THE EFFECTS OF INOCULUM SPORES CONTENT ON BOTH *PENICILLIUM CHRYSOGENUM* PF (PELLETIZED FORM) MORPHOLOGY AND PENICILLIN BIOSYNTHESIS AT INDUSTRIAL SCALE

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Keywords: *Penicillium chrysogenum*, pelletized form, spore, morphology, penicillin, biosynthesis.
Abstract: The present paper is intended to provide a correlation/relationship between the morphological aspect of the mycelia produced by *Penicillium chrysogenum* strain (pelletized form) and the spores concentration of the inoculum utilized to seed the culture medium for antibiotic biosynthesis. In case of *Penicillium chrysogenum* strains, the increase of the biosynthesic potential is determined by the close relationship between the genotype features/characteristics and the cultivation conditions.

INTRODUCTION

In submerged cultivation, just after spores germination, hyphae begin to occur with dichotomic branching thus resulting in thick, spherical agglomerations known as pellets or glomerules. Under normal circumstances, a germinating spore generates just a single pellet, that gradually increases in size (diameter) and density as the penicillin biosynthesis stages proceed. Toward the end of the biosynthesis cycle, fragments of terminal hyphae separate themsellves from the pellet structure with no further new development process being started, so that the total numer of pellets remains unchanged. In close relationship with the number of spores in the inoculum, during the biosynthesis stage the pellets follow various morphological evolutions, ranging from fluffy, lax and spherical shapes with borders formed of elongated, thickened hyphae to dense, spherical pellets with boundaries formed of short, thick hyphae with growing button-like tips. The fine structure of resting and germinating conidia of *Penicillium chrysogenum* has been examined by electron microscopy (Jünsten P. *et all.,* 1998). In addition to enlargement of the cells, a number of changes in ultrastructure become evident as morphogenesis proceeds. The newly synthesized germ tube is continuous with the corresponding layers of the conidial wall. Some conidial wall layers, however, do not extend into the hyphal wall. Several sections showing initial septum synthesis suggest that a septal pore is not a necessary structural entity. A characteristic orientation of the initial septum formed after germination is described. Aside from numerical considerations, no significant changes occur in nuclei, mitochondria, or ribosomes. Image analysis has been used to investigate the effect of spore inoculum concentration on the subsequent morphology of *Penicillium chrysogenum* pelletized form and on the penicillin biosynthesis at industrial scale grown in both shake flasks and a 6L agitated bioreactor. Even with the same inoculum concentration, differences were found between the two systems in the eventual morphology of both the freely dispersed mycelia and the mycelial aggregates or „clumps” (Taguki, H. *et all.,* 1968).

MATERIALS AND METHODS

In order to ensure genetic stability of the *Penicillium chrysogenum* PF*,* the storage of the sporulated biological material is carried out/performed in a deep-freezer at -80^{o}C.

The deep-freezer working parameters are continuously recorded on a diagram. The maximum storage period is 5 years; the storaged biological material is monitored for viability and genotypal stability studies are performed during every calendar year.

Spores suspension preparation. The harvest of spores from the cultures grown on specific culture media in glass containers is carried aut by means of sterile Tween 80 solution. In every container to be washed, an adequate amonut of sterile solution is aded in 2 portions for every container. The procedure is as follows:

- in the container to be washed is added sterile Tween 80 solution, then the culture content is homogenized by means of a metal spatula under sterile conditions;
- the glass container is slowly rotated so the washing solution cover the entire inner surface, then the solution is poured in the stock container;

- the remaining amount of Tween 80 solution is added so that the culture surface is thoroughly washed out of spores;

Thus a final volume of harvesting solution is obtained. The spores should be harvested in the proximity of the utilization place.

Working parameters, i.e. temperature, humidity, maintained during the incubation period are important factors for a good sporulated biological material (Lazăr, V. *et all.,*1979).

Spore counting technique

Materials: sterile, calibrated glass pipettes; Neubauer counting chamber; microscope equipped with objective lens (10x or 40x); spore counting standard form; etanol solution 70% for counting camera and microscope slide disinfection; sterile glass tubes 15 ml or 18/180 mm for dilution preparation.

Spore counting procedure. Prior the use, the Neubauer counting chamber and the cover device should be cleaned and dried.

In case the Neubauer counting chamber is not fitted with a covering evice, dry and clean thin slides (22 x 22 x 0.7mm) may be used (*Figure 1*).

The spores counting is performed in several phases (Vardar, F., Lilly., M.D., 1982) as follows:

1. A serial dilution of spore suspensions (e.g.: of 10^{-1}, 10^{-10}) is prepared with 0.01% Tween-80 solution.
2. The cover of the counting chamber (or the thin glass lamella) is applied so that both counting areas to be covered.
3. The dilution of spore suspension is thoroughly homogenized.
4. By means of a pipette, a drop of suspension is spread over both niches of the counting chamber ensuring that the glossy areas are entierely covered by suspension, but not in excess.
5. The counting chamber is fitted on the microscope table and adjust the objective lens (10x or 40x) for an accurate visualization.
6. Focus the image on the overlapped lines under the objective lens.
7. Before the counting, allow the spores to settle down for few minutes for image stabilization.
8. Only the spores dwelling within the the 25 squares of the counting area should be counted.

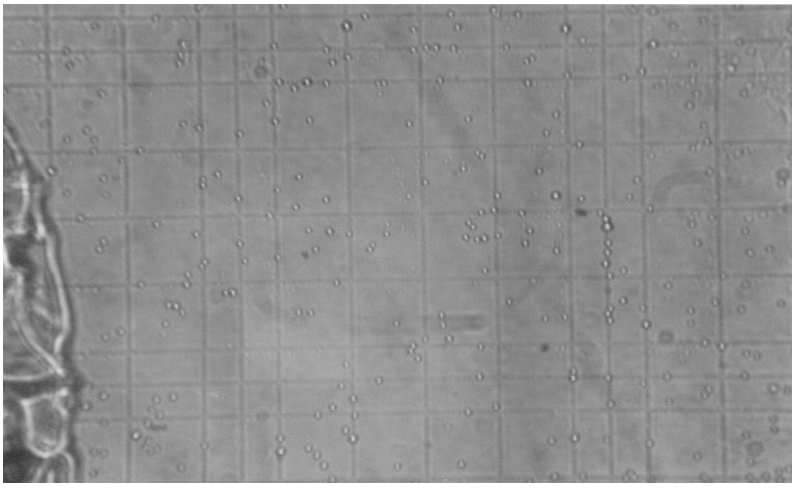

Figure 1. Microscopic aspect of the spore content counting in the spore suspension utilized for *Penicillium chrysogenum* PF inoculum with a Neubauer counting chamber

9. The spores number for each side of the counting chamber is recorded.

10. Calculate the average value of 2 spores counting's. At least 200 spores are selected for counting. Should be less than 200, another dilution is to be checked. A working standard form is used in order to facilitate the spores counting and subsequent calculations imposed by the optimum number of spores required for inoculum's preparation.

The number of spores per ml (N) is calculated by the formula:

$$\mathbf{Z \times 1/10^{-4} \times 1/Y = spores\ count\ /ml\ (N)}$$

where:

Z = average of spores counts / square (16 small squares/largesquare)

10^{-4} = overall volume of all 25 squares of the Neubauer counting chamber (ml)

$V = 1mm \times 1mm \times 0,1\ mm = 0,1\ mm^3$

$1mm^3 = 0,001$ ml

$V = 0,1\ mm^3 = 0,0001$ ml (10^{-4} ml)

Y = utilized dilution (e.g.: 1/10, 1/100 etc.)

The performed counting is overchecked, including the calculations, according to the working formula (Demain, A.L., Solomon, N.A., 1985).

RESULTS AND DISCUSSION

During the first stage of germination of spores from *Penicillum chrysogenum* PF*,* the break of spore wall may be initiated at several sites accompanied by occurence of sprouts of vegetative growth that will lead to formation of pellets, glomerular structures of short, thickened, dichotomised branched hyphae (*Figure 2*).

The morphological evolution of pellets over a penicillin biosynthesis cycle may differ and it is strictly related to the concentration and viability of the spores present in inoculum used to seed the culture broth (Crueger, W., Crueger, A., 1992).

The optimum spores concentration to be used for a penicillin biosynthesis stage, in order to ensure a maximum antibiotic potency as related to the mycelium mass in the culture broth (also correlated with the working parameters and nutritional factors) ranges from 1×10^{10} to 1×10^{12} spores/mc. The assessment of an adequate spores concentration (taking into account a 85% viability of the sporulated biological material stored by freezing at -80^{o}C) represents a controlling factor for the growth dynamics during the penicillin biosynthesis stage.

As compared to the filamentous morphology, the pelletized one favourably influences the culture broth viscosity and, implicitly, the biosynthesis kinetics due to much improved characteristics of dissolved oxygen, nutrients and precursors transfer at the hyphae level.

Due to fluctuations that might occur through an improper corelation in the ratio spores number vs culture broth volume, the morphological structure of the pellets may exhibit the following features:

- laxed pellets (fluffy pellets), whose aspect seem close to the filamentous morphology, are formed by an inoculum with spores concentration under 1×10^{10} spores/mc (*Figure 3*).

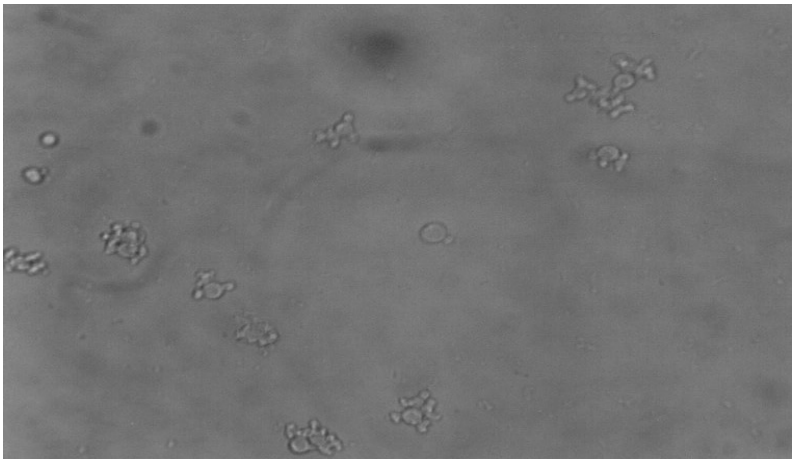

Figure 2. Germination of spores from *Penicillium chrysogenum* PF

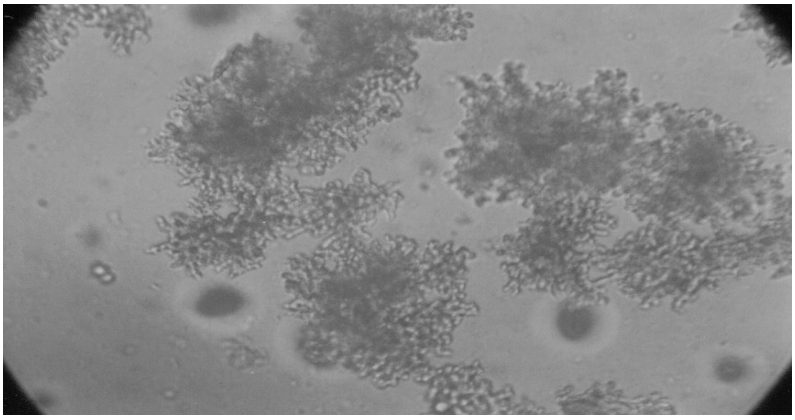

Figure 3. Laxed morphological structure pellets

- large sized pellets with compacted consistency (250 - 500 μm) result from the use of an inoculum having a spore concentration of 1 x 10^{10} ÷ 1 x 10^{12} spori/mc (*Figure 4*).

Figure 4. Large pellets with compacted morphological structure

- small sized pellets with compacted consistency (100 ÷ 150 μm) produced by inocula with spores concentrations above 1 x 10^{12} spores/mc (*Figure 5*).

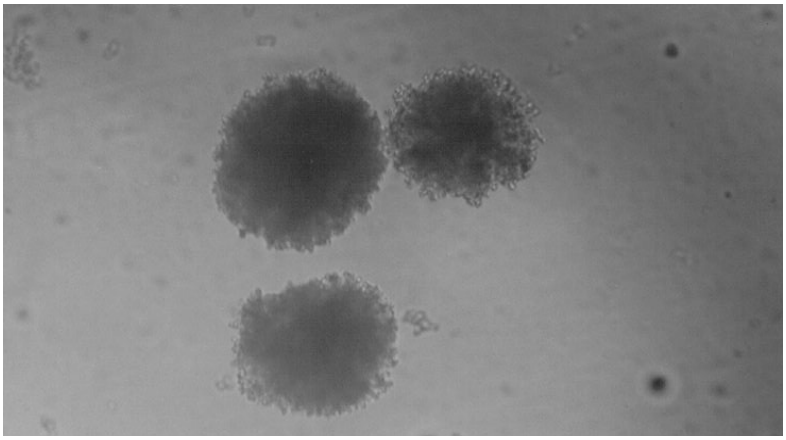

Figure 5. Small sized pellets with compacted morphological structure

The volume of inoculum utilized for seeding the culture broth for penicillin biosynthesis by the *Penicillium chrysogenum strain – Pelletized Form* is established by means of the ratio between inoculum spores concentration and the culture broth volume.

If, by accident, due to an improper assessment of the spores count/culture broth volume ratio, then the morphological structure of the pellets may exhibit the following peculiar features:

- pellets with laxed consistency (fluffy pellets), closely related to the filamentous morphology (spores concentration is under 1 x 10^{10} spores/mc).

- small sized pellets (diameter 100 ÷ 150 μm) with compacted consistency (spores concentration is above 1 x 10^{12} spores/mc).

CONCLUSIONS

The storage of the sporulated biological material resulting from the *Penicillium chrysogenum strain Peletized Form,* spread on glycerol-based cryoprotecting media at -80^{o}C have a 5 years valability and up to 85% viability. The check and remaking of the sporulated biological material stock is carried out at every 3-5 years.

Implementation of the spore counting technique by means of a Neubauer counting chamber in the industrial inoculum preparation technology for the penicillin biosynthesis ensures a proper reproduction of the cultivation conditions also an increaased antibiotic concentration at the end of the biosynthesis cycle.

With reference to the micelia mass growth dynamics, the main feature of the *Penicillium chrysogenum strain Peletized Form,* can be expressed by the relationship: **one spore = one pellet.**

As compared to the filamentous morphology, the pelletized one favourably influences the culture broth viscosity and, implicitly, the biosynthesis kinetics due to much improved characteristics of dissolved oxygen, nutrients and precursors transfer at the hyphae level.

The pellet morphological structure is directly influenced by the ration between spores concentration of inoclum and the culture broth volume. In case the spores concentrations fall within the range $1 \times 10^{10} \div 1 \times 10^{12}$ spores/mc, the ratio biomass/pellet diameter/antibiotic concentration reaches a maximum.

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