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Production of Antitumoral Retamycin During Fed-Batch Fermentations of *Streptomyces olindensis*

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Abstract

Fed-batch runs were performed in order to correlate the production of retamycin, an anthracycline antibiotic produced by *Streptomyces olindensis* in submerged cultures, with the specific growth rate. Maximum retamycin production was achieved with an exponential feed rate, controlling the specific growth rate at a low value (0.03 h^{-1}, about 10% of the maximum specific growth rate). Control of the specific growth rate at higher values (0.10 and 0.17 h^{-1}) caused a decrease in antibiotic production. Morphology, assessed by image analysis, was shown to be highly relevant in this process. Cell growth mainly in the form of clumps (90% clumps and 10% free filaments) led to better results than growth as clumps (75%) and free filaments (25%).

Index Entries: *Streptomyces*; fed batch; morphology; antibiotic; retamycin.

Introduction

Filamentous microorganisms such as *Actinomycetales* and filamentous fungi are used in several industrial fermentations. In particular, *Streptomyces* spp. are very important in antibiotic production.

Retamycin is an anthracycline antibiotic complex with potent antitumor activity *(1)*, similar to daunorubicin and doxorubicin (adriamycin), showing positive results in the treatment of human leukemias *(2)*. Retamycin complex is a red powder with low solubility in water and high solubility in organic solvents (methanol, chloroform), and it is produced by

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submerged cultivations of *Streptomyces olindensis (3)*. Like the other anthracyclines used in chemotherapy of cancer, retamycin intercalates into DNA and presumably inhibits DNA replication and transcription *(3,4)*. The isolation of new anthracyclines has been pursued in attempts to overcome its undesirable cardiotoxicity *(4)*. Only a few studies have been published about this antibiotic, but retamycin showed promising results in the intercalation into DNA, which makes this antibiotic suitable for use as a chemotherapeutic agent *(5)*.

Like the majority of secondary metabolites, antibiotic production can be strongly influenced by specific growth rate (μ_x), where production is favored by lower specific growth rates *(6)*. Several studies of antibiotic production have been done in fed-batch processes, since the specific growth rate can be controlled during the feed period, if an exponential feed rate is implemented *(7)*. Many studies to improve penicillin and other antibiotic production (streptomycin, tetracycline, cephalosporin) have been done in fed-batch fermentations *(7,8)*, showing that fed-batch is a good strategy for the production of such metabolites.

In addition, morphology plays an important role in the production of metabolites by filamentous microorganisms *(8–11)*. Quantification of morphology is important in studies of metabolite production, and it can be assessed by image analysis. Image analysis is the obtainment of information (measurements) from an image by a computer in a reproducible and semiautomated or automated manner. The main advantages of image analysis are that repetitive measurements can be automated and that measurements can be made accurately, reproducibly, and more quickly than manual methods based on human observations *(12)*. Additionally, fed-batch fermentation has been used as a good tool to study morphologic characteristics of microorganisms *(8,9)*.

This article focuses on the production of the antibiotic retamycin in fed-batch cultivations of *S. olindensis*, using different exponential feed rates, in order to control the specific growth rate in different values and analyze its influence on the production of retamycin.

Theoretical Considerations

Fed batch is defined as a technique in microbial processes in which one or more nutrients are supplied to a bioreactor during cultivation and the products remain in the containment until the end of the run *(7)*.

Biomass and growth-limiting substrate balances lead to the following equations:

$$\frac{d(X \cdot V)}{dt} = X \cdot \frac{dV}{dt} + V \cdot \frac{dX}{dt} = \mu_X \cdot X \cdot V \tag{1}$$

$$\frac{d(S \cdot V)}{dt} = S \cdot \frac{dV}{dt} + V \cdot \frac{dS}{dt} = \phi \cdot S_{feed} - \frac{\mu_X \cdot X \cdot V}{Y_{X/S}} \tag{2}$$

in which S is the growth-limiting substrate concentration (glucose) in the bioreactor (g/L), S_{feed} is the growth-limiting substrate concentration (glucose) in the feed (g/L), V is the volume of culture broth in the bioreactor (L), X is the biomass concentration in the bioreactor (g/L), $Y_{X/S}$ is the growth yield (g/g), ϕ is the medium flow rate (L/h), and μ_x is the specific growth rate (h^{-1}).

If the feed rate of the growth-limiting substrate is increased in proportion to the exponential growth rate, it is possible to maintain a constant rate of growth for a long time, while keeping the substrate concentration in the culture broth at a constant level ($S = S_0$) *(7)*.

From Eq. 1, considering the necessary conditions for exponential growth, specific growth rate can be assumed constant (μ_{X0}), which leads to

$$X \cdot V = X_0 \cdot V_0 \cdot \exp[\mu_{X0} \cdot (t - t_0)] \tag{3}$$

in which V_0 is the volume of culture broth in the bioreactor in the instant t_0 (L), X_0 is the biomass concentration in the bioreactor in the instant t_0 (g/L), and t_0 is the instant of the beginning of feed (h).

Since $\phi \cong dV/dt$ and considering the substrate concentration constant ($S = S_0$ and $dS/dt = 0$), from Eqs. 2 and 3, it can be obtained

$$\frac{\mu_{X0} \cdot X \cdot V}{Y_{X/S}} = \phi \cdot S_{feed} - \phi \cdot S_0 \tag{4}$$

$$\phi = \frac{\mu_{X0} \cdot X_0 \cdot V_0 \cdot \exp[\mu_{X0} \cdot (t - t_0)]}{Y_{X/S} \cdot (S_{feed} - S_0)} \tag{5}$$

Thus, if an exponential feed rate is implemented, according to Eq. 5, both specific growth rate and substrate concentration can be maintained at fixed levels. The feed equation may be calculated from previous results, and at the instant of the beginning of feed (t_0), the specific growth rate should be near the desired value μ_{X0}.

Materials and Methods

Microorganism

A mutant strain of *S. olindensis* ICB20 was supplied by Laboratório de Genética de Microrganismos, Instituto de Ciências Biomédicas, Universidade de São Paulo. The cells were stored in cryotubes containing 20% glycerol at –20°C *(13)*.

Preparation of Inoculum

The inoculum for the fermentor runs was prepared in a New Brunswick rotary shaker at 200 rpm and 30°C in two steps. The first step was carried out for 16 h and the second for 24 h *(13)*. A 10% inoculum was used in the bioreactor inoculation.

Culture Medium

The medium for the inoculum preparation and bioreactor cultivations contained the following nutrients *(13)*: glucose (10.0 g/L), yeast extract (5.0 g/L), tris(hydroxymethyl)-aminometan (3.09 g/L), casein hydrolysate (0.10 g/L), K_2SO_4 (0.25 g/L), and $MgCl_2 \cdot 6H_2O$ (10.12 g/L). The pH was adjusted to 7.0 using 2 *N* HCl. After medium sterilization, the following sterile solutions were added (for 250 mL of culture medium): 0.5% (w/v) (2.5 mL) KH_2PO_4, 5 *M* (1.0 mL) $CaCl_2$, and 0.5 mL of a trace elements solution (40 mg of $ZnCl_2$, 200 mg of $FeCl_3 \cdot 6H_2O$, 10 mg of $[NH_4]_6Mo_7O_{24} \cdot 4H_2O$, 10 mg of $CuCl_2 \cdot 2H_2O$, 10 mg of $MnCl_2 \cdot 4H_2O$, and 10 mg of $Na_2B_4O_7 \cdot 10H_2O$ in 1000 mL of distilled water). The culture medium for the fed-batch operation had fourfold this composition, in order to obtain suitable periods of feed.

Culture Conditions

Three fed-batch runs were performed in a 5-L New Brunswick Bioflo fermentor under the following conditions: Initial volume = 3.5 L; feed volume = 1.0 L; agitation rate = 500 rpm; air flow rate = 4 L/min; pH = 7.0; and temperature = 30°C. The fermentor was provided with two flat-blade turbine disk impellers, with four blades each.

After fermentor inoculation, there was an initial batch period cultivation (between 13 and 21 h, according to the desired value of μ_{X0}). In run FB-1, the exponential feed rate used was $\phi = 0.0256 \cdot e^{0.03 \cdot (t - 21)}$ L/h, in order to control the specific growth rate in 0.03 h^{-1}, between 21 and 47 h of cultivation. In run FB-2, the exponential feed rate used was $\phi = 0.0569 \cdot e^{0.10 \cdot (t - 14)}$ L/h, in order to control the specific growth rate in 0.10 h^{-1}, between 14 and 24 h of cultivation. In run FB-3, the exponential feed rate used was $\phi = 0.0775 \cdot e^{0.17 \cdot (t - 13)}$ L/h, in order to control the specific growth rate in 0.17 h^{-1}, between 13 and 19 h of cultivation. The equations for the exponential feed rate were determined from Eq. 5, using previous results of *S. olindensis* cultivations *(13)*.

Methodology

Samples collected periodically from the fermentor were evaluated for biomass, glucose concentration, retamycin concentration, and morphology. Biomass (*X*) was evaluated after vacuum filtration and drying, and glucose concentration (*S*) was determined by glucose oxidase method *(13)*. The antibiotic retamycin is a pigmented compound (red). Therefore, its concentration was measured as the absorbance at 547 nm, after sample vacuum filtration and adjusting the pH to 6.3 using 0.02 *N* HCl, because there is no commercial pure product to establish a calibration curve *(13)*.

Morphology was evaluated by image analysis by classifying the microorganism into four morphologic classes (unbranched free filaments, branched free filaments, clumps, and pellets, as shown in Fig. 1) *(14)* using a LEICA Q550IW Image Analyser attached to a LEICA DM/LS optical microscope. The images were acquired with a Sony DXC-950P charge-coupled device camera. The microscopic images were recorded with a reso-

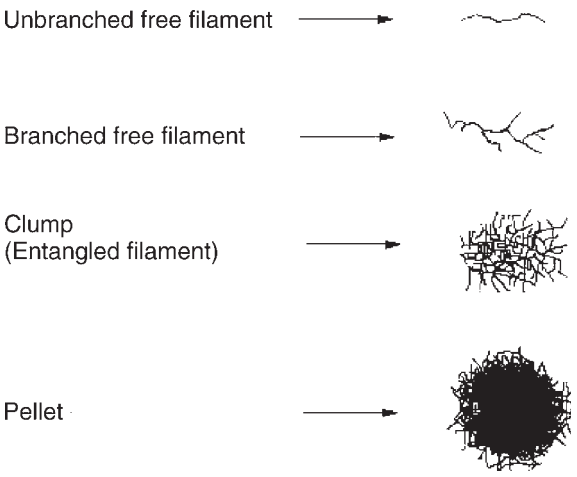

Fig. 1. Morphologic classes in an *S. olindensis* cultivation (unbranched free filament, branched free filament, clump, and pellet).

lution of 764×574 pixels and 256 gray values. The gray image was corrected using erosion and dilation, before binarization. The image was then segmented by two thresholds to obtain a binary image. In the segmentation process, pixels that have a gray scale between the two thresholds are picked out and the others are eliminated, to form the binary image *(12)*. After binarization, the binary image was corrected using erosion, dilation, skeletonization, and pruning. Skeletonization and pruning were useful to obtain the number of tips of the hyphal elements. Samples from the culture broth were diluted 5- to 100-fold with distilled water. A 20-µL aliquot of the diluted sample was pipetted onto a slide glass, dried and fixed over a Bunsen flame, and stained with methylene blue (0.3 g of methylene blue and 30 mL of 95% ethyl alcohol in 100 mL of distilled water) *(15)*. For each sample, a minimum of 100 objects was measured, and the percentage of each morphologic class, in terms of projected area, was determined *(14)*.

Results and Discussion

Figure 2 shows the specific growth rate during runs FB-1, FB-2, and FB-3. In run FB-1, the specific growth rate had an oscillation around 0.03 h^{-1} (desired value) during the feed period (from 21 to 47 h of cultivation). In run FB-2, the specific growth rate was controlled at the desired value (0.10 h^{-1}) during the feed (from 14 to 24 h of cultivation). Finally, in run FB-3, the specific growth rate was controlled around 0.16 h^{-1}, between 13 and 19 h of cultivation (feed period), very near the desired value (0.17 h^{-1}), because of the exponential feed rate implemented.

Figure 3 shows biomass profiles during runs FB-1, FB-2, and FB-3. The biomass concentration reached a maximum value of about 7.8 g/L in run FB-1, similar to the other runs (7.3 g/L in run FB-2 and 7.6 g/L in run

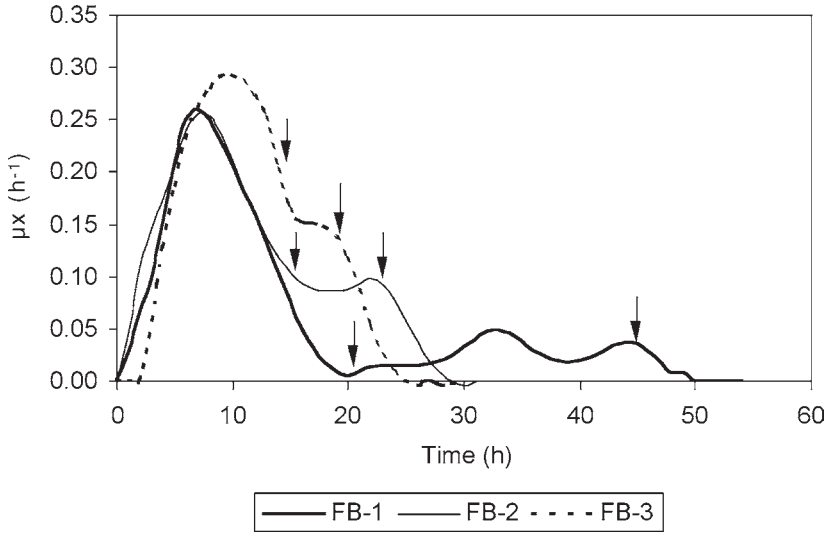

Fig. 2. Specific growth rate (μ_X) during fed-batch cultivations of *S. olindensis* (runs FB-1, FB-2, and FB-3). Arrows indicate the beginning and end of feed, in each run. Feed was performed between 21 and 47 h, between 14 and 24 h, and between 13 and 19 h of cultivation for runs FB-1, FB-2, and FB-3, respectively.

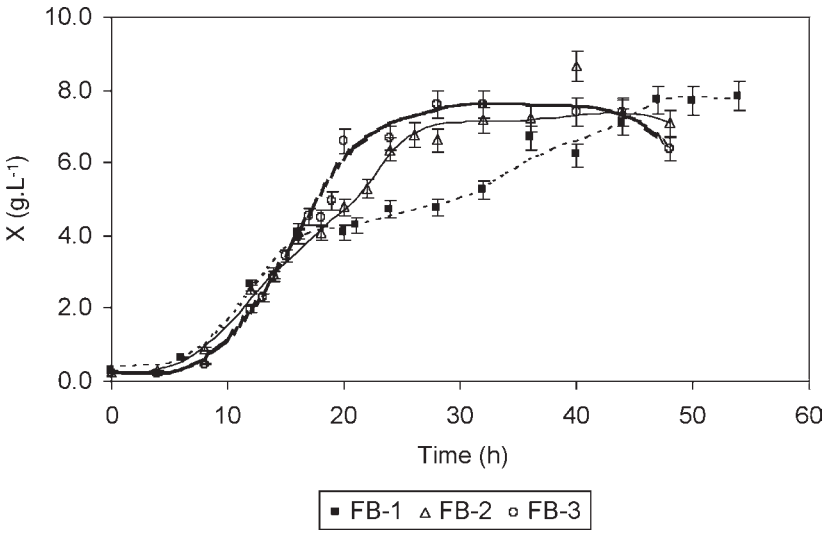

Fig. 3. Biomass concentration (*X*) during fed-batch cultivations of *S. olindensis* (runs FB-1, FB-2, and FB-3). Feed was performed between 21 and 47 h, between 14 and 24 h, and between 13 and 19 h of cultivation for runs FB-1, FB-2, and FB-3, respectively.

FB-3). In run FB-1, growth was slower than in the other runs during the feed period, because of the medium feed rate used, which led to a lower specific growth rate (0.03 h^{-1}) during the feed, in this run.

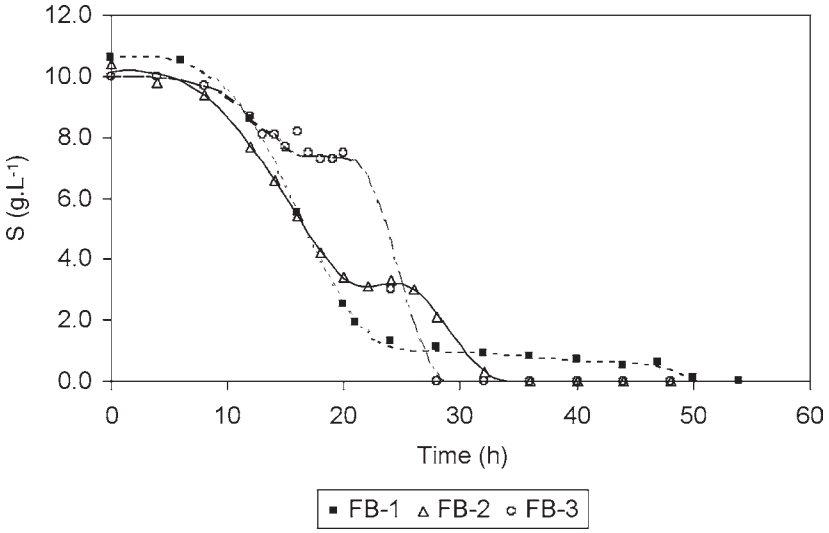

Fig. 4. Glucose concentration (*S*) during fed-batch cultivations of *S. olindensis* (runs FB-1, FB-2, and FB-3). Feed was performed between 21 and 47 h, between 14 and 24 h, and between 13 and 19 h of cultivation for runs FB-1, FB-2, and FB-3, respectively.

Regarding glucose concentration, the use of an exponential feed rate led to an almost constant limiting substrate concentration (glucose) during the feed period, as shown in Fig. 4. In run FB-1, the glucose concentration was controlled in a low value (0.6 g/L) during the feed. On the other hand, in run FB-2, glucose concentration was controlled in an intermediate value (about 3.4 g/L) during the feed. In addition, in run FB-3, in which a high specific growth rate was implemented, glucose concentration was controlled in a high value (about 8.0 g/L) during the feed. These results show that the use of higher exponential feed rates led to higher glucose concentrations in the bioreactor and, consequently, to higher specific growth rates. So, fed-batch processes are suitable to control the limiting substrate supply (glucose) to the microorganism during feed, which permits the control of the specific growth rate, since Monod kinetic growth model can be considered valid *(7)*.

Figure 5 shows retamycin concentration, in terms of absorbance units. In run FB-1, in which the specific growth rate was controlled in 0.03 h^{-1} (low value, corresponding to about 10% of the maximum specific growth rate), final retamycin production was about 3.0 units of absorbance. In run FB-2, in which the specific growth rate was controlled in an intermediate value (0.10 h^{-1}, corresponding to 33% of the maximum specific growth rate), final retamycin concentration was lower than in run FB-1 (about 2.4 units of absorbance). In run FB-3, the specific growth rate was controlled in a high value (0.17 h^{-1}, about 56% of the maximum specific growth rate), which led to a lower antibiotic production (0.7 units of absorbance). As a secondary metabolite, specific growth rate influences antibiotic production. Increas-

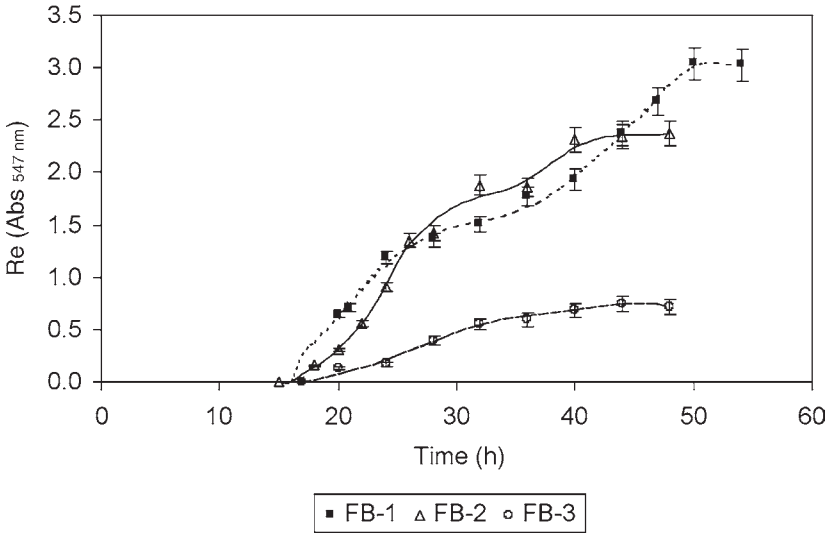

Fig. 5. Retamycin concentration (*Re*), in terms of units of absorbance, during fed-batch cultivations of *S. olindensis* (runs FB-1, FB-2, and FB-3). Feed was performed between 21 and 47 h, between 14 and 24 h, and between 13 and 19 h of cultivation for runs FB-1, FB-2, and FB-3, respectively.

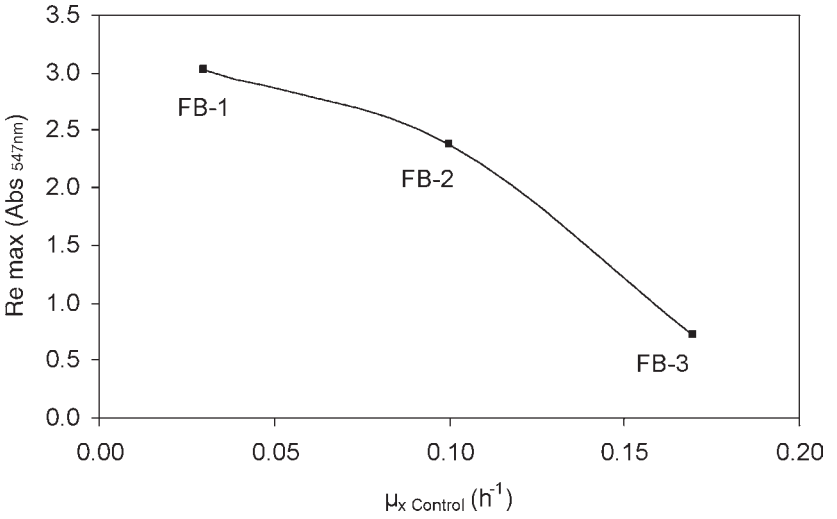

Fig. 6. Maximum retamycin concentration (Re_{max}), in terms of units of absorbance, during fed-batch cultivations of *S. olindensis* (runs FB-1, FB-2, and FB-3) as a function of specific growth rate of control.

ing specific growth rate usually reduces the secondary metabolite production *(6)*. This behavior is shown in Fig. 6, which represents the final retamycin concentration as a function of the controlled specific growth rate ($\mu_{x\,control}$).

Note that the feed period in run FB-3 represented only 20% of the period of retamycin production, different from runs FB-1 and FB-2, in which

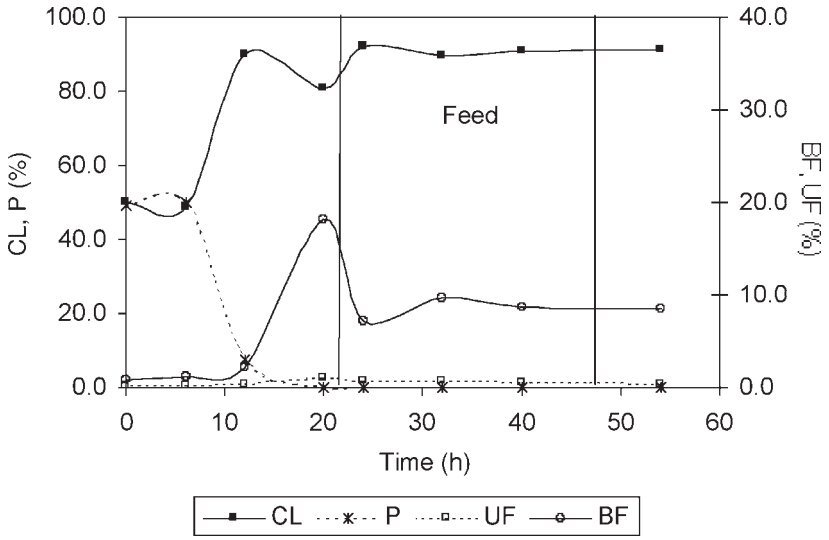

Fig. 7. Percentage of morphologic classes during run FB-1. Vertical lines represent the beginning (21 h) and end (47 h) of the feed period. CL, clumps; P, pellets; BF, branched filaments; UF, unbranched filaments.

the feed period represented 80 and 40% of the period of retamycin production, respectively. These feed periods, in which the specific growth rate was fixed, had to be necessarily distinct in order to guarantee that a same total amount of nutrients could be supplied in the three fed-batch runs. However, it should be emphasized that although the feed period in run FB-3 was shorter, the entire production process was prejudiced by the high specific growth rate fixed during the feed.

Figures 7, 8, and 9 show the evolution of each morphologic class (pellets, clumps, branched and unbranched filaments) during runs FB-1, FB-2, and FB-3, respectively. In a general manner, the percentage of pellets decreased during cultivation, owing to shear in the reactor caused by the impellers. On the other hand, the percentage of clumps increased during cultivation owing to disruption of the pellets (pellets were present in the bioreactor only up to 20 h of cultivation). At the end of runs FB-1 and FB-2, growth was mainly in the form of clumps (about 90% clumps and 10% free filaments). In addition, at the end of run FB-3, in which retamycin production was lower, the percentage of clumps was about 75% and the percentage of free filaments was about 25%. This difference in morphology observed in the fed-batch cultivations is an important factor in the antibiotic production, besides the specific growth rate, since the results obtained indicate that a high clump percentage favors retamycin production *(16)*.

In conclusion, fed-batch cultivations were shown to be a good strategy for conduction of the retamycin production process by *Streptomyces* cultivation. This strategy permitted the control of both the specific growth rate and glucose concentration during the feed period. Control of the specific

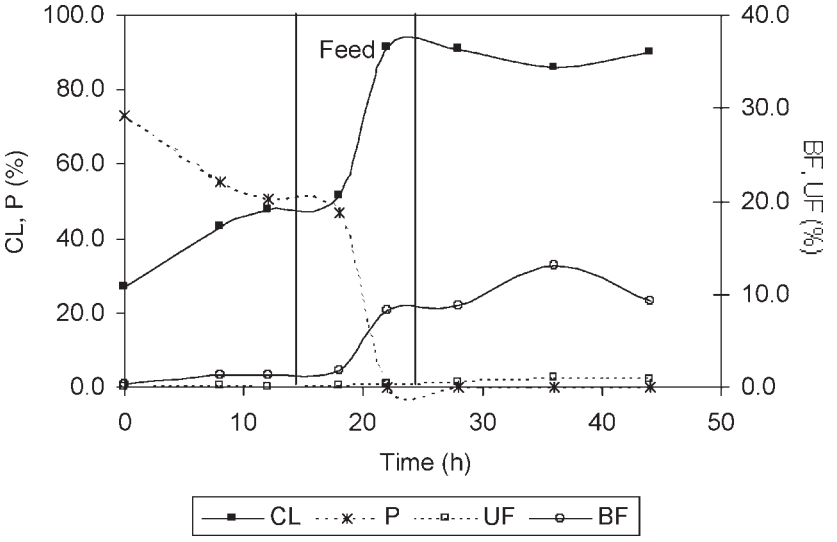

Fig. 8. Percentage of morphologic classes during run FB-2. Vertical lines represent the beginning (14 h) and end (24 h) of the feed period. CL, clumps; P, pellets; BF, branched filaments; UF, unbranched filaments.

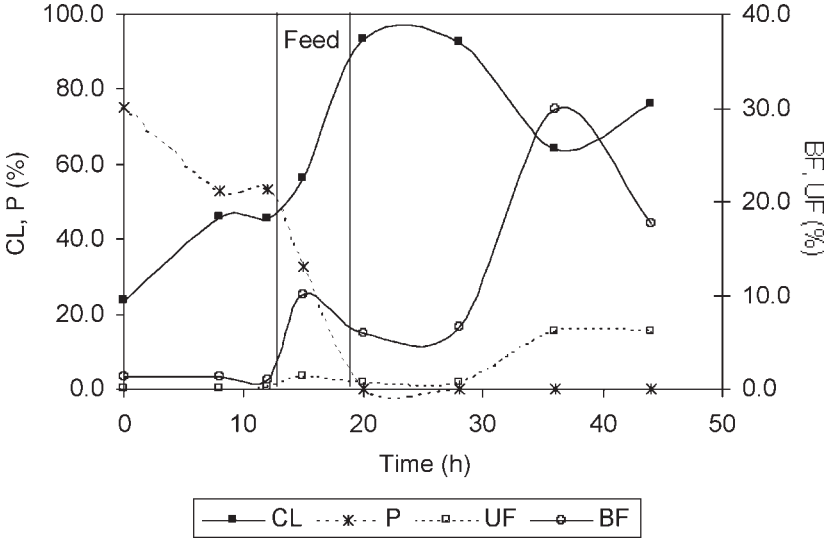

Fig. 9. Percentage of morphologic classes during run FB-3. Vertical lines represent the beginning (13 h) and end (19 h) of the feed period. CL, clumps; P, pellets; BF, branched filaments; UF, unbranched filaments.

growth rate at a low value (0.03 h^{-1}) led to a higher antibiotic production when compared with higher specific growth rates. In addition, the morphology predominantly in the form of clumps was favorable for the retamycin production process.

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