evident at lower concentrations (data not shown). More than 5 biological replicates were performed for each culture, and monitored morphologically and biochemically. However, extensive quantitative measurements were performed in only two of these biological replicates, and the quantitative data presented in the figures of this work correspond to the average ± SD of these two independent fermentations (biological replicates).

### 2.2. S. coelicolor repeated batch cultivations

"Dense cultures" ($10^7$ spores/ml), growing in R5A sucrose-free medium amended with antifoam (1% of Biospumex 153K, BASF), and using the growth parameters indicated above were grown in the bioreactor for 66 h. After that time-point, the full bioreactor contents were extracted into a 2-L sterile bottle using a peristaltic pump connected to the inoculation port. Mycelial pellets were allowed to sediment in a static state for 5 min, after which supernatant was removed under sterile conditions. The volume of medium extracted was replaced by the same volume of fresh, sterile, R5A medium amended with antifoam, and the whole culture was reintroduced into the bioreactor using again a peristaltic pump connected to the inoculation port.

### 2.3. Determination of the oxygen uptake rate (OUR) and oxygen transfer rate (OTR)

OUR was obtained from the slope of the plot of dissolved oxygen concentration over time following a momentary interruption of the air supply to the bioreactor. OTR was estimated according to the slope of dissolved oxygen recovery after aeration (fixed value of 1 L/min) and agitation (interval of between 300 and 800 rpm) was restored to the values present at the time at which the air supply was interrupted. OUR and OTR values were only estimated at developmental time points at which DOT values were less than 7 mg/L (90% saturation).

### 2.4. Streptomyces sampling throughout the differentiation cycle

Samples of *S. coelicolor* obtained from liquid cultures were centrifuged (7740*g*, 10 min at 4 °C). Supernatants of the culture medium were used to estimate extracellular proteins. Cellular extracts were obtained as follows: the mycelium pellets were resuspended in a known volume of buffer A (Tris–HCl 20 mM, pH 8, EDTA 1 mM, β-mercaptoethanol 7 mM, and complete EDTA-free Protease Inhibitor Cocktail Tablets from Roche) and ruptured using Fast-Prep (MP™ Biomedicals) with ⩽106 μm beads (Sigma, G8893500G) and three 20-s force 6.5 cycles, with 1 min on ice between each run. Finally, samples were centrifuged at 7740*g* in an Eppendorf microcentrifuge for 15 min at 4 °C; the resulting supernatant fraction was used as the cellular fraction.