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Importance of Growth Form on Production of Hybrid Antibiotic by *Streptomyces lividans TK21* by Fed-Batch and Continuous Fermentation

MONTSERRAT SARRÀ,[1] JOSEP A. PÉREZ-PONS,[2] FRANCESC GÒDIA,[1] AND CARLOS CASAS ALVERO*,[1]

[1]*Departament d'Enginyeria Química, Facultat de Ciències, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain;* [2]*Institut de Biologia Fonamental and Departament de Bioquímica i Biologia Molecular, Universitat Autónoma de Barcelona, 08193 Bellaterra, Barcelona, Spain*

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ABSTRACT

A fermentation strategy, based on the controlled feeding of growth-limiting nutrients in order to maintain metabolic activity for extended periods, has been examined in the case of the production of a hybrid antibiotic by a transformed strain of *Streptomyces lividans* TK21. The fed-batch operation did not improve the results obtained with batch operation. Continuous cultures on defined medium showed stable levels of biomass concentration, but antibiotic production ceased when continuous operation was started. The results obtained indicate the critical influence that morphology of the cell aggregates has on metabolic activity. The antibiotic is produced only in culture conditions providing growth in compact mycelial pellets.

Index Entries: Hybrid antibiotic; *Streptomyces lividans;* fed-batch; continuous cultivation.

*Author to whom all correspondence and reprint requests should be addressed.

INTRODUCTION

The advent of recombinant DNA technology and its application to the study of antibiotic production by *Streptomyces* and related bacteria has made the discovery of new antibiotics possible, as well as the creation of higher-producing strains and cheaper fermentation processes *(1–2)*.

Batch fermentations for secondary metabolism products, such as antibiotics, often follow a biphasic pattern. During the first phase, there is a rapid growth of cell mass without significant production of secondary metabolites. The second phase involves the production of secondary metabolites, and takes place without significant cell growth *(3)*. Therefore, cells often retain their catalytic activity at the time of nutrient depletion at the end of a typical batch fermentation, and this potential is discarded upon termination of the fermentation. Additionally, the time spent in emptying, cleaning, and refilling the fermentation vessel reduces volumetric productivity for this type of operation.

Although limiting cell growth may be essential for secondary metabolites production, the complete suppression of growth may be unfavorable. The fed-batch operation permits researchers to control feeding of a growth-limiting nutrient, and to extend the antibiotic production for a longer period, and, as a result, a higher final concentration can be obtained *(4–5)*.

Continuous operation can overcome some disadvantages of the batch operation, especially low productivity, and it provides a useful means of studying the effect of nutrients and growth rate on secondary metabolite production. In spite of these potential advantages, there is little research reporting on continuous antibiotic production by *Streptomyces*. One example, however, is the production of nikkomycins by *Streptomyces tendae*, which has been studied by different authors *(4,6–7)*.

On the other hand, continuous antibiotic production by genetically modified microorganisms may present an extra difficulty. The segregational instability, caused by the loss of plasmids during the cell division, leads to poor yields of cloned gene products, and makes continuous culture impracticable.

This article describes the behavior of a hybrid antibiotic fermentation during fed-batch and continuous process with free cells of a recombinant strain of *Streptomyces lividans* TK21. Not only do the experiments presented here support previous results on the importance of mycelial morphology in successful secondary metabolite production, but they also suggest the suppressive effect of dispersed growth over limiting nutritional conditions in the expression of cloned genes for hybrid antibiotic production in *S. lividans* TK21.

METHODS

Strain

The transformed strain of *S. lividans* TK21 was provided by F. Malpartida (Centro Nacional de Biotecnologia, Madrid). It was maintained as spores at −20°C. For strain construction, an 18.6 kb fragment of DNA from *Streptomyces antibioticus*, containing the *act* I loci, was subcloned into the low-copy-number vector pIJ941 (25 kb) to yield plasmid pMH9410. When this plasmid was introduced by transformation into *S. lividans* TK21, a strain previously not known to produce antibiotics, the transformed strain produced a new pigmented compound with some analogous chemical characteristics and possibly chemical structures to the antibiotic actinorhodin *(8)*. The new hybrid antibiotic was active against some bacterial species, such as *Bacillus subtilis* and *Vibrio alginolyticus*.

Medium

The defined culture medium contained, per liter of distilled water: 30 g starch, 52 mmol glutamic acid, 10 mmol phosphate (as a 7:3 mixture of K_2HPO_4 and KH_2PO_4), 0.2 g $MgSO_4 \cdot 7H_2O$, 18 mg $FeSO_4 \cdot 7H_2O$, 1 mg $CaCl_2$, 1 mg NaCl, and 9 mL trace mineral solution, as described previously *(9)*. The pH of the medium was adjusted to 6.5 with 2 *M* NaOH.

Fermentation Equipment and Conditions

All fermentations were performed in a stirred-tank reactor of 4 L working volume (Bioflo III, New Brunswick Scientific Limited, Piscataway, NJ). Temperature was maintained at 29°C and sterile air was supplied at 1 vvm. The agitation rate was set at 400 rpm and pH control was achieved by addition of 1 *M* NaOH or 1 *M* HCl, in order to maintain pH at 6.5. The bioreactor was inoculated with a known volume of a spore stock suspension to give a final concentration of 10^4 spores/mL.

Fed-Batch Cultures

Two types of fed-batch experiments were performed. In the first type, half of the volume of the fermenter, filled with the double-concentrated defined medium, except for glutamic acid, was inoculated. When the antibiotic production ceased, the feeding was initiated by pumping a 52-m*M* glutamic acid solution at a flow rate of 20.8 mL/h. The second kind of fed-batch fermentation was carried out in a defined medium containing 4 m*M* phosphate and 30 m*M* glutamic acid. When the concentration of both compounds was below 0.5 m*M*, pulse additions of 1 m*M* phosphate or 5 m*M* glutamic acid solutions were performed.

Continuous Cultures

When the maximum antibiotic concentration in a batch run was reached, the system was switched to continuous operation. The feeding solution consisted of the defined medium containing 0.5 m*M* phosphate, and was pumped at a flow rate of 41.6 mL/h. In this case, the working volume of the fermenter (3 L) was maintained constant by continuously pumping out the overflow volume.

Repeated Batch Cultures

At the end of a batch fermentation, 95% of the working volume was withdrawn and replaced with fresh medium. The same operation was repeated at the end of this second batch fermentation.

Analytical Methods

Biomass concentration in liquid cultures was determined as dry wt by filtering 10 mL centrifuged samples (3500*g*, 10 min) and drying at 90°C to a constant weight. Starch concentration was determined by the anthrone method *(10)* in the supernatant obtained after centrifugation. Inorganic phosphate was analyzed by test kits (Merkotest Anorganisches Phosphat no. 3331). Glutamic acid was determined by high-performance liquid chromatography (Hewlett-Packard Aminoquant).

The hybrid antibiotic present in the culture medium was determined by an agar diffusion assay using *B. subtilis* ATCC506 as the test organism. Moreover, antibiotic concentration was estimated in the culture supernatant by measuring the absorbance at 530 nm after dilution with a 0.5 *M* carbonate buffer (pH 10.0). Measures of absorbance were correlated with antibiotic activity by the expression: $D = 1.4858 + 2.311 \ln A$, where D is the diameter of the inhibition zone, and A the antibiotic concentration measured as absorbance units at 530 nm.

DNA Isolation

Total and plasmid DNAs were prepared according to the procedure of Fisher and Kieser, respectively, as described by Hopwood et al. *(11)*, from aliquots of mycelia withdrawn at different times of fermentation. DNA samples were analyzed by agarose gel electrophoresis and ethidium bromide staining.

RESULTS

Batch Fermentations

During a batch fermentation, the exponential growth phase (μ = 0.11/h) started 25–30 h after spore inoculation. Biomass concentration

reached 5.5–6 g/L after 110–120 h of fermentation. The onset of antibiotic biosynthesis was associated with other metabolic changes, even when rapid growth occurred. In a typical batch fermentation, the glutamic acid added to the medium as the nitrogen source was depleted during the first 60 h, and its consumption could be related to the consumption of acid used for pH control. This behavior was caused by ammonia release from glutamic acid deamination. Antibiotic accumulation in the medium started at the point when the metabolism shifted to ammonia consumption. Again, this change could be tracked by a shift in the pH-control system, which at this point changed to base addition. Antibiotic production was even initiated with a high growth rate, and stopped a few hours after the stationary phase had been reached. At the end of a batch fermentation (140–150 h), the antibiotic concentration was about 2 absorbance units.

Fed-Batch Fermentations

A first type of fed-batch fermentation was initiated with a limiting concentration of glutamic acid (26 m*M*), and, when the antibiotic production ceased, a solution of glutamic acid was supplied intermittently. Figure 1 shows typical results obtained from this type of fermentation, in which the maximum biomass concentration was about 5.5 g/L, not higher than the value obtained in batch experiments, and the antibiotic concentration declined rapidly, following the dilution curve because of the increase in working volume produced by limiting substrate addition. These data suggested that antibiotic production did not recover at all, and, therefore, the production phase was not extended.

Phosphate is an essential nutrient in antibiotic production, and it is believed that secondary metabolism is triggered by limiting phosphate concentrations *(3)*. Thus, a second type of fed-batch fermentation was performed by pulsing phosphate and glutamic acid independently, when their concentrations were detected as being lower than 0.5 m*M* (Fig. 2). Continuous accumulation of the antibiotic occurred, but the time required to reach the same antibiotic concentration in the bioreactor was always higher than in the batch process.

Continuous Fermentations

The central question in developing a method for continuous fermentation is whether the microorganisms can grow and simultaneously produce antibiotics, and under what conditions they do this. The continuous operation was initiated from a batch culture very early in its stationary phase, by feeding with a basal medium with low phosphate concentration (0.5 m*M*). The dilution rate initially selected was 0.014/h, and after four retention times it was reduced to 0.007/h. Figure 3 shows the progress of

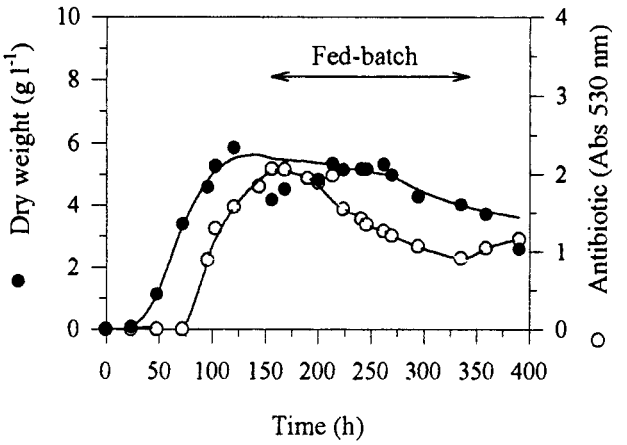

Fig. 1. Time-courses of hybrid antibiotic and biomass concentration during a fermentation with fed-batch addition of 52 m*M* glutamic acid. Fed-batch was initiated at the end of a batch run, when the antibiotic production ceased.

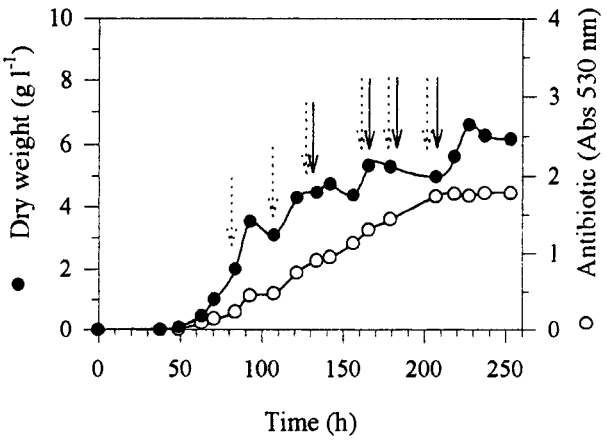

Fig. 2. Time-courses of hybrid antibiotic and biomass concentration during a fermentation with fed-batch addition of glutamic acid and phosphate; continuous arrow and dotted arrow are 1 m*M* phosphate and 5 m*M* glutamic acid pulse, respectively.

biomass and antibiotic concentration vs fermentation time. Although biomass remained approximately constant at its steady-state value of about 6.5 g/L, antibiotic concentration in the bioreactor decreased according to the dilution curve. This means that the antibiotic production ceased when the continuous process was switched on, and it was not recovered even when experiments at lower dilution rate were conducted.

As shown in Fig. 4, glutamic acid was exhausted in the first 60 h, and remained at the zero level for the rest of the fermentation. Inorganic phosphate had already disappeared after 80 h, and followed a similar pattern

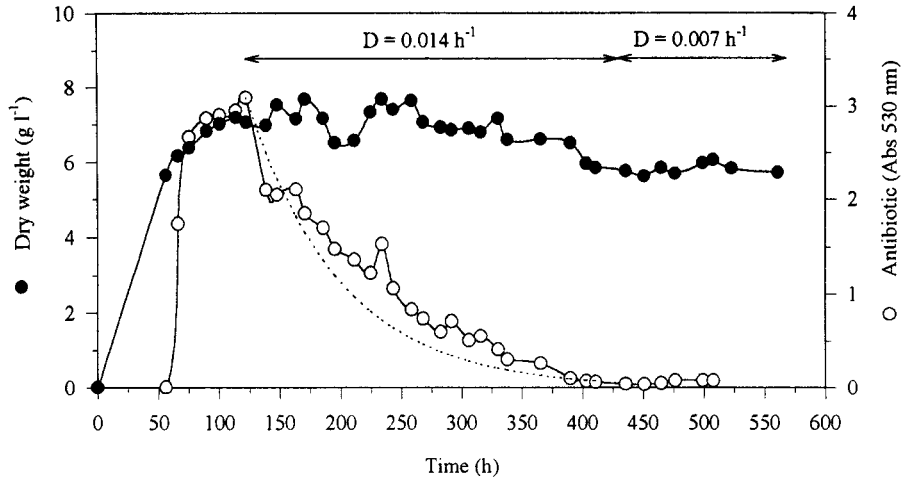

Fig. 3. Antibiotic and biomass concentration during a continuous fermentation fed with phosphate-limited medium (0.5 m*M*) at two different dilution rates. Dotted line represents dilution curve.

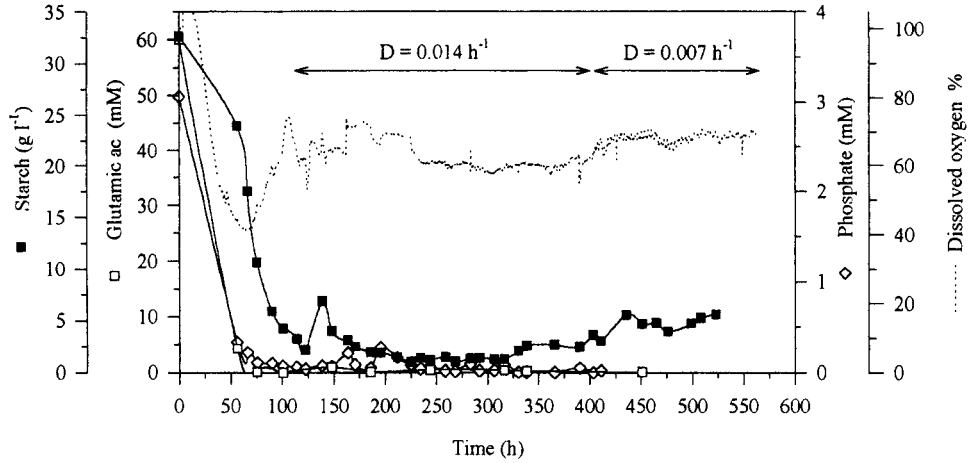

Fig. 4. Time-courses of dissolved oxygen and carbon, nitrogen, and phosphorous sources in a fermentation continuously supplied with phosphate-limited medium.

until the end of the process. Dissolved oxygen was mostly consumed during the initial growth phase (μ = 0.11/h), but, after reaching steady-state conditions, its concentration was greater than 60% of saturation. During the continuous operation, starch concentration was between 2 and 5 g/L.

In order to show the effect of limiting amounts of glutamic acid in the continuous production of the hybrid antibiotic, further experiments were performed. Thus, continuous fermentation with 30 and 10 m*M* glutamic

acid in the feed medium were carried out. In all cases, no improvement could be observed. Figure 5 shows the results of a continuous fermentation performed at a dilution rate of 0.009/h, under phosphate (0.5 m*M*) and glutamic acid (10 m*M*) limitations. Antibiotic production stopped when the continuous process started, and did not recover, even when it was once again shifted to batch conditions process: after 580 h, a 90% working volume (3 L) was withdrawn from the fermenter and replaced with fresh unlimited medium. Under these conditions, mycelia grew rapidly, and biomass concentration reached a higher level than in the initial batch process. However, antibiotic production never switched on again.

Repeated Batch Fermentations

As a result of these experiments, it can be assumed that cells growing under strong limited conditions for long periods may lose their ability to produce secondary metabolites. In order to assess this effect, three repeated batch fermentations were carried out. During the first batch culture, the biomass concentration reached 5.6 g/L in 80 h; the antibiotic concentration was 2.6 absorbance units at the end (140 h). After emptying and refilling with fresh medium, the cells reacted immediately by growing rapidly (0.11/h), and 60 h later the biomass concentration obtained was 6.4 g/L. Although this second-stage fermentation was maintained for 120 h, the antibiotic concentration reached was only 30% of that obtained after the first-stage culture. When the emptying–refilling operation was repeated again, the biomass level achieved was 6.7 g/L after 70 h, but the antibiotic production did not switch on during the whole process (120 h). Figure 6 shows the time-courses of biomass and antibiotic production in these repeated batch fermentations.

Although starch had been the best carbon (C) source for cell growth and antibiotic production in batch cultures, the results obtained using glycerol as C source were also significant *(9)*. When pellets were used as immobilized bioparticles in a fluidized-bed bioreactor, the continuous antibiotic production could be maintained for long periods by feeding phosphate-limited medium containing glycerol as C source *(19)*. As a consequence, two repeated batch fermentations were carried out with 30 g/L glycerol concentration. The results obtained did not shown any difference from the results obtained with starch as C source. The biomass concentration reached 4.8 g/L after 90 h inoculation; the antibiotic concentration was 2.2 absorbance units at the end of the first batch culture (145 h). After emptying and refilling with fresh medium, the biomass concentration reached 5.2 g/L in 70 h. Although this second batch fermentation was maintained for 120 h, the antibiotic concentration at the end of the culture was only 38% of that obtained after the first batch culture.

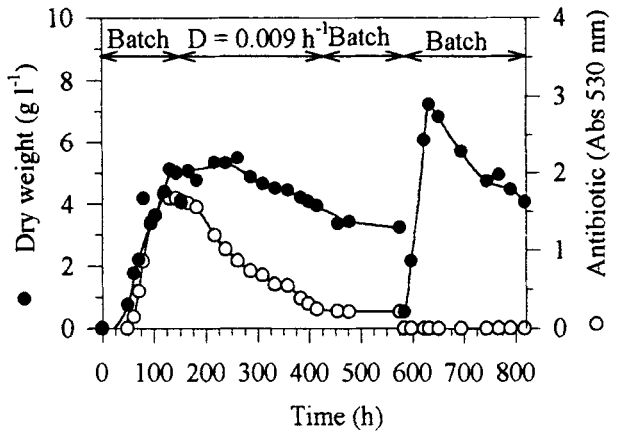

Fig. 5. Evolution of the cell mass and antibiotic concentration in a continuous fermentation, in which the feeding solution was limited in phosphate and glutamic acid. After 420 h, feed was stopped (first batch period), and, 160 h later, 90% working volume was replaced with unlimited medium (second batch period).

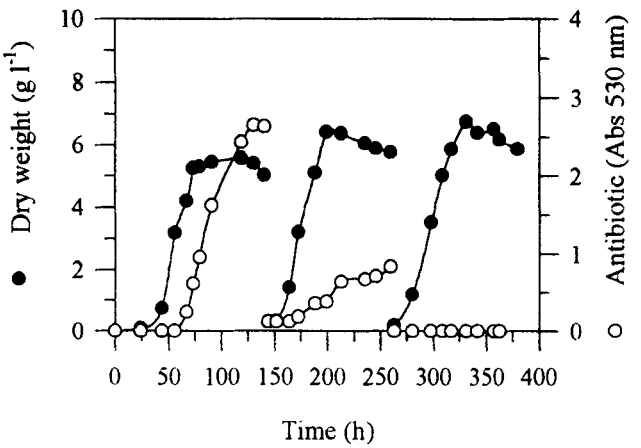

Fig. 6. Antibiotic and biomass concentration during a repeated batch fermentation series. In each fermentation, the inoculum used was obtained from the previous one.

Plasmid Stability

The authors attempted to determine the plasmid stability along continuous and repeated-batch fermentations by quantifying the plasmid DNA/chromosomal DNA ratio in total DNA preparations from mycelium samples withdrawn at different culture times. However, this approach was not suitable (*see* Discussion) probably because of the high molecular mass and the low copy number of the recombinant plasmid pMH9410, which yielded DNA ratios too low for an accurate determination. Thus, the

authors checked the presence of pMH9410, especially at long culture times, by specific plasmid-DNA-isolation method. In order to allow a semiquantitative comparison between samples from the same fermentation batch, equal (in weight) amounts of mycelium were processed for plasmid extraction. Figure 7 shows the electrophoretic analysis of some representative samples in which a covalently closed circular plasmid band is visible even at later stages of culture. Note that significant differences in the plasmid content are not observed when samples early and late in the cycle (Fig. 7) were analyzed. The plasmid content shown in lane 2 belongs to a sample withdrawn when the biomass concentration had been duplicated approximately six times from the early exponential growth phase; lanes 1, 3, and 4 show the plasmid content when the biomass concentration had been duplicated 11, 15.5, and 10.5 times, respectively.

DISCUSSION

Several hypotheses could be drawn from these experiments. First, during continuous process, growth under strong limiting conditions could irreversibly damage the cells and hamper the efficient expression of secondary metabolism. This means that growth conditions would not allow the generation or accumulation of the primary metabolites needed for the synthesis of idiolites. Second, the sudden decrease of antibiotic production may be caused by a progressive plasmid loss during continuous operation. Finally, a further hypothesis is proposed, based on the significant changes in the morphology of the culture from dense pellets to loose mycelia, after shifting to continuous culture.

When present in Gram-negative or Gram-positive unicellular bacteria, many plasmids show segregational instability, mostly caused by plasmid loss during cell division. This eventually leads to loss of the plasmid-borne phenotype and poor yields of the cloned gene product. Because *Streptomyces* are filamentous microorganisms, the traditional method of following segregational plasmid instability, by assessing the proportion of colony-forming units exhibiting a plasmid-borne phenotype, is unsuitable. In this way, Wrigley-Jones et al. *(12)* developed a new method for filamentous microorganisms, in which the number of plasmid copies is related to the chromosome equivalents, and it seems suitable for small-size plasmids (<12 kb) with a high copy number. The plasmid pMH9410 (43 kb) is derived from the low-copy-number vector pIJ941. In the authors' experience, when total DNA extraction was performed, it was impossible to identify plasmid bands in the agarose gels, probably because of the low amount of plasmid DNA present in total DNA samples. Therefore, specific plasmid DNA preparations were used for runs in agarose gels. Plasmid bands, corresponding to covalently closed circular

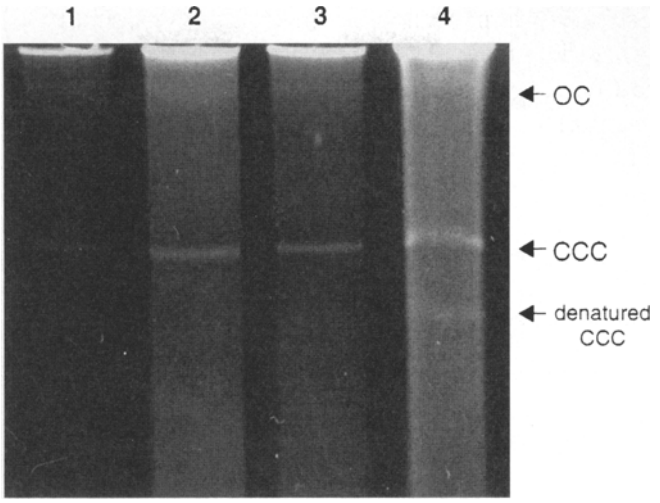

Fig. 7. Presence of pMH9410 DNA (43 kb) in different fermentation samples taken at times indicated between brackets. Lane 1, continuous culture (694 h); lane 2, repeated batch fermentation with starch as C source (70 h); lane 3, repeated batch fermentation with starch as C source (357 h); lane 4, repeated batch fermentation with glycerol as C source (220 h). Electrophoretic analysis was done in a 0.8% agarose gel using Tris-acetate buffer. OC and CCC stand for the open circular and the covalently closed circular forms of plasmid DNA, respectively.

DNA, were visible in all of the samples obtained from continuous and repeated-batch fermentations. Although band intensities were not measured, plasmid loss was not qualitatively observed in any of the samples in which analysis revealed no antibiotic production. These results show that complete segregational loss was not attained under continuous and repeated-batch culture conditions in which biomass concentration was maintained at normal values. Therefore, antibiotic production could be affected by other regulatory mechanisms involved in the gene expression.

Morphological changes in mycelia were a common feature in all the experiments performed. During batch operation, spores inoculated at low concentration ($<10^5$ sp/mL) developed compact pellets with a diameter between 350 and 450 μm, and a smooth surface (Fig. 8A). When the culture reached the stationary-growth phase, the surface structure of the pellets changed to a fluffy, loose form from which, finally, free mycelia are detached. When fermentations were extended, either by fed-batch or continuous operation, the detached mycelia grew quickly, developing small aggregates (Fig. 8B). As a result, viscosity of the medium increased, and heterogeneous morphology was observed. By the end of a fed-batch fermentation, the compact pellet percentage gradually decreased. In contrast, during the continuous and repeated batch operation, compact pellets were

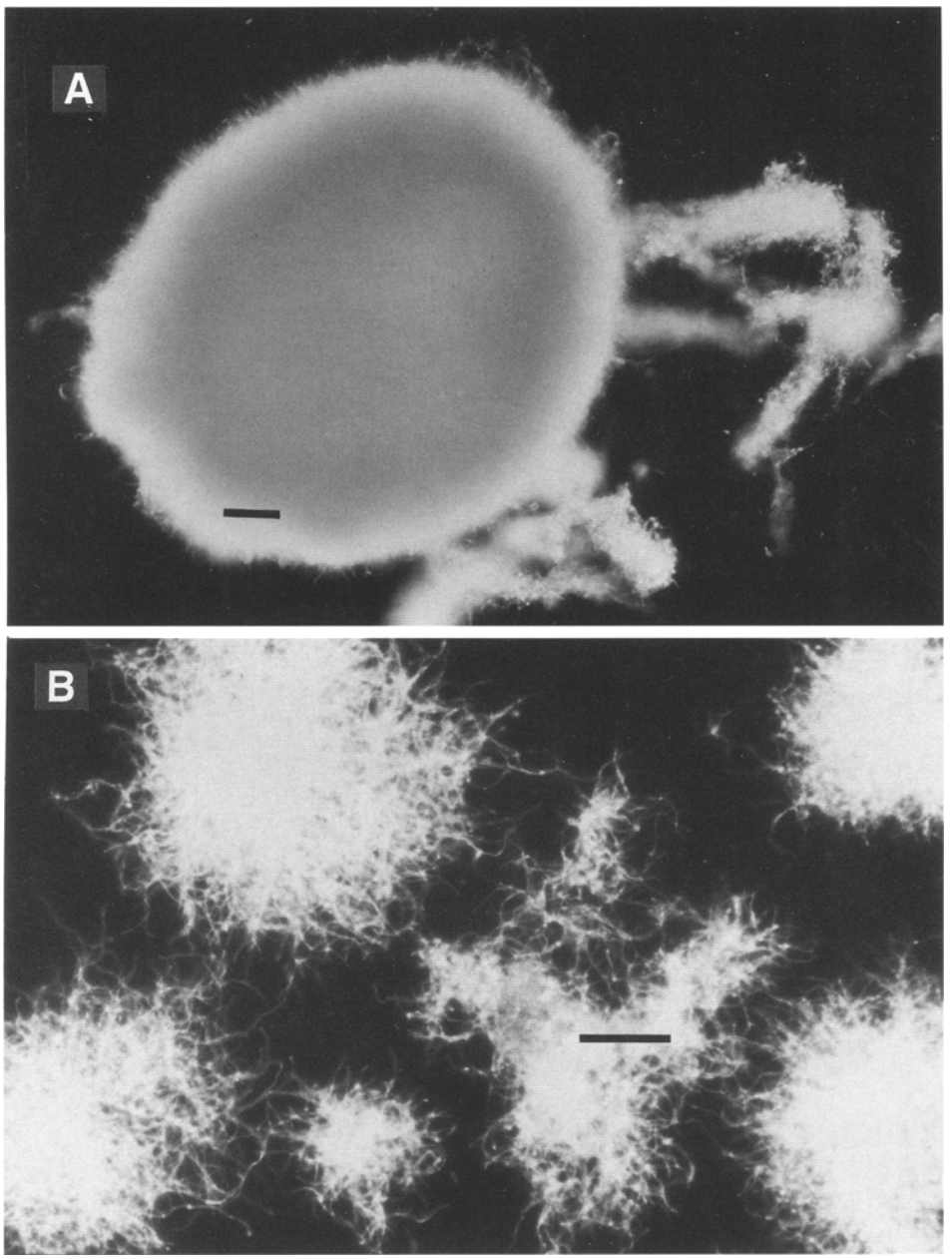

Fig. 8. Two typical morphologies of *S. lividans* TK21: **(A)** compact pellets obtained after 90 h of the batch culture and **(B)** small mycelia aggregates obtained after 300 h of continuous cultivation. Bar = 50 μm.

washed out, and loose mycelium morphology was predominant. Such morphological changes in defined medium cultures are in accordance with previous findings for *S. noursei* and *S. lividans (13)*, and for *S. tendae (7)*.

Compact pellets may be responsible for antibiotic production: They are subject to internal mass-transfer limitations, which may result in a shift-down effect at their center *(14,15)*. As a result, mycelial aggregates appeared to operate, not merely as mechanical conglomerates, but rather as complex differentiated tissues, phenotypically characterized by a specific set of metabolic activities *(16)*. The authors' results indicate that the formation of compact mycelial pellets is an inevitable prerequisite for successful antibiotic production by the transformed *S. lividans* TK21 strain. Not only the pellet heterogeneity in different layers, but also their formation during the growth phase seem to be responsible for secondary metabolite production.

The other major factor influencing antibiotic production is likely to be phosphate concentration. Under growth conditions with excess phosphate, this compound can be stored as polyphosphate in amounts from 0.1 up to 20% of the cell dry wt *(17)*. After phosphate depletion in the medium, internal phosphate can be used for cell growth *(18)* or secondary metabolite production. Although extracellular phosphate represses antibiotic production, internal phosphate directly enhances it when pellets are used as immobilized bioparticles *(19)*. Dispersed mycelial growth would not allow internal phosphate accumulation, and this would result in the decay of antibiotic production. Production would be maintained only if the compact pellet morphology does not change during continuous operation.

The work reported above describes the critical effect of growth form on antibiotic production as an additional factor to be considered in the complex regulatory systems that influence antibiotic biosynthesis in *Streptomyces*.

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