STABILIZATION OF THE *PENICILLIUM CHRYSOGENUM* STRAIN WITH PELLETIZED MORPHOLOGICAL STRUCTURE BY SELECTION, MULTIPLICATION, AND CONTROL OF THE VARIABILITY OF PURE CULTURES

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Abstract: This study showed that *Penicillium chrysogenum* exhibits during submerged cultivation two distinct features: a filamentous structure characterized by long, branched, loose hyphae, and a pelletized morphological structure characterized by short, thickened hyphae clustered in a glomerule-like formation called pellet. With a view to maintaining the potential to biosynthesize the pelletized strain penicillin to a maximum, the operations involving the selection, multiplication and variability control of pure culture should be conducted periodically to prevent phenotypic and genotypic deviations to parental forms.

INTRODUCTION

This technological process yielding the biological material for penicillin biosynthesis uses a highly productive strain of *Penicillium chrysogenum* with pelletized morphological structure achieved by an integrated program of mutagenesis and cultivation on growth media under strong selective pressure (Crueger, W., Crueger A., 1992).

To be able to reproduce the penicillin biosynthesis results at the highest potential of the strain, the operations involving selection, multiplication, and control of the variability of pure cultures are performed periodically in order to prevent phenotypic and genotypic deviations to the parental forms (Veenstra, A.E. *et all.,* 1991).

MATERIALS AND METHODS

Of the techniques used to preserve the biological material of *Penicillium chrysogenum* with pelletized morphological structure, three can be employed, as follows:

- refrigeration of the spore suspension prepared from the 1st or 2nd generations at -80ºC;
- lyophilization of the spore suspension;
- suspension of conidia grown on crushed corn grains or rice grains on quartz crystals.

The three conservation methods are recommended for most laboratories producing biological material used in penicillin biosynthesis (Taguki, H. *et all.,* 1998).

Strain selection and multiplication

According to the method applied for strain preservation, the selection operations are conducted as follows:

- if a vial containing spore suspension refrigerated at -80ºC is used, take the vial out of the freezer, transfer it to the laminar air flow hood, keep at room temperature for approximately 1 hour, and then prepare serial tenfold dilutions using sterile distilled water;
- if a vial containing lyophilized material is used, suspend the contents of the vial in 5 ml of sterile distilled water, stir until complete dissolution of the spore suspension, and prepare serial tenfold dilutions using sterile distilled water;
- if spores suspended on quartz powder are used, collect 1 g of quartz powder using a spatula, transfer to a test tube containing 9 ml of sterile distilled water, stir the contents of the test tube for 2-3 minutes using a tube shaker; a spore suspension forms while the quartz sand settles immediately on the bottom of the test tube. Prepare from the resulted suspension serial tenfold dilution with sterile distilled water.

From the three strain preservation techniques, the conidia suspension on quartz is the most commonly encountered one when selecting the clones with high potential for penicillin biosynthesis.

Materials and equipment needed for the selection operations: Conidia suspension on quartz powder (spore deposit on sand); sterile test tubes (18/180 mm); sterile distilled water; Petri dishes (Ø = 10 cm); specific agar medium; sterile pipettes and spatulas; VELP-type test tube shaker; laminar air flow hood; thermostat with temperature adjustable to 25ºC; dehumidifier for humidity control.

Procedure:

Using a calibrated stainless steel spatula collect 1 g of spore-containing quartz powder in sterile conditions, suspend in 9 ml of sterile distilled water, stir the contents for 2- 3 minutes using a VELP type test tube shaker. Prepare

serial dilutions from 10^{-1} to 10^{-7}. The optimal concentration of pure colonies grown on Petri dishes is obtained with dilutions between 10^{-3} and 10^{-5}. At the 10^{-3} dilution, 30-40 pure colonies are obtained (Figure 1), while the 10^{-5} dilution yields 12-15 pure colonies (Figure 2). Inoculate the specific growth medium in the Petri dishes with 0.15 ml of spore suspension spreading it evenly onto the surface of the solid medium using a sterile triangular glass spatula. For each dilution, the spore suspension should be spread on 6-7 Petri dishes. Wrap the dishes, label them and incubate in the thermostat at 25ºC and 50% (± 5%) humidity for 8-9 days. Store the dishes for 24 hours in normal position, then invert them and keep them as such for the following 7-8 days (Crueger, W., Crueger A., 1992).

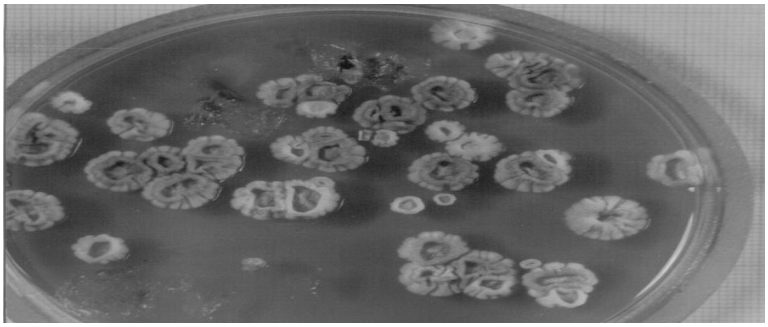


Figure 1. Appearance of pure *Penicillium chrysogenum* colonies with pelletized morphological structure at 10^{-3} dilution stored in the thermostat for 9 days

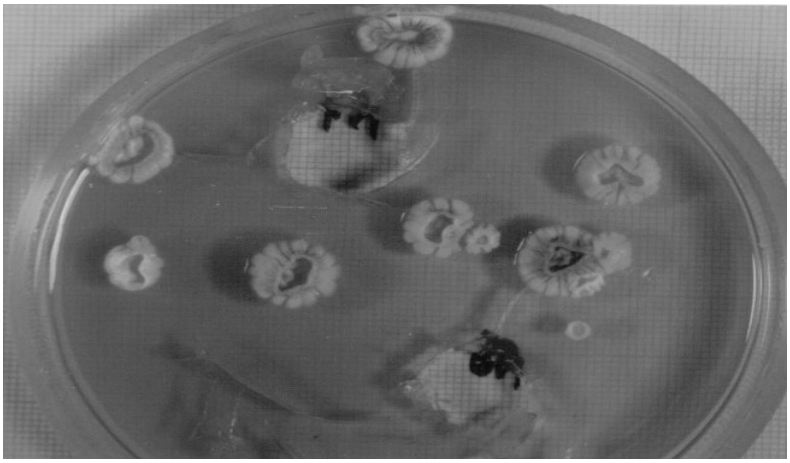
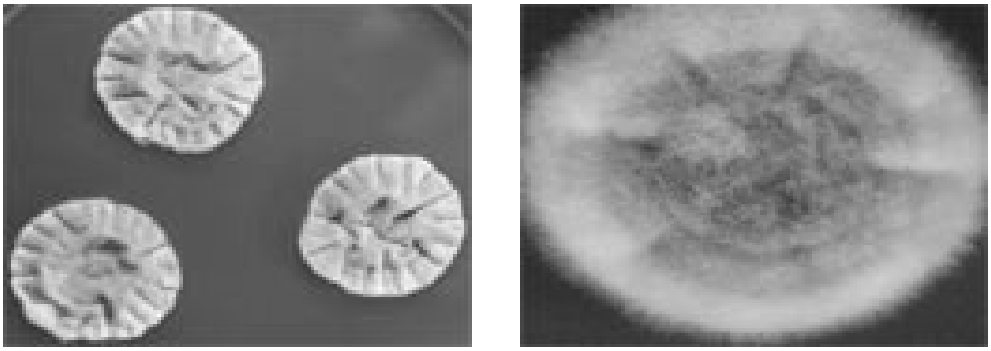


Figure 2. Appearance of pure *Penicillium chrysogenum* colonies with pelletized morphological structure at 10^{-5} dilution stored in the thermostat for 9 days

RESULTS AND DISCUSSIONS

Compared to the *Penicillium chrysogenum* strain with filamentous morphological structure (Figure 3), the *Penicillium chrysogenum* strain with pelletized morphological structure exhibit distinct morphological features important for the selection of pure cultures.



(a) (b)

Figure 3. Appearance of pure *Penicillium chrysogenum* cultures with filamentous morphological structure. Five- (a) and seven-day (b) cultures

Description of the pure *Penicillium chrysogenum* cultures with pelletized morphological structure (Figure 4):

- The colonies have a basal diameter of 9-12 mm and a height of 3-4 mm. In the middle, they exhibit a high volcanic cone-like prominence with a 4-5 mm wide crater with a depth that reaches the immediate proximity of the growth medium;
- Only the upper part of the volcanic cone exhibits green colored sporulation, while the margins of the colony are colored in white on a 2-3 mm surface;
- The colony reverse is colorless. The substrate of the growth medium is not pigmented;
- Only the colonies exhibiting a volcanic cone developed in depth to the proximity of the agar medium are considered appropriate pure colonies;
- The flat colonies are not selected as it is an indication of colony aging;
- The pure colonies are selected after 9 days of cultivation under controlled conditions of temperature and humidity.

Controlling the variability of the *Penicillium chrysogenum* strain with pelletized morphological structure:

The genetic stability of the strain is controlled by selecting the specific morphological characters and HPLC testing for penicillin biosynthesis potential in submerged culture.

To stabilize the morphological characters and examine variability, use a spore deposit on quartz powder. Prepare dilutions as described above. For each dilution, inoculate 10 Petri dishes containing specific agar medium with 0.1 ml of spore suspension in dilution from 10^{-1} to 10^{-7}. To prevent errors in counting the pure colonies grown on the agar medium, follow strictly the procedure for counting the spores in the suspension and accurately prepare the serial dilutions.

At the end of the 9 days of incubation, count the colonies taking into consideration that at least 800 colonies should be counted to reach an accurate result. The average number of viable conidia found in a gram of quartz is 2.5 to 3.5 million. The number of conidia in the suspension depends on the conditions of spore cultivation on the crushed corn or rice grains.

Figure 4. Appearance of pure *Penicillium chrysogenum* cultures with pelletized morphological structure after 9 days of growth

The number of colonies exhibiting atypical morphology should not exceed 8-10% of the total colonies grown on the agar medium.

If the above criteria are not met, the vials containing spores on quartz powder will be removed from the production cycle.

CONCLUSIONS

From the morphological point of view, *Penicillium chrysogenum* exhibits during submerged cultivation two distinct features: a filamentous structure characterized by long, branched, loose hyphae, and a pelletized morphological structure characterized by short, thickened hyphae clustered in a glomerule-like formation called pellet.

The morphological variability of the microorganisms producing active pharmaceutical ingredients is determined by their genetic structure.

The *Penicillium chrysogenum* strain with pelletized morphological structure is achieved by an integrated mutagenesis program and cultivation on growth media with strong selective pressure.

With a view to maintaining the potential to biosynthesize the pelletized strain penicillin to a maximum, the operations involving the selection, multiplication and variability control of pure culture should be conducted periodically to prevent phenotypic and genotypic deviations to parental forms.

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