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Cell immobilization of *Streptomyces coelicolor*: effect on differentiation and actinorhodin production

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Summary. Streptomycetes are mycelium-forming bacteria that produce two thirds of the clinically relevant secondary metabolites. Despite the fact that secondary metabolite production is activated at specific developmental stages of the *Streptomyces* spp. life cycle, different streptomycetes show different behaviors, and fermentation conditions need to be optimized for each specific strain and secondary metabolite. Cell-encapsulation constitutes an interesting alternative to classical fermentations, which was demonstrated to be useful in *Streptomyces,* but development under these conditions remained unexplored. In this work, the influence of cell-encapsulation in hyphae differentiation and actinorhodin production was explored in the model *Streptomyces coelicolor* strain. Encapsulation led to a delay in growth and to a reduction of mycelium density and cell death. The high proportion of viable hyphae duplicated extracellular actinorhodin production in the encapsulated cultures with respect to the non-encapsulated ones. [**Int Microbiol** 2014; 17(2):75-80]

Keywords: *Streptomyces coelicolor* · encapsulation · differentiation · antibiotics · cell death

Introduction

Streptomycetes are Gram-positive, environmental soil bacteria that play important roles in the mineralization of organic matter. *Streptomyces* species are extremely important in biotechnology, given that approximately two thirds of all clinical antibiotics and several bioactive compounds are synthesized by members of this genus [25]. Streptomycetes are mycelial microorganisms with complex developmental cycles that include programmed cell death and sporulation [7,32–34]. A young, compartmentalized vegetative mycelium (MI) differentiates into a multinucleated mycelium (MII); MII corresponds to the differentiated stage expressing genes and proteins related to secondary metabolism and sporulation [7,32–34].

Most processes for secondary metabolite production are performed in liquid cultures, conditions in which most streptomycetes do not sporulate, but in which differentiation remains fundamental for secondary metabolite production [19,24]. Different streptomycetes have different behaviors in liquid cultures. Some of them, such as *S. coelicolor* or *S. griseus,* form large pellets and clumps [13], while others, such as *S. clavuligerus,* grow in dispersion [23], and some species such as *S. venezuelae* [11], can even sporulate. Fermentation conditions need to be optimized for each specific strain and secondary metabolite, and alternatives to the classical fermentations in liquid and solid cultures are highly demanded

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in industry in order to improve the production of secondary metabolites produced in small amounts, or to activate the production of cryptic clusters [3,9,17,20,26].

Cell-encapsulation constitutes one fermentation alternative which was demonstrated to be useful in bacterial and fungal fermentations [22,30], but whose potential applications have only just begun to be explored in *Streptomyces*. Cell-immobilization was demonstrated to be useful in the production of cephamycin C [8] or clavulanic acid [14,27] by *S. clavuligerus*; neomycin by *S. marinensis* [1,28], or *S. fradiae* [16]; and α-galactosidase by *S. griseoloalbus* [2]; to name just a few examples. However, the influence of encapsulation in *Streptomyces* hyphae differentiation has remained unexplored.

The main objective of this work was to analyze *Streptomyces* differentiation growing into alginate capsules, focusing on their potential industrial applications. *Streptomyces coelicolor*, one of the best-characterized *Streptomyces* strains [6], was used as a model. *S. coelicolor* produces various secondary metabolites, including undecylprodigiosines and actinorhodines, two colored antibiotics (red and blue respectively) whose production can be easily visualized [5]. In addition, γ-actinorhodin is secreted to the extracellular medium [5] and can be easily recovered from the extracellular medium between the mycelial capsules.

Materials and methods

Strain, media and pre-culture conditions. The strain used in this work was *S. coelicolor* M145. Spores were harvested from 7 days SFM plates [12] grown at 30 ºC, and stored in 30 % glycerol for long-term preservation at –80 ºC. Pre-cultures for *Streptomyces* immobilization were performed in liquid medium as previously reported [19]: freshly prepared spores were used to inoculate 100 ml sucrose-free R5A medium [10] at a final concentration of 10^7 spores/ml. Flasks were incubated at 30 ºC and 200 rpm for 72 h.

***Streptomyces* immobilization in sodium alginate.** Sodium alginate (Sigma, A0682) was prepared in sucrose-free R5A medium to a final concentration of 2 %. The solution was heated at 45 ºC under agitation, until alginate was completely dissolved. The alginate solution could not be autoclaved [15], but was boiled for 5 min which was enough to allow sterilization. Alginate stock solution was cooled to room temperature before use.

All of the encapsulation steps were performed at room temperature under sterile conditions. With the exception of the alginate solution (see above), all of the solutions and pumping tubes were autoclaved. Mycelium from 100 ml of the pre-culture (72 h, inoculated with spores) was harvested by centrifugation (7900 ×*g*), washed with fresh sucrose-free R5A medium, and resuspended in 10 ml of sucrose-free R5A, which were further diluted with other 90 ml of sucrose-free R5A-2 % alginate (final volume of 100 ml, alginate concentration of 1.8 %). This mixture was pumped using a peristaltic pump through six hypodermic syringes with 0.9 mm diameter and 40 mm length, and dropped into sterile 2.5 % $CaCl_2$ solution to solidify the alginate and form the capsules (3 mm average diameter). Capsules were hardened in the $CaCl_2$ solution for one hour, before being washed with 0.9 % NaCl solution to remove any excess $CaCl_2$. As discussed below, modification of these standard encapsulation conditions did not significantly change the development of *Streptomyces*.

***Streptomyces coelicolor* encapsulated and non-encapsulated cultures.** In the case of encapsulated cultures, 10 ml of mycelial capsules (7.5 ml intra-capsule volume, 2.5 ml of dead volume) were used to inoculate 90 ml of R5A sucrose-free culture medium. In the case of the control non-encapsulated cultures, the original pre-culture (72 h cultures, inoculated with spores) was processed as reported for the encapsulated cultures (see above), but was resuspended in fresh R5A sucrose-free medium without alginate; 7.5 ml were used to inoculate 92.5 ml of R5A sucrose-free culture medium. Flasks of 500 ml, were incubated at 30 ºC and 200 rpm in both cases. Two biological replicates were performed for each condition.

***Streptomyces* sampling throughout the fermentations.** Samples (one milliliter) were collected at different developmental time points and centrifuged at 7740 ×*g* for 10 min at 4 ºC. Supernatants were collected and constituted the extracellular samples. The pellets were resuspended in 1ml of 0.5M NaOH, boiled for 10 min, and cellular debris was removed by centrifugation (at 7740 ×*g* for 15 min at 4 ºC), in order to obtain the intracellular samples.

Actinorhodin quantification. Actinorhodin was quantified spectrophotometrically according to Bystrykh et al. [5]. Sodium alginate interfered with spectrophotometric measurements, and an extraction step was necessary to eliminate the alginate. Supernatants from 1ml of cultures (extracellular samples) were extracted twice with 1 volume of ethyl acetate containing 1 % formic acid. Extracts were vacuum-dried, and resuspended in 1 ml of NaOH 1 N. Actinorhodin was quantified spectrophotometrically with a UV/visible spectrophotometer (Shimadzu, Model UV-1240), applying the linear Beer–Lambert relationship to estimate concentration ($\varepsilon_{640} = 25{,}320$).

Protein quantification. Protein was quantified using the Bradford method [4] with bovine serum albumin (Sigma) as the standard.

Viability staining. Culture samples were obtained and processed for microscopy at different incubation times. In the case of non-encapsulated control cultures, samples were stained directly, while in the encapsulated cultures, capsules were cut manually into slices with a scalpel. Cells were stained with a cell-impermeable nucleic acid stain (propidium iodide, PI) in order to detect the dying cell population and with SYTO 9 green fluorescent nucleic acid stain (LIVE/DEAD Bac- Light Bacterial Viability Kit, Invitrogen, L-13152) to detect viable cells. The SYTO 9 green fluorescent stain labels all of the cells, i.e. those with intact membranes, as well as those with damaged membranes. In contrast, PI only penetrates bacteria with damaged membranes, decreasing SYTO 9 stain fluorescence when both dyes are present. Thus, in the presence of both stains, bacteria with intact cell membranes appear to fluoresce green, whereas bacteria with damaged membranes appear red. After incubating them for at least 1 minute in the dark, the samples were examined under a Leica TCS-SP2-AOBS confocal laser-scanning microscope at a wavelength of either 488 nm or 568 nm excitation and 530 nm (green) or 630 nm (red) emission, respectively (optical sections about 0.2 μm). Images were mixed using Leica Confocal Software. In some cases, samples were also examined in the differential interference contrast mode, which is available using the same equipment.

Unstained samples were used as controls to determine the minimum photomultiplier tube (PMT) gain necessary to detect autofluorescence in the confocal microscope. The interference of green autofluorescence [31] was negligible when compared to the SYTO9 green fluorescence and did not interfere appreciably with the green fluorescent fluorochromes. Tenconi et al. [29] have recently demonstrated the existence of red autofluorescence associated with undecylprodigiosin that displays an excitation-emission spectrum similar to PI. Under the experimental conditions used in this work,

red autofluorescence was significantly weaker than PI fluorescence and the minimum PMT gain necessary to observe it was 860 volts (using the 63x objective), which is 60 % more than the PMT gain used to observe PI fluorescence (535 volts under the 63x objective) (data not shown). Despite this, red autofluorescence was not negligible, and some of the red fluorescent background detected at later time points in the centers of mycelial pellets and capsules may have been derived from undecylprodigiosin.

More than 30 images were analyzed for each developmental time point using a minimum of three independent cultures.

Results and discussion

Confocal laser-scanning fluorescence microscopy (CLSM) analysis of *Streptomyces coelicolor* encapsulated and non-encapsulated liquid cultures. In order to facilitate a comparison of *Streptomyces* differentiation in encapsulated liquid cultures with differentiation previously reported in non-encapsulated cultures, the culture conditions used were similar to those previously used in laboratory flasks [19]. Liquid cultures were inoculated with spores; they were grown until differentiation of the second multinucleated antibiotic-producing mycelium (72 h) [19], and were then split into two: the first sample was inoculated directly into fresh liquid medium (non-encapsulated control culture) (Fig. 1), and the second was encapsulated prior to inoculation in fresh liquid medium (encapsulated culture) (Fig. 2). (See Methods for details.)

Development in the non-encapsulated control cultures was comparable to that previously observed in liquid cultures inoculated with spores [19]. Viable hyphae resumed growth, increasing the diameter of mycelial pellets from 480 µm to 600 µm (compare Fig. 1F with Fig. 1J), and there was a high proportion of dead hyphae in the center of mycelial pellets at any time (red staining in Fig. 1F–J). Hyphae showed the appearance of the second multinucleated mycelia (they lacked the discontinuities characterizing MI hyphae) [19] (Fig. 1K–O). The most important differences with respect to liquid cultures inoculated

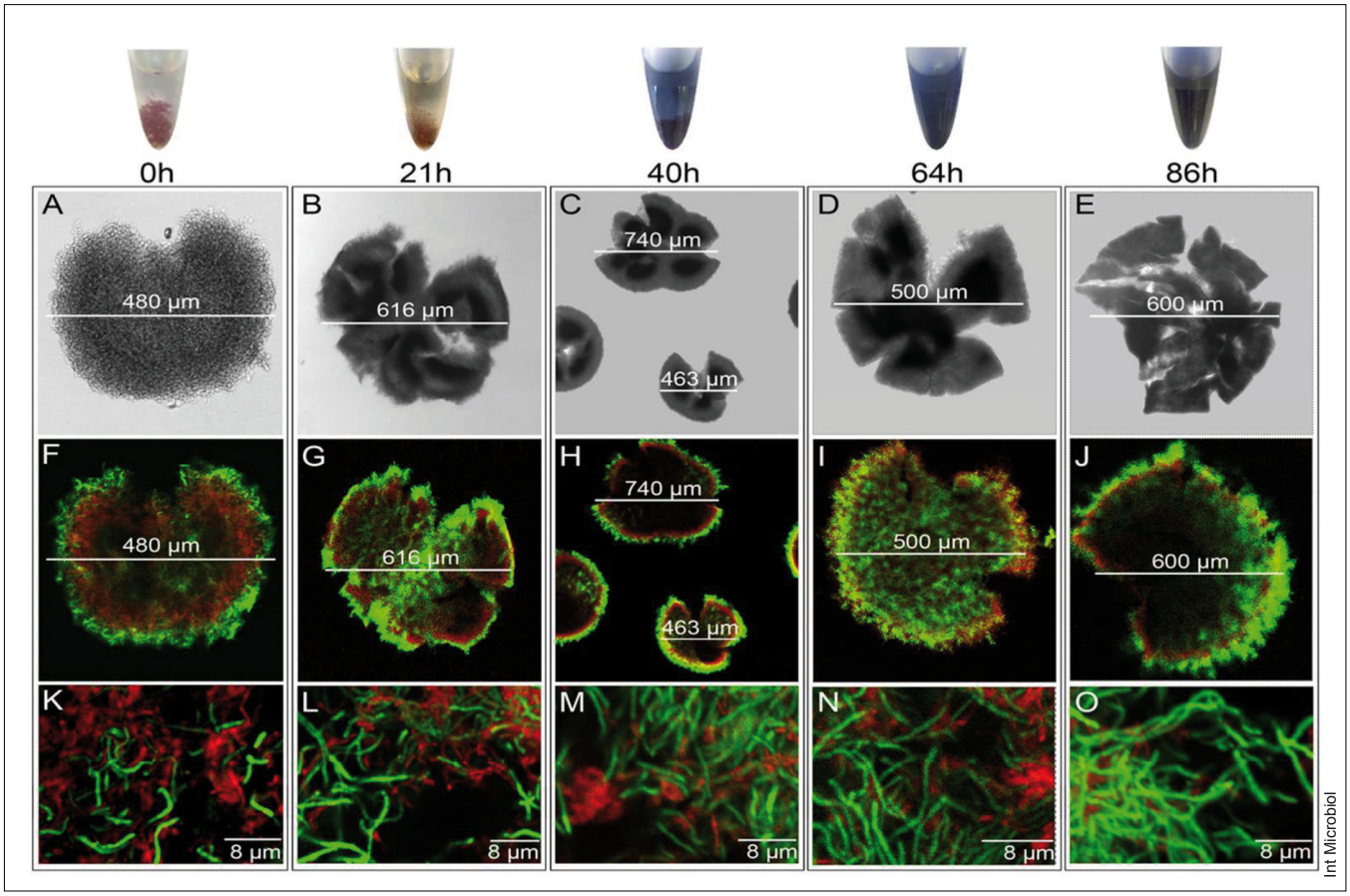

Fig. 1. Development of *Streptomyces coelicolor* M145 control non-encapsulated cultures. Upper pannels: macroscopic view of cultures. Undecylprodigiosin (red) and actinorhodin (blue) productions are visible. Lower panels: CLSM microscopy analysis. (**A**–**E**) interference contrast mode images; (**F**–**O**) fluorescence images (SYTO9/PI staining). Developmental time points are indicated.

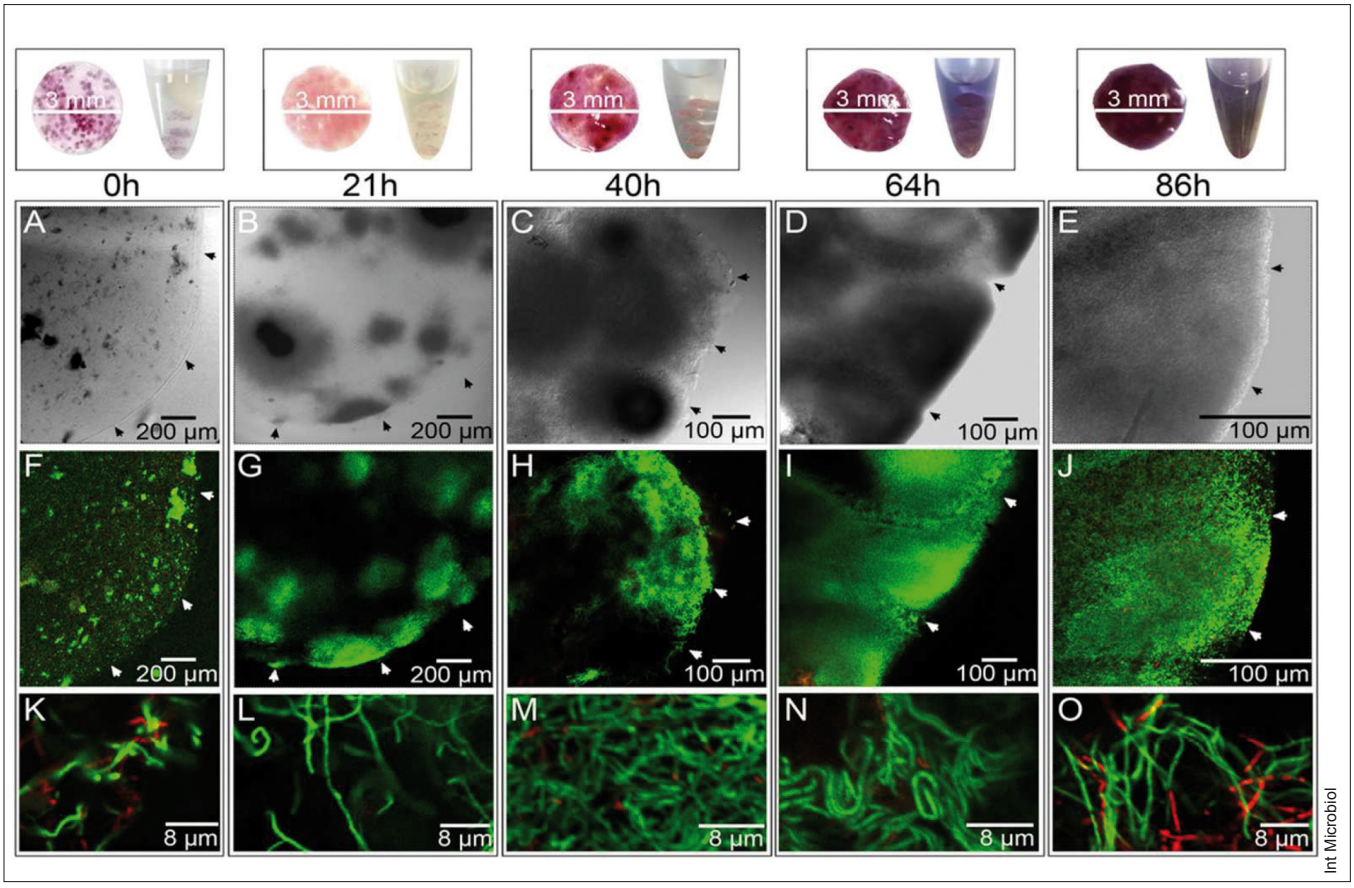

Fig. 2. Development of *Streptomyces coelicolor* M145 encapsulated cultures. Upper panels, macroscopic view of the capsules. Undecylprodigiosin (red) and actinorhodin (blue) productions are visible. Lower panels, CLSM microscopy analysis. (**A–E**) interference contrast mode images; (**F–O**) fluorescence images (SYTO9/PI staining). Developmental time points are indicated. Arrows indicate the border of the capsules.

with spores were: first, most of the mycelial growth was observed in the center of the pellets in the form of spots of viable hyphae (green staining in Fig. 1I) growing between dead hyphae (red staining in Fig. 1 and Fig. 1I); and second, mycelial pellets were much more fragile and appeared highly fragmented (Fig. 1B–E). This instability is probably due to the aging of the pellets, and the subsequent disintegration of dead hyphae which formed an important part of the pellet structure that had been formed in the original cultures used for inoculation [19].

Growth in the encapsulated cultures showed important differences with respect to the non-encapsulated ones. Mycelial pellets were completely destroyed during the encapsulation, and viable hyphae were dispersed around the entire capsule (green spots in Fig. 2F) at the beginning of the culture. The extension of cell death was lower, and the presence of dead hyphae only was significant at 0 h, derived from the original inocula (Fig. 1K), and at late time points (86 h) (red staining in Fig. 2O). Note that, despite the fact that the mycelia were growing embedded in a solid surface (alginate), there was no sporulation. As in the case of the control non-encapsulated cultures, viable hypha showed the appearance of the second multinucleated mycelia (Fig. 2K–O). In summary, the most spectacular difference between encapsulated and non-encapsulated cultures was, that capsules were fully of viable second multinucleated mycelia (Fig. 2F–J), while in the case of the non-encapsulated cultures, a high proportion of hyphae in the center of the mycelial pellets were dead (Fig. 1F–J) [19]. As discussed below, this interesting result might be related to the growth of the hyphae inside the alginate matrix which maintain them at lower densities than in the non-encapsulated cultures, reducing stresses related to low nutritional/oxygen levels.

Growth and extracellular actinorhodin production of *Streptomyces coelicolor* M145 in encapsulated and non-encapsulated liquid cultures. Growth rate was 1.5-fold lower in the encapsulated cultures (0.0077 mg ml^{-1} h^{-1}) than in the non-encapsulated ones (0.0117 mg ml^{-1} h^{-1}) (Fig. 3A), and maximum growth was also lower

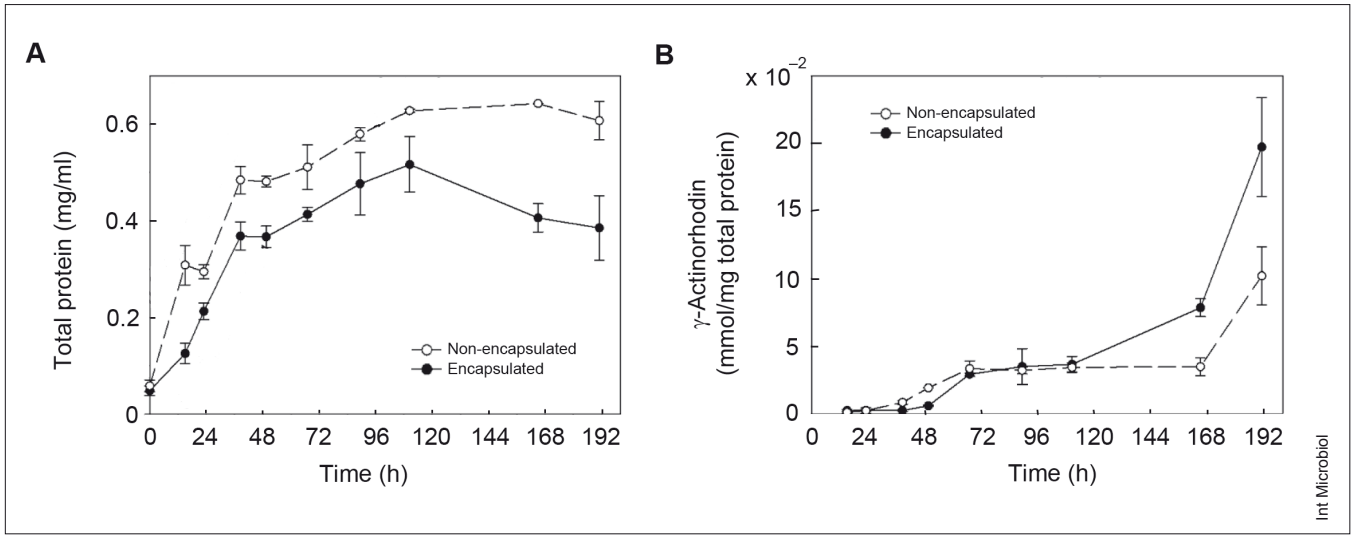

Fig. 3. Time-course of growth (**A**) and γ-actinorhodin production (**B**) in encapsulated and non-encapsulated cultures. Values are the average ±SD from two biological replicates. Growth rates (slopes) in (A) to non-encapsulated: 0.0117 mg ml^{-1} h^{-1}; to encapsulated: 0.0077 mg ml^{-1} h^{-1}.

in the encapsulated cultures (0.47 mg of protein per ml) than in the non-encapsulated cultures (0.62 mg of protein/ml) (Fig. 3A). The slow growth observed in encapsulated cultures might be a consequence of the difficulty for hyphae to grow inside the alginate matrix, and it might be the reason for the low levels of cell death observed in these conditions, since low cell densities might reduce the stresses triggering cell death [18].

Second mycelium hyphae (MII) produced antibiotics in the encapsulated and non-encapsulated cultures from the beginning of the experiment (red and blue colors in the macroscopic views shown in Figs. 1 and 2). Mycelium encapsulation has obvious advantages for the production of extracellular secondary metabolites, but not in the case of intracellular ones, since the recovery of intracellular compounds from encapsulated cultures is more complicated than from non-encapsulated ones. For this reason, extracellular levels of γ-actinorhodin [5] were analyzed (Fig. 3B). The production of γ-actinorhodin was slower in the encapsulated cultures (starting at 48 h) than in the non-encapsulated ones (starting at 38 h) (Fig. 3B). However, the maximum levels of production were duplicated in the encapsulated cultures (Fig. 3B).

These data correlate with growth curves and microscopic observations: encapsulation led to a slower rate of mycelial growth (Fig. 3A), and to larger proportion of viable MII antibiotic producing hyphae (compare the proportions of green and red stained hyphae in Figs. 1J and 2J) which were producing more antibiotics than the non-encapsulated cultures (Fig. 3B).

Different encapsulation conditions (concentrations of alginate and $CaCl_2$, time of capsule hardening in $CaCl_2$) were tested, but they did not significantly modify the development of *S. coelicolor* described above (data not shown). Inoculation density is one of the most well-known fermentation variables, that is usually conducive to modifications in growth and production [19,24]. Different inoculations modify the final actinorhodin production levels and growth kinetics, but not the general development described in this work in which cell death was reduced and antibiotic production increased in the encapsulated cultures with respect to the non-encapsulated ones (data not shown).

Results overview and conclusions. One important drawback of *Streptomyces* fermentations is the need to optimize culture conditions in order to obtain the most optimal equilibrium between differentiation, cell death and secondary metabolite production [19,21,24]. Cell-encapsulation constitutes an alternative to classical fermentations in liquid or solid cultures, which, in *S. coelicolor,* was demonstrated to maximize the proportion of viable antibiotic producing hyphae (MII), reporting the maximum levels of actinorhodin production even with lower levels of total biomass. In addition, cell immobilization has intrinsic advantages facilitating the separation between culture medium and cells, and recycling the productive biomass. Further work will be necessary to evaluate the applicability of cell immobilization in order to improve and/or activate secondary metabolite production in other streptomycetes.

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Conflit of interests. None declared.

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