Storage of Conidia of *Penicillium chrysogenum* in Liquid Nitrogen

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Conidiated slope cultures of a derivative of *Penicillium chrysogenum* Wis 54-1255 were stored at -196 or +4 C for a period of 3.5 years. After this time, the viability fell to 68% in the former case and to 4% in the latter. At the end of the experiment, 65 single conidial isolates from each series were tested for penicillin yield. Among those from conidia stored at -196 C, the spread of penicillin yields did not differ markedly from that of 65 single conidial isolates made as controls prior to storage. However, 18% of those from conidia stored at +4 C formed a subpopulation with substantially lower penicillin titers than those of control isolates. Storage at -196 C may reduce or prevent a possible source of penicillin yield decay, namely, the selection of spontaneous mutants of low titer present in small numbers in the original culture and selected, as viability decreased, by virtue of their increased longevity relative to that of the parental culture.

Microbial mutants giving increased amounts of commercially useful products are of obvious interest, and it is clearly important that their genetic integrity be maintained in culture. Whether or not this can be done will depend on their spontaneous mutability and the environmental pressures to which they are subjected. For example, such mutants can be susceptible to yield decay during replication so that serial subculturing leads to a reduction in productivity (1, 2, 4, 14). Even the preservation of a culture in the quiescent state does not necessarily avoid a drop in product yield if viability decreases during storage (4).

It is normal industrial practice to avoid serial subculturing, and, from a master culture, subcultures are made in parallel to establish separate fermentations. The preservation of a culture under conditions where there is little or no loss of viability might be a way of preventing yield decay during storage (4, 6, 10).

Previous work indicated that when a strain of *Penicillium chrysogenum* producing a relatively high yield of penicillin was stored at +4 C, mutants with low titers accumulated as viability fell (4). Heterokaryon tests (3) showed that these were nuclear rather than cytoplasmic in origin. There were two possibilities, neither being mutually exclusive: the selection of pre-existing mutants with low penicillin productivity because of their increased longevity relative to the parent or the induction of such mutants by the storage conditions. The present paper reports results with the same strain of *P. chrysogenum* when a comparison was made of preservation at temperatures of +4 and -196 C. Studies by Wellman (13) indicated that when conidiated slope cultures of two strains of *P. chrysogenum* were stored for 38 months in liquid nitrogen, conidial survival approached that of control cultures, when estimated as per cent germination. Fermentations derived from mass conidial inocula of material stored in liquid nitrogen yielded similar amounts of penicillin to controls. The work reported here is an extension of Wellman's studies in that a strain with a higher penicillin titer was used and also tests were made of single conidial isolates after storage to discover whether there were population changes in the pattern of penicillin yield not detectable by mass conidial transfer.

**MATERIALS AND METHODS**

**Organism.** From *P. chrysogenum* Wis 54-1255 (11), a mutant was produced after three serial ultraviolet-light treatments (5) which had brown conidia and requirements for biotin and nicotinamide. This mutant was used in the work reported here, and conidia from a lyophilized culture were dispensed on CM (see below) so that separate colonies grew after incubation. A single colony was then sown on to 10 CM slopes. After incubation, one was set aside as master culture and the remaining nine when tested
for penicillin yield, as described later, averaged about 3,000 units/ml, which was similar to that of the parental culture Wis 54-1255 tested under the same conditions.

**Media.** Minimal medium agar (MM) had the following composition (9): NaNO₃, 6 g; KCl, 0.52 g; MgSO₄·7H₂O, 0.52 g; KH₂PO₄, 1.52 g; FeSO₄·7H₂O, 0.01 g; ZnSO₄·7H₂O, 0.01 g; glucose, 10 g; agar (Oxoid no. 3), 12 g; distilled water to 1 liter. The pH was adjusted to 6.5, and the media was autoclaved at 115 C for 10 min. When necessary, MM was supplemented with biotin and nicotinamide at levels of 1 and 100 µg per liter, respectively. Complete medium agar (CM) was made as follows (5): KCl, 0.5 g; MgSO₄·7H₂O, 0.5 g; FeSO₄·7H₂O, 0.01 g; KH₂PO₄, 1 g; corn steep liquor, 10 g; 3 ml each of thymus and yeast nucleic acid hydrolysates (9); DL-methionine, 0.06 g; phenylacetyl-ethanolamine, 1 g; riboflavin, 2.6 mg; sucrose, 30 g; agar (Oxoid no. 3), 12 g; distilled water to 1 liter. The medium was adjusted to pH 6 and autoclaved at 115 C for 10 min. Fermentation medium (FM) had the following ingredients (5): lactose, 55 g; CaCO₃, 10 g; MgSO₄·7H₂O, 4.5 g; KH₂PO₄, 7 g; phenylacetyl-ethanolamine, 3.75 g; corn steep liquor nitrogen, 2.1 g (corn steep liquor was added at a concentration determined by its total nitrogen content on a weight-to-volume basis); distilled water to 1 liter. The medium was adjusted to pH 5.2 and distributed in 16-ml amounts into 100-ml Erlenmeyer flasks; to each flask was added 1.6 ml of white mineral oil (Esso Petroleum Co.) and 0.33 ml of soya bean oil (Clyde Oil Refineries) prior to autoclaving at 120 C for 15 min.

**Incubation.** All slope cultures were incubated at 24.5 C for 7 days.

**Penicillin yield testing.** Each isolate for test was grown on a slope of CM prepared in a 1-oz (ca. 31.1 g) universal container. Conidia from a slope were inoculated into a 100-ml Erlenmeyer flask containing FM which was placed on a rotary shaker (2- inch ca. 5.0 cm) throw, 220 rev/min) at 24.5 C for 6 days before assaying the filtered broth. The assay method in its essentials was the arselenomolybdate technique as described by Pan (8).

**Methods of storage.** Cultures were stored on slopes of CM either in a refrigerator at +4 C or in liquid nitrogen at −196 C in a Union Carbide LR-10A-6 unit. Those stored in liquid nitrogen were on slopes in 0.5-dram (ca. 0.58 g) vials (Johnson and Jorgensen Ltd., London) measuring approximately 3.5 by 1 cm with screw caps. The latter were tight- ened before immersion in liquid nitrogen. Care had to be taken when removing slopes from liquid ni- trogen because of the possibility of leakage of the liquid into slopes and the danger of explosion when they were thawed. No trouble was experienced, but as a precaution slopes removed from liquid nitrogen were placed in a large metal container and allowed to rise to room temperature before use.

**Estimation of viability.** Conidia were dispersed in a wetting agent consisting of 0.02% (v/v) calsolene (ICI Ltd) in distilled water and plated on at least 10 plates of CM at dilutions to give approximately 100 colonies per plate.

**RESULTS**

Conidia from the master culture of the auxo- troph of *P. chrysogenum* were inoculated on to several CM slopes for storage, after incubation, at +4 and −196 C. Conidial viabilities of slope cultures were estimated immediately before preservation, after one week at +4 and −196 C and then at intervals throughout a storage pe- riod of 42 months. There was a drop in viabili- ty after 1 week at −196 C which could be attri- buted to the effects of freezing and thawing (Table 1). Slope cultures were simply immersed in liquid nitrogen and removed to room temperature as required. No attempts were made to establish controlled conditions of cooling or heating. The results in Table 1 showed that, after 42 months, viability was much better preserved at −196 than at +4 C.

At the beginning of the experiment when slope cultures were inoculated from the master culture for preservation, conidia from this master culture were also plated on CM, and 65 single conidial isolates were tested for peni- cillin yield with the results shown in Fig. 1. When viabilities were estimated after 4 and 21 months, 10 single conidial isolates from each series at both periods retained the penicillin yield of the parental auxotrophic culture. After 30 and 42 months, 65 single conidial isolates were made from each series and tested for peni- cillin yield. At 30 months, single conidial iso- lates from both series included a small propor- tion with low penicillin yields (Fig. 1). This was substantiated after 42 months among iso- lates from material stored at +4 C but not from isolates grown from conidia held at −196 C (Fig. 2). After 42 months, 100 single conidial isolates of each series were also tested for growth requirements. With one exception, all 200 retained the three genetic markers carried by the parental strain determining brown spore color and requirements for biotin and nicotinamide. The exception was a single co- nidial isolate from material stored at +4 C which had lost its requirement for nicotin- amide and had a penicillin titer of less than 500 units/ml. None of the three genetic mar- kers had a substantial effect on penicillin yield, so, unless this isolate also bore an inde- pendent mutation which reduced penicillin titer, the loss of its requirement for nicotin- amide was probably due to a suppressor muta- tion having a pleiotropic effect which lowered penicillin yield rather than a reversion at the original site of the mutation determining the vitamin requirement. The ‘evidence was min- imal but suggested storage at −196 rather than at +4 C to prevent loss of auxotrophy.
TABLE 1. Conidial viabilities after storage at +4 and −196 C

| Time (months) | Viabilities at +4 C relative to: | Viabilities at −196 C relative to: |
|---------------|---------------------------------|-----------------------------------|
|               | Hemocytometer count | Initial viability | Viability after storage for 1 week | Hemocytometer count | Initial viability | Viability after storage for 1 week |
| 0 (prior to preservation) | 77.4 | 100 | 77.4 | 100 |
| 0.25 | 76.5 | 98.8 | 100 | 60.7 | 78.4 | 100 |
| 4 | 72.9 | 94.2 | 96.3 | 61.4 | 79.3 | 101.1 |
| 21 | 52.4 | 67.7 | 68.5 | 37.7 | 48.7 | 62.1 |
| 30 | 14.9 | 19.3 | 19.5 | 60.3 | 77.9 | 99.4 |
| 36 | 3.2 | 4.1 | 4.1 | 46.7 | 60.3 | 76.9 |
| 42 | 3.1 | 4.0 | 4.0 | 52.3 | 67.6 | 86.2 |

DISCUSSION

Some mutants yielding more of a useful metabolite than their parent may not be stable and range from those so unstable as to escape discovery, because of poor viability or an extreme liability to yield decay on replication, to those whose instability only becomes evident after their operational use on an industrial plant. The latter sort may be of the kind which show yield decay on storage, a characteristic not amenable to test immediately after a mutant’s isolation. As yields continue to be raised by serial mutagenic treatments, it may become increasingly necessary to use unstable mutants in industry. If so, methods will have to be sought to minimize the effects of instability both on replication and during storage.

Obviously an attempt could be made to reduce or prevent any decreases in productivity during replication by using fewer seed stages in industrial fermentations. However studies with Streptomyces griseus indicated that when an iron salt was omitted from the culture me-
dium loss of streptomycin yield was avoided during serial subculturing (7). Presumably sufficient Fe"⁺ was available as a contaminant in other medium ingredients to allow growth. It was suggested that the presence of porphyrin-containing enzymes, involved in the biosynthesis of streptomycin, was dependent on a genetically labile step inactive in the absence of sufficient Fe"⁺ and that possibly the chances of deleterious mutations were then limited (7). Perhaps further investigations in the field of nutrition may indicate other ways of yield stabilization during replication. Attention to the effects of media ingredients may also be important in preserving viability during storage. For example, raising the level of Fe"⁺ above that required for growth increased the longevity of Pseudomonas cultures (12). It was proposed that when cells have ceased to divide pools of primary metabolites may have to be converted to innocuous secondary metabolites to prevent distorted growth and loss of viability, and that the metal was required for the operation of synthases in these conversion processes (12).

The present work investigated the possibility of precluding or reducing instability by preserving cultures of P. chrysogenum at −196 C. When conidia were stored at this temperature over a period of 42 months, they retained good viability. Furthermore, 65 single conidal isolates had penicillin yields similar to that of the same number made prior to storage. That a reduction in viability can be correlated with loss of penicillin yield (4) has been supported by the demonstration that storage of conidia at +4 C for 42 months resulted in poor survival and a decrease in penicillin titer in a proportion of 65 isolates grown from individual surviving conidia. It has not been possible to decide whether mutants of low titer were selected as viability fell or storage per se induced mutation to low titer. Proof of the latter event would require a demonstration of an absolute increase in the number of low-yielding mutants during storage at +4 C. When after storage for 42 months at +4 C, the viability had dropped to 4%, then among 65 conidial isolates 18% had low penicillin titer (Table 1, Fig. 2). Assuming that mutants with low yields survived preservation, this represented a level of 0.72% in the original sample prior to storage. No single conidal isolates of low yield were present among a sample of 65 made before storage (Fig. 1 and 2). The results were not incompatible with a selection hypothesis, although they did not disprove that preservation at +4 C could induce mutations to low titer, particularly if such mutants died off during storage, albeit at a slower rate than the parent. However, even if storage at +4 C did induce mutation to low titer, storage at −196 C, as was pointed out previously (4), should tend to inhibit metabolic reactions including those involved in mutation.

Whether, in the future, precautions will become necessary in mutational screening programs with highly developed industrial strains to preserve the viability of putative mutants, immediately after mutagenic treatment, will be a matter for the individual experimenter to decide in the light of his experience and rate of success in producing mutants with increased yields.

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