Refinement of Lyophilization Methodology for Storage of Large Numbers of Bacterial Strains

R. D. GITAITIS, Associate Professor, Department of Plant Pathology, Coastal Plain Experiment Station, University of Georgia, Tifton 31793

ABSTRACT

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A method was developed for the lyophilization of large numbers of bacterial strains. A heavy loopful of bacteria picked from a 48- to 72-hr culture on an agar medium was mixed with 0.2 ml of sterile evaporated milk in a well of a 96-well plastic microwell tissue culture plate. Bacterial suspensions were frozen for 1 hr at −73 C, then placed in a lyophilizer chamber. Samples were lyophilized for either 5 or 24 hr. After removal from the lyophilizer chamber, the microwell plates were placed under vacuum and heat-sealed in polyethylene bags. Samples were stored at room temperature or at −73 C. Viability of bacteria was checked periodically. A lyophilized pellet was mixed with 0.2 ml of sterile distilled water, and the suspension was streaked on a suitable agar medium. The bacteria have remained viable for at least 1 yr.

Additional key words: freeze-dry, *Clavibacter michiganense* subsp. *michiganense*, *Xanthomonas campestris* pv. *vesicatoria*

Lyophilization is a method frequently used to preserve bacteria, including phytopathogenic strains (1,2,5). The technique is advantageous for long-term storage of bacteria because fewer undesirable mutations occur than with storage in screw-cap water tubes or on refrigerated agar slants (3). Although lyophilization is a relatively simple technique, it can become laborious and an encumbrance if a large number of bacterial samples have to be processed in a relatively short time. Commonly, a 10–20% skim milk solution is used as a cryoprotective agent (2). Sterilization of the milk solution can be a nuisance because overheating can cause carmelization. Care also has to be taken when the milk is pipetted into the cryophile ampules, because milk residue on the neck of the ampule will scorch and produce fumes when the ampules are cut off the lyophilizer. Although these problems are not difficult to solve, they do add to the total time and labor required to lyophilize a large number of strains of bacteria. Another major problem in lyophilizing large numbers of bacterial strains is that most lyophilizers have at the most a 24-port manifold. If the bacterial suspensions were lyophilized for 18 hr (overnight) as recommended (3), it would take at least 10 days to lyophilize 240 strains.

During a recent study, it was necessary to store several hundred strains of bacteria in a relatively short time. Conventional methods of lyophilization would have been laborious and time consuming. Therefore, the following rapid method was developed for the lyophilization of numerous strains of bacteria.

MATERIALS AND METHODS

Canned evaporated milk (Winn Dixie Grocery, Jacksonville, FL) was used as a cryoprotectant. The lid of the can was wiped with an ethanol-soaked tissue, flamed, and opened with the triangular-shaped end of a sterile punch-type can opener. Aliquants of 0.2 ml of evaporated milk were pipetted aseptically into the wells of a 96-well, plastic, flat-bottom Microtest III plate treated for tissue culture use (Becton Dickison & Co., Oxnard, CA). Strains XV 83-38 of *Xanthomonas campestris* pv. *vesicatoria* and CM 84-1 of *Clavibacter michiganense* subsp. *michiganense* were selected as test strains representing gram-negative and gram-positive bacteria, respectively. Strain XV 83-38 was grown on nutrient agar, and CM 84-1 was grown on CNS medium (4). Bacterial strains were incubated for 48–72 hr at 30 C, and heavy loopfuls of bacteria were mixed with the sterile 0.2 ml of evaporated milk. The bacteria and milk suspension were thoroughly mixed, and the microwell plates were covered and placed at −73 C for 1 hr. After their removal from the

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freezer, lids were removed from the plates and samples were placed in the lyophilizer chamber of a Unitrap II lyophilizer (Virtis Co., Gardiner, NY). Samples were lyophilized for either 5 or 24 hr under vacuum (0.01 kPa) with the sink temperature at −55 C. After lyophilization was completed, plates were heat-sealed in evacuated polyethylene bags (15.2 cm wide and 0.15 mm thick) with a Sealboy bag sealer (Packaging Aids Corp. San Francisco, CA). Bag evacuation was accomplished by cutting the corner of the plastic bag to create a hole to insert a Pasteur pipet vacuum line. As the vacuum developed, indicated by a tightening of the polyethylene bag around the plate, the bag was heat-sealed in front of the Pasteur pipet tip. Sealed plates were tested for leaks with a vacuum leak detector/tesla coil (Electro-Technic Products, Chicago, IL). Samples were stored either at room temperature or at −73 C. Bacterial viability was tested for three replicates of each bacterial strain at each storage temperature for each lyophilization period after 1, 2, 6, 8, and 12 mo of storage. A 0.2-ml volume of sterile distilled water was slowly added to each well and the contents were carefully mixed with the pellet. When the pellet was well suspended, loopfuls were removed and streaked onto appropriate media. Plates were incubated at 30 C, and bacteria that grew were identified.

RESULTS AND DISCUSSION

The lyophilizer chamber of the Unitrap II could accommodate 32 microwell plates, each plate capable of holding 96 samples. Theoretically, more than 3,000 samples could be lyophilized at once. The identity of each culture is readily maintained by recording the species and the alphabetical and numerical position.

Although the microwell plate lids had to be removed during the lyophilization process, there was no contamination in any of the samples during the test period. All cultures stored equally well at room temperature or at −73 C. Over time, however, some of the polyethylene bags lost their vacuum. Although no discernible effects were observed during the test period, loss of vacuum could affect long-term storage. Bags maintained in the ultra-low-temperature freezer at −73 C were less likely to regain positive pressure. Alternate methods of storing plates under a vacuum such as in a vacuum jar or by double-bagging could alleviate this problem.

A makeshift lyophilizer chamber was evaluated for use if a regular chamber was not available. A vacuum desiccator jar was used as the chamber and connected to one of the ports of a 12-port manifold on the lyophilizer. The constructed chamber method proved unsuccessful because sublimation was too slow and the bacterial samples thawed (J. D. Schmidt, *personal communication*). The small diameter (2–3 mm) of the outlet port connecting the chamber to the manifold and the long (>1 m) path from the chamber to the cold sink are most likely what prevented sublimation. In addition, the makeshift glass chamber could allow a buildup of heat radiated from lights, equipment, and personnel in the laboratory. Packing the chamber in dry ice, covering it with aluminum foil, and increasing the outlet port diameter to at least 1–2.5 cm could create conditions for proper lyophilization.

A regular lyophilizer chamber and the microwell plate method of lyophilization were found convenient for rapid processing of a large number of strains over a short time. Retention of bacterial viability was excellent for at least 1 yr (the maximum length of the test period). In all cases, all replicates of both bacterial strains, XV 83-38 and CM 84-1, survived the initial lyophilization process of either 5 or 24 hr. There was no apparent loss of viability with a 24-hr period of lyophilization as previously reported for certain lactic-acid bacteria (5). In addition, using canned evaporated milk as a cryoprotective agent was more convenient than preparing and "sterilizing" skim milk. Several samples of lyophilized strains have been successfully sent to co-workers around the United States on microwell plates, which have proven to be a convenient and safe means of transporting large numbers of cultures.

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