

STUDIES ON VACUUM-DRYING FOR THE PRESERVATION OF PLANT PATHOGENIC BACTERIA

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Summary

A comparison of freeze-drying and vacuum-drying of plant pathogenic bacteria showed no short-term differences in survival. Storage long term with vacuum-drying suggests that this simpler method offers similar survival rates to freeze-drying. A modification of the procedure in which the condenser is by-passed in the latter stages of drying offers the prospect of achieving lower pressures which are not limited by the vapour pressure of ice.

Introduction

The practices of bacterial preservation are largely the subject of heuristic development. The very long periods over which information is gathered and the absence of formal experimentation in relation to practice means that the methods are not well documented. Reviews on the method are written by Lapage et al. (1970a, 1970b) Alexander et al. (1980) and Rudge (1991) [1, 2, 3, 4].

One of the favoured methods of preserving bacterial cultures is by freeze-drying. The procedure considered best to ensure the protection of labile organisms involves a two-stage process. (1) Ampoules containing bacterial cultures as suspensions are cooled to -60°C , air pressure in the ampoules is reduced, and water - including that inside bacteria - is removed by ablation as the temperature is allowed to rise. (2) The ampoules are then placed on a manifold for secondary drying, evacuated, and sealed. Factors in the freeze-drying procedure that

are considered to favour long-term preservation of bacteria are (1) that water is removed from frozen cells by ablation, resulting in less cell injury than if water is removed in the liquid phase, and (2) that water is removed rapidly from cells.

Vacuum-drying is a simpler procedure involving drying by a single-step process. Bacterial cultures are prepared as suspensions in ampoules which are attached to a manifold coupled to an evacuated condenser maintained at low temperature (below -45°C). When the manifold is opened and pressure in the ampoules falls rapidly, water in the ampoules boils and the bacteria are cooled. Water is removed from the cultures at low temperature and pressure, and collected in the condenser. When the water content in the ampoules has stabilized, they are sealed under vacuum and stored. Although water in the ampoules may be frozen initially, it is not certain that it remains

frozen, particularly at high ambient temperatures. It is possible that the temperature at which water removal occurs is only briefly below 0°C. In this process, the pressure in the ampoules cannot be less than the vapour pressure of water at the temperature of the condenser (usually -45°C). This limits the amount of water that can be removed from cultures.

Preservation of bacteria for the International Collection of Microorganisms from Plants (ICMP) has always been by vacuum-drying. Examination of samples of ampoules in which bacterial survival was low suggested that failure to achieve low pressures in ampoules was a major factor. Therefore, minimum standards adopted before sealing ampoules were that a condenser temperature reading of less than -57°C (equivalent to a vapour pressure of ice of 0.0122 mm Hg; 1.63×10^{-2} mbar) and a pressure reading on a Pirani gauge less than 1.63×10^{-2} mbar was achieved.

Originally, our single-stage refrigerator used in our vacuum-drying equipment employed a refrigerant which was environmentally harmful. This refrigerant was necessary if the low temperatures associated with acceptable preservation were to be achieved. The decision was taken to modify the unit to use a safe refrigerant avoiding, if possible, the complexities and cost of a two-stage cascade refrigeration unit to achieve low temperatures (less than -55°C). A vacuum line with a stop-valve was introduced which by-passed the condenser, and two stop-valves were placed into the vacuum line above and below the condenser so that it could be isolated (Fig. 1). In the initial stages of drying, the by-pass line was closed and water vapour was trapped in the condenser at -45°C. After 3 hours, when the manifold

pressure was 10^{-1} mbar, the condenser was isolated by closing the valves and the by-pass was opened to allow ampoules to be evacuated directly. Drying was continued overnight after which time pressure had fallen to $<10^{-2}$ mbar.

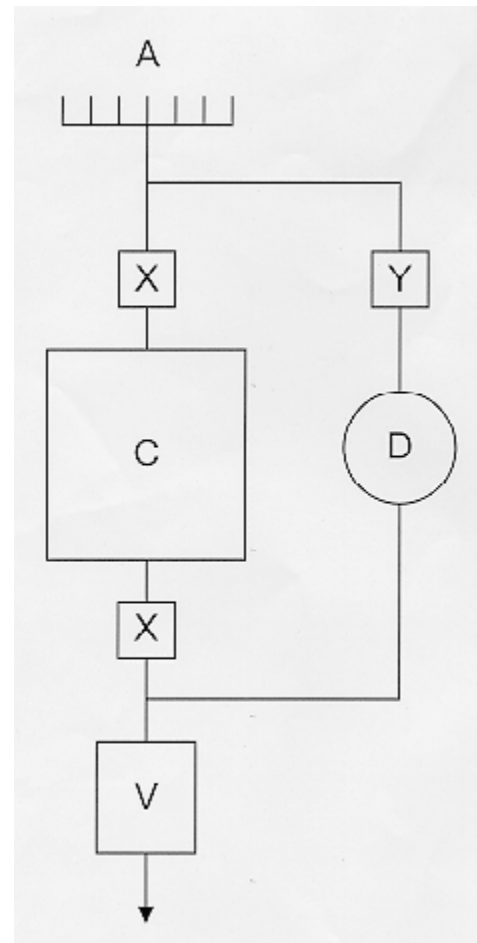


Fig. 1. Schematic diagram of vacuum-drying equipment at ICMP.

- A - ampoule manifold
- C - condenser
- D - oil diffusion pump
- V - rotary vacuum pump
- X - condenser isolating valves
- Y - diffusion pump isolating valve

In this paper we report a comparison of freeze-drying and vacuum drying and the effect of the modification to our vacuum-drying equipment to obviate the need to reduce condenser temperatures below -50°C.

Materials and Methods

Preparation of bacterial strains.

Strains were from the ICMP, Landcare Research, Auckland. Strain numbers are reported in Table 1. Full details are recorded in Young and Fletcher [5]. Strains were grown on agar slopes. Growing media were as follows: for *Erwinia amylovora*, *E. carotovora*, *Pseudomonas syringae*, *Xanthomonas axonopodis*, *X. campestris* pv. *campestris*, *X. campestris* pv. *phaseoli*, and *X. fragariae* - yeast extract nutrient agar (YNA; nutrient agar (Difco) - 23 g, yeast extract (Difco) - 3 g, deionized water - 1 l); for *Burkholderia*

solanacearum - glucose peptone casamino acids agar (GPCA; glucose - 5 g, Bacto-peptone - 1 g, casamino acids plus vitamins (Difco) - 1 g, agar - 15 g, deionized water - 1 l) and for *X. albilineans* - yeast sucrose peptone agar (YSPA; yeast extract - 5 g, sucrose - 20 g, peptone - 20 g, agar - 15 g, deionized water - 1 l). Turbid suspensions with target values of 10^{10} colony-forming units per ml (cfu/ml) were prepared in glucose:peptone (7% : 7% w/v) solution and ampoules were charged with approximately 0.18 ml and constricted using standard procedures [1].

Table1. Counts of *Erwinia*, *Pseudomonas*, *Ralstonia* and *Xanthomonas* bacteria inoculated into ampoules (a), after freeze-drying (b) and after vacuum-drying (c)

Taxon and ICMP №	Log numbers of bacteria per ml		
	a *	b **	c **
<i>B. solanacearum</i> 5712	10.56 ± 0.090	9.96 ± 0.195	9.62 ± 0.806
<i>E. amylovora</i> 1540	10.78 ***	9.65 ± 0.049	9.83 ± 0.129
<i>E. carotovora</i> 5702	10.85 ± 0.098	8.99 ± 0.170	9.89 ± 0.141
<i>P. syringae</i> 3023	9.38 ***	8.29 ± 0.175	8.11 ± 0.382
<i>X. albilineans</i> 196	8.21 ***	7.25 ± 0.222	8.27 ± 0.209
<i>X. axonopodis</i> 50	10.12 ± 0.208	8.57 ± 0.106	8.58 ± 0.115
<i>X. c. campestris</i> 13	10.67 ***	7.47 ± 0.216	8.58 ± 0.136
<i>X. c. phaseoli</i> 5834	8.84 ± 0.000	8.03 ± 0.120	8.72 ± 0.508
<i>X. fragariae</i> 5715	9.38 ± 0.092	8.72 ± 0.506	9.26 ± 0.095

* Average of three dilution series from initial suspensions;

** Average of dilution series from five ampoules;

*** From a single dilution series.

Freeze-drying. The procedure is a modification of that described in Alexander et al. [1].

Prepared ampoules were refrigerated in a vacuum desiccator to -70°C for 1 hour. The desiccator was then attached to a vacuum pump and placed in a dry-ice/ethanol slurry and pressure was reduced to 2×10^{-2} mbar. This was maintained until all dry ice had ablated. Ampoules were removed and attached to a vacuum manifold which was evacuated until a pressure of 10^{-3} mbar (Pirani gauge) was achieved, and then sealed.

Vacuum-dry procedure. Prepared ampoules were attached directly to the vacuum manifold. The condenser was refrigerated to -60°C , evacuated to 10^{-3} mbar, and then opened to the manifold. Evacuation was continued until a pressure of 10^{-3} mbar (Pirani gauge) was achieved, and ampoules were then sealed.

Estimation of bacterial numbers. Suspensions were prepared by adding 0.5 ml nutrient broth to the ampoule contents and decimally diluting them in 0.1%

peptone (w/v). Numbers of bacteria in the initial inocula and the final populations from freeze-dried and vacuum-dried ampoules were estimated by counting the numbers of colonies produced following the inoculation of measured volumes as drops from dilutions series onto plates containing appropriate supporting agar. Counts of bacteria were made 2 - 4 weeks after preparation. Changes in numbers of bacteria following freeze-drying and vacuum-drying were examined and compared.

Effect of by-passing the condenser. Eighteen prepared ampoules were weighed before and after filling with a standard suspension of *P. fluorescens* ICMP 3512. They were then placed on the vacuum manifold, dried for 3 hours (10^{-1} mbar) with the condenser in operation, and re-weighed. The ampoules were then replaced on the manifold and dried overnight, without the condenser in series (10^{-2} mbar), and weighed. From these weights, the total weight of inoculum and the liquid volume removed could be calculated.

Results

Reductions in the initial populations of 1 - 2 orders of magnitude occurred after bacteria were freeze-dried or vacuum-dried. Although some (e.g. *E. carotovora*, *X. albilineans*) differed in their reactions to freeze-drying and vacuum-drying, there was no systematic difference between the

effects of the two treatments. Results are shown in Table 1.

The mean weight of inoculum fluid was 0.180 ± 0.044 g/ampoule. The quantity extracted into the vacuum pump was 0.0013 ± 0.0008 g which amounts to 0.15 g water per 120 ampoules, the usual load.

Discussion

Our results indicate that there is no difference in short-term survival rates between cultures that are freeze-dried or vacuum-dried in the weeks after preparation. There will be a benefit from the extra work involved in freeze-drying if long-term survival is higher in the freeze-drying process, and if the difference in survival relative to vacuum-

drying is a selective and not a stochastic process. The fact that most bacterial species can be revived from vacuum-dried preservation after 20 years in our collection suggests that the two-stage freeze-drying process is cost-effective only for species for which there may be a biased selection of surviving cells in the long term.

Although lower vacuum levels are reported as satisfactory [4], we believe that the long storage lives achieved at ICMP are due the high vacuum set for routine preservation. The disadvantage of vacuum-drying in a single step is that the manifold pressure is limited by the vapour pressure of ice in the condenser; at -45°C this is 7.2×10^{-2} mbar (0.054 mm Hg). Pressures will not fall below this value until all water vapour has been ablated from ice in the condenser. The isolation of the condenser after preliminary vacuum-drying allows pressure

to be reduced to that which can be achieved directly by the vacuum pump. We have introduced a vacuum line from the manifold to the vacuum pump with valves which allow the condenser to be by-passed. Initial evacuation into the condenser removes most water from the unit. With our equipment, this means that a final pressure of 10^{-2} mbar (0.0075 mm Hg), equivalent to a partial pressure over ice of -60°C , can routinely be achieved. The small amounts of water (0.15 g per load) carried into the pump are purged during gas-ballasting.

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