ACTA STEREOL 1994; 13/1: 215-220
PROC 6ECS PRAGUE, 1993
ORIGINAL SCIENTIFIC PAPER

CHARACTERIZATION AND GROWTH MONITORING OF FILAMENTOUS MICROORGANISMS BY IMAGE ANALYSIS

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ABSTRACT

The growth of filamentous antibiotic productive bacteria *Streptomyces ambofaciens* in submerged culture is monitored via analysis of light microscopy images. The method provides data on the size of the microorganisms and classifies their morphological types into three populations: single filaments, entangled filaments and pellets. Two different growth media are studied, the first one contains valin which is a precursor of the antibiotic (spiramycin) and the second contains an ammonium salt as nitrogen source. Both kinetics of the distribution in number and in projected area of the morphological types during the fermentation are obtained.

Key words : antibiotic, filamentous microorganisms, growth monitoring, image analysis.

INTRODUCTION

Filamentous microorganisms present a commercial interest for production of secondary metabolites. This microorganisms exhibit various types of morphology in submerged culture: free filaments, entangled filaments and pellets. Production of secondary metabolites has been linked, in some cases, to the morphological state of these filamentous microorganisms (Matsumura et al., 1980). The efficient control of bioengineering processes is severely limited by the lack of reliable sensors. More specially automatic devices able to determine the morphological state are still missing. Cell morphology is often linked to the process efficiency and on line process optimization could be done based on the control of the morphological changes of the microorganisms. Then it is necessary to develop tools to monitor shape of the cells in order to act on control variables to maintain the morphological state at its optimal set point.
Filamentous growth has been extensively explored by Packer and Thomas (1990) using image analysis. Pichon et al., (1991) describe some parameters to caracterize different types of pellets. The pellet morphology during submerged culture has been studied by image analysis (Reichl et al., 1992).
Our purpose is to study the morphological evolution of the microorganisms during culture using a descriptive approach in order to quantify the existing morphologies. A set of parameters (area, Euler number,...etc) has been selected to permit classification in three main populations. This technique has been applied to a culture of filamentous bacteria *Streptomyces ambofaciens* wich produces a macrolide antibiotic: spiramycin. Two different growth media have been studied. The first one contains valin as nitrogen source which is a precursor of spiramycin. The second medium contains ammonium salt as nitrogen source.

MATERIALS AND METHODS

Culture conditions

The filamentous bacteria *Streptomyces ambofaciens* have been cultured in different growth media. The inoculum culture and the culture are carried out in Erlenmeyer flasks. The carbohydrate source is glycerol at 10-g/L in all cases. The nitrogen source of the first medium is an amino acid: valin at 6-g/L. This medium allows the production of spiramycin. In the second medium the nitrogen source is an ammonium salt which does not allow the antibiotic formation in the choosen conditions.
A 2-mL sample is regularly withdrawn from the flask of each cultures. 1-mL is used for dry weight measurments. 1-mL is diluted with water. 0,5-mL of the diluted sample is stained with 50-µL of an alcoholic Cristal Violet solution and with 50-µL of Ziehl Fuschin solution. A 100-µL solution is transfered on a glas slide.

Image capture

The images are captured by a Bosch™ video camera (chalnicon tube) with 256 grey levels through a professionnal package (Visilog™ by Noesis, Velizy, France). The digitized images (512 lines of 512 pixels, rectangular grid) are processed by means of Visilog™ or locally developed software on a SUN 3/110 colour workstation (8Mb memory, matrox VIP 1024 acquistion board) connected to the laboratory network.

Image analysis

The purpose of image analysis is to classify objects in the image into three classes: free filaments, entangled filaments and pellets. The first stage is aimed to the determination of the core of pellets. A grey level opening increases the dark part of the pellet, which corresponds to the core, darker than the filamentous elements. Thresholding, erosion and opening finally give a binary image (A) containing cores of the pellets. Different cores are referenced by a label (image A_{lab}).

In The second stage, the filamentous part is retrieved by applying a top-hat transformation: the resulting image B is the thresholded difference between the closed and original images.

Image C is a result of a logical OR operation between images A and B: image C contains objects existing in A or in B. The objects of C are referenced by a label. They are analyzed and their compacity [perimeter/(4π.area)] is computed. A filter is applied to eliminate all the objects in C with a compacity lower than a preset threshold. This procedure eliminates the broth debris with a spherical or ovoidal shape. The corresponding labelled image is D_{lab}. The results of analysis of images A_{lab} and $D_{lab}$by Visilog™ is stored into two respectives ASCII files which contain all the information necessary for classification.
A locally-developed software reads the ASCII files and calculates classification of the microorganisms in three classes.
- an object of D_{lab} is a pellet if it has a corresponding core in A_{lab}.
- if an object of D_{lab} is not a pellet it is an entangled filament if it has holes. The number of holes of an object is calculated from the Euler number.

Fig. 1. presents the main three different types of morphological shapes.

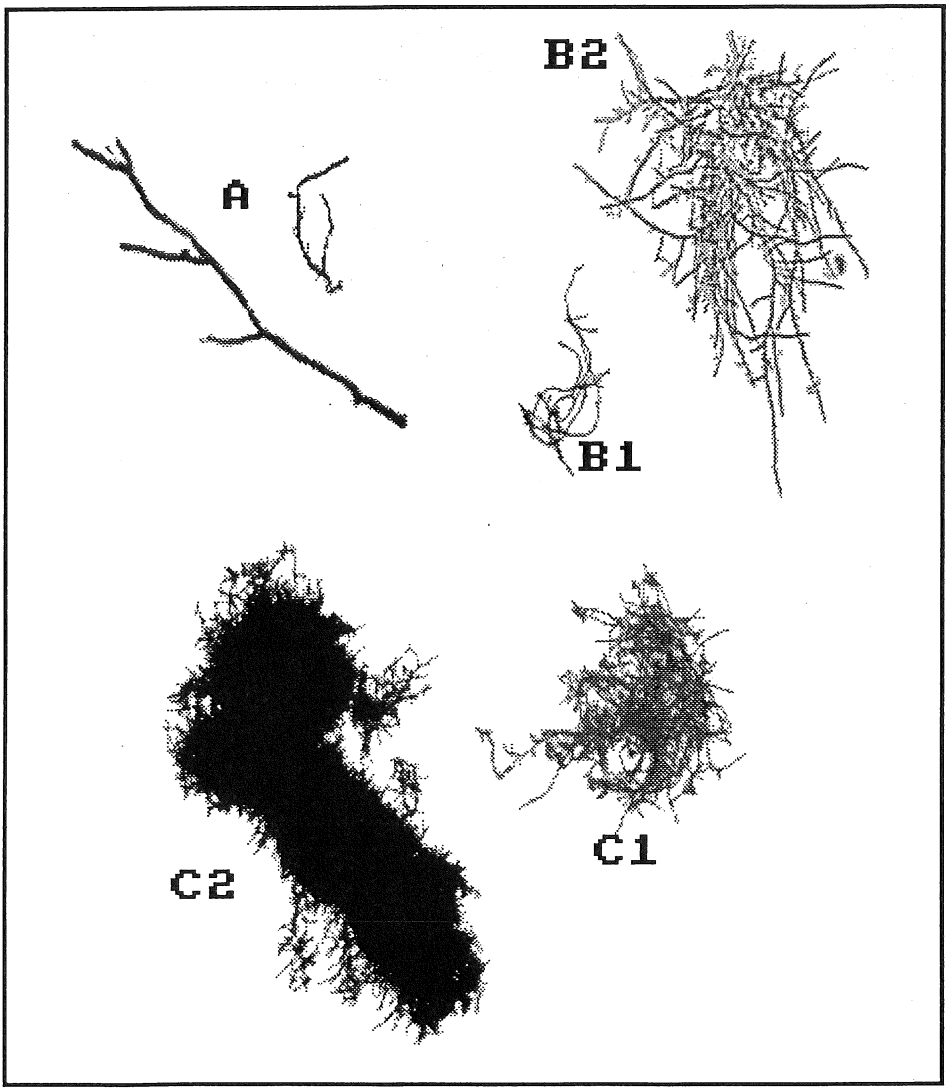


Fig. 1. The main three types of recognized morphological shapes:
single filaments (A), entangled filaments [small (B_1) and large (B_2)], pellets [pellet with small core and large hair (C_1) and pellet with large core and small hair (C_2)]

RESULTS

The stirred flask culture of *Streptomyces ambofaciens* exhibits three phases: growth, stationnary and death (Fig.2). Generally the behaviour of both cultures is different because the rate of growth is faster in the ammonium salt medium (Fig.2.b) and the stationnary phase is longer in the valin-based medium. The death phase is the same in both cultures. In the medium with valin, the production of spiramycin appears in the middle of the stationnary phase (Fig. 2.a).

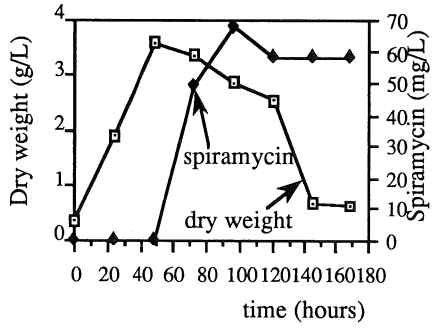
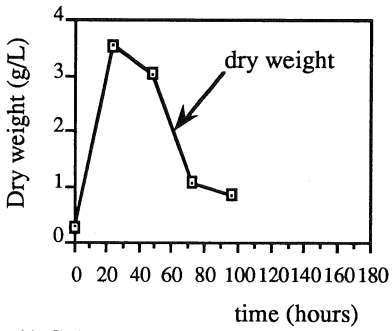


Fig. 2. a)Culture with valin growth medium and spiramycin concentration



b) Culture with ammonium salt growth medium

Kinetics of the three morphological types is shown in Fig.3 in terms of projected area distribution in both cultures. For the three stages (growth, stationnary and death) pellets occupied more than 90 % of the biomass whereas filaments and entangled filaments occupied the other part. Just after the death stage the non-pellet population occupied a significative part of the biomass (≈ 20%).

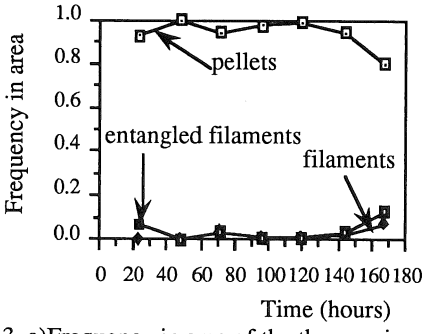
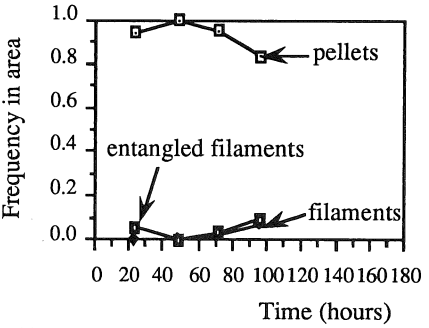


Fig. 3. a)Frequency in area of the three main shapes in culture medium with valin



b) Frequency in area of the three main shapes in culture medium with ammonium

Fig.4 presents the kinetics of the three morphological types in terms of frequency in number in the culture with valin-based medium.

The frequency in number of pellets is maximum (80%) in the end of the growth phase, but during the stationnary phase it decreases severely (20%). It is close to zero at the end of the culture.

The frequency in number of filaments increases between the end of the growth phase and the beginning of the stationnary phase. During the stationnary phase the frequency in number of filaments remains constant (70%). The frequency in number of the entangled filaments remains nearly constant during the culture. Increase of the frequency in number of filaments is concomitant to the production of spiramycin.

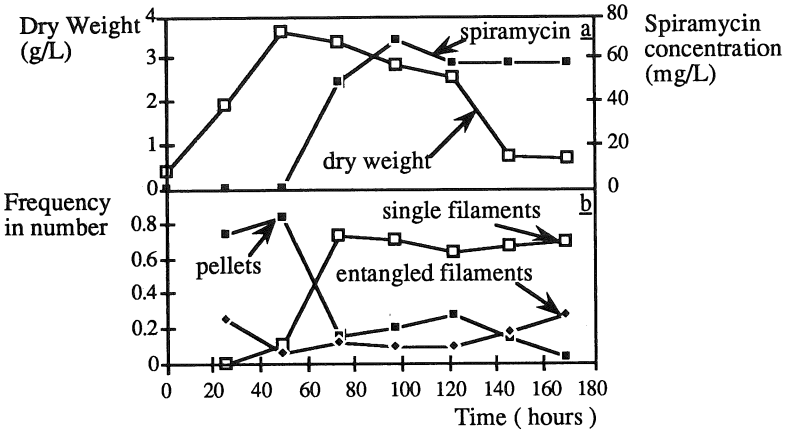


Fig.4. Culture on valin growth medium
a) kinetics of growth and production of spiramycin
b) kinetics of the frequency in number of the three main shapes

Kinetics of the three morphological types on the ammonium salt medium is shown in Fig.5. In the end of the growth stage the frequency in number of pellets is 48%. Filaments and entangled filaments are present (28% and 24% respectively). During stationnary and death phases the frequency in number of filaments increases and reaches its maximum at the end of the culture. The frequency in number of pellets decreases during the culture whereas the frequency in number of entangled filaments remains almost constant.

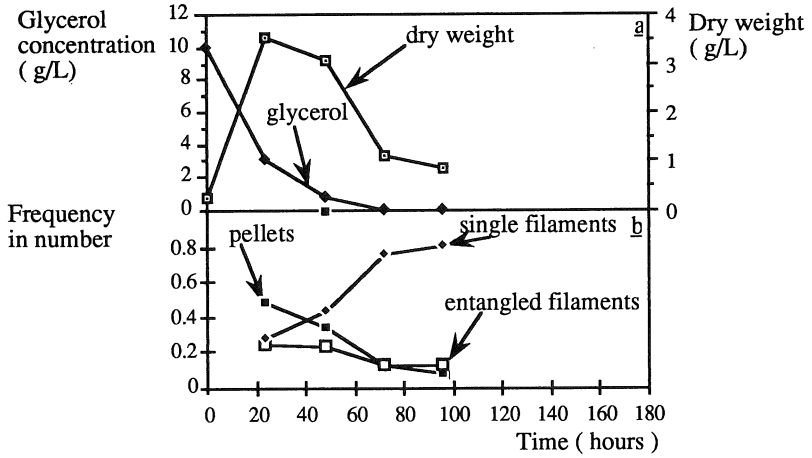


Fig.5. Culture on ammonium salt growth medium
a) kinetics of growth and consumption of glycerol
b) kinetics of the frequency in number of the three main shapes

DISCUSSION

The behaviour of the frequencies in number of the main three populations in both cultures is similar. However there is a difference because these behaviours do not appear in the same phases of the culture. Evolution of the frequency in number of filaments on the ammonium growth medium is slower than on valin-based medium.
Kinetics of the morphological families are different according to the nitrogen source: valin or ammonium salt.
In the valin-based medium, spiramycin is produced in the stationnary phase where a large increase of the number of filaments indicates a very important morphological modification.
There is no correlation between the increase in the number of filaments and spiramycin production in view (Fig.4).
There is no basic difference between both morphological evolutions. Nevertheless, when valin is used as nitrogen source the increase in the number of filaments is a response to physiological modifications leading to spiramycin production.

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