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Silica Gel Media for Isolating and Studying Bacteria Under Hydrostatic Pressure

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Individual colonies of *Micrococcus euryhalis* and of a marine bacterial isolate were grown in pour tubes under hydrostatic pressure. The medium was prepared in a silica sol, and gelation was effected at 4°C by addition of salts to achieve concentrations found in seawater.

The cultivation of bacteria under hydrostatic pressure and low temperatures is conventionally done in a liquid medium (13). At atmospheric pressure, solid media have proved to be the most useful for the isolation of pure cultures of bacteria. The preparation of pour tubes by inoculating warm liquid agar places marine bacteria under the risk of thermal inactivation. Agar spread plates are conveniently used to isolate marine bacteria at low temperatures under atmospheric pressure. But to use spread plates at high pressures requires either that the pressurization be done with gases or that the pressurizing hydraulic fluid be separated from the agar surface by an interface, such as a plastic film or an inert liquid. These constraints make the use of plates inconvenient, costly, or hazardous.

Culture preparation using a silica gel-based medium would obviate the risk of thermal shock because the gelation can be effected at low temperature. Silica gels have not been commonly used, possibly because of an apparent tediousness of preparation. In this communication, we describe a simple method for the preparation of sterile silica gel-based media for use in ordinary high-pressure incubation vessels.

The silica sol is prepared essentially as described by Taylor (10). A strong cation exchange resin (Amberlite 120-H-C-P, medium porosity, Rohm and Haas, Mallinckrodt) was packed to form a column 4.4 cm in diameter by 43 cm high. This was regenerated with 250 ml of 4 N HCl and rinsed with 1 liter of distilled water. The water was drained to the top surface of the resin bed, whereupon 500 ml of 0.5 M $Na_2SiO_3 \cdot 9H_2O$ meta (technical grade; Fischer Scientific Co.) was added to the column. Ion exchange was effective up to a flow rate of 100 ml per min. The first 200 ml of the effluent was discarded. The effluent was then collected in a 1-liter graduated cylinder. When the sodium silicate solution had completely entered the column, distilled water was added until a total of 800 ml of effluent was collected. Additions to the column were made carefully to minimize mixing of eluants.

The effluent volume was brought up to 1,000 ml with distilled water, and the pH was adjusted to 1.5 with 5 ml of 4 N HCl. The sol was then autoclaved at 15 lb/in^2 for 20 min. The sterile sol was stored until required.

According to the manufacturer, the cation exchange resin that we used has a capacity of 1.7 meq/ml of packed resin. The column which we described has a total exchange capacity of 1,034 meq. We advise taxing only half of this capacity. As the resin approaches saturation with sodium, gelation may occur within the column. Washover of sodium ions into the effluent may cause gelation before or during autoclaving. A pH above 2.0 may also result in spontaneous gelation during autoclaving (5). Sodium ions in the sol and a pH greater than 2.0 also shorten the shelf life of the sterile sol. Problems with spontaneous gelation may occur if a sodium silicate solution more concentrated than 0.5 M is used (10). Ion exchange capacity of the resin decreases with use, especially when spontaneous gelation occurs, but may be reactivated with 5% KOH (8). The procedure described allows for preparation of 1,250 ml of medium and for clean and easy regeneration of the column. The final sterile sol is stable at room temperature or at 3°C for at least 1 month. Refrigeration increased the shelf life of the sol.

After collection of the silicic acid effluent, the column was rinsed with an additional 500 ml of distilled water. The column was then repacked by backwashing with distilled water and then regenerated with 250 ml of 4 N HCl. If the column was not to be reused immediately, it was flushed with an additional 250 ml of 4 N HCl and stored with the resin soaked in this condition.

To make media, silica sol was chilled to 4°C,

and the pH was adjusted with 5 ml of sterile 1 N NaOH to 8.7. A 10-ml amount of tryptone-glucose-yeast extract broth (5 g of tryptone, 2.5 g of yeast extract, and 1.0 g of glucose in 100 ml of water) were added to 100 ml of the sol, followed by addition of 10 ml of artificial seawater solution (24 g of NaCl, 0.7 g of KCl, 5.3 g of $MgCl_2 \cdot 6H_2O$, and 7.0 g of $MgSO_4 \cdot 7H_2O$, in 100 ml of water). The medium was added to test tubes containing 0.1 ml of inoculum. The tubes were covered tautly with a wax film (Parafilm) and incubated at 23°C at atmospheric pressure or in pressure vessels at elevated hydrostatic pressures. Duplicate spread plates of 2216 marine agar at 4°C were inoculated with 0.1 ml of each dilution.

Serial dilutions of *Micrococcus euryhalis* and of a marine isolate from the central North Pacific Ocean (CNP1-2) were made in 2216 marine broth (Difco). Duplicate tubes were inoculated with 0.1 ml of each dilution and placed in an ice bath until addition of the silica gel media. Empty sterile petri plates were inoculated in a similar manner. The silica gel medium was prepared and added to tubes and plates as described below. Tryptone was substituted for peptone because the latter nutrient caused a precipitate to form when it was added to the silica sol. This precipitated final product would not support growth. The salt solution must be added separately from the nutrient solution. Premixing of these two constituents results in precipitation. Additions to the silica sol must be made in the prescribed order. The final pH of the silica gel medium was 7.2 to 7.5.

Nutrient composition other than that described may be substituted, but the final pH of the media should be monitored. At 4°C, gelation occurred at approximately 10 min after addition of the salt solution. Gelation time will vary depending upon concentration of salts and silicic acid and upon pH and temperature (5). A protocol is presented in Table 1.

Syneresis of the gel may result in a thin layer of water at the surface of the gel medium in pour tubes. If colony numbers are high or if a colony develops at the top surface of the gel, this liquid layer becomes contaminated and develops a dense suspension of bacteria. If this occurs, this layer should be removed along with some of the underlying gel before the gel is penetrated for removal of colonies.

The silica gel media described can also be used for freshwater application because 0.5% NaCl will effect gelation in about 15 min at 23°C. Information for modification of this media for use in estuarine systems may be obtained by referring to the study on the effects of salinity,

TABLE 1. *Protocol for preparation of silica gel media*

Step no.	Procedure	Observation/comments
1	Regenerate column with 250 ml of 4 N HCl.	Column contracts slightly.
2	Rinse with 1 liter of distilled water.	Flow rate approximately 100 ml/min. Do not allow column to go dry.
3	Add 500 ml of 0.5 M $Na_2SiO_3 \cdot 9H_2O$; flush through with distilled water.	Ion exchange generates heat. Add distilled water after all sodium silicate has entered column.
4	Discard first 250 ml of effluent; then collect 800 ml of effluent (silica sol).	Void volume of described column is 250 ml.
5	Bring effluent volume to 1,000 ml with distilled water.	The silica sol now contains 1.5% (wt/vol) SiO_2.
6	Add 5 ml of 4 N HCl per 1,000 ml of sol.	Adjust pH to 1.5 with 4 N HCl.
7	Autoclave at 15 lb/in^2 for 20 min.	This may be stored until required.
8	Add 5 ml of 1 N NaOH per 100 ml of sol.	Adjust pH to 8.7 with sterile 1 N NaOH.
9	Add 10% tryptone-glucose-yeast extract concentrate.	pH drops to 8.2.
10	Add 10% artificial seawater solution concentrate.	pH drops to 7.4. Gelation occurs in 2 to 3 min at 23°C or 10 to 12 min at 0°C.
11	Wash column with 500 ml of distilled water and backwash with distilled water.	Clean and repack column.
12	Regenerate column with 250 ml of 4 N HCl.	See step 1.
13	If column is not to be reused immediately, add another 250 ml of 4 N HCl.	Store column in this condition to maintain resin and to avoid bacterial growth in the column.
14	If gelation has occurred in the column or ion exchange capacity is noticeably reduced, then wash column with 1 liter of 5% KOH, rinse with distilled water, and regenerate with HCl.	See step 1.

TABLE 2. *Comparison of agar and silica gel media*

Bacterium	No. of bacteria (CFU/ml of inoculum) on:[a]			
	2216 agar		SiTGYE[b]	
	Spread plates	Pour plates	Pour plates	Pour tubes
M. euryhalis	2.8×10^6	2.4×10^6	2.5×10^6	2.2×10^6
CNP1-2	7×10^6	ND[c]	8.2×10^6	6.7×10^6

[a] Colonies numbered 30 to 300 per tube or plate. The standard error was from 0.24×10^6 to 0.7×10^6 CFU/ml.

[b] SiTGYE, Silica gel-tryptone-glucose-yeast extract medium.

[c] ND, Not done.

temperature, and pH on gelation conducted by Pramer (5). Lower salinities result in slower gelation.

The marine bacterial isolate from the central North Pacific Ocean and *M. euryhalis* grew well and comparably at atmospheric pressure on 2216 marine agar and on silica gel-tryptone-glucose-yeast extract medium (Table 2).

The silica medium also proved to be suitable for growth and isolation of bacteria under hydrostatic pressure. Serial dilutions of *M. euryhalis* inoculated into silica gel tubes and incubated at atmospheric pressure indicated 4.1×10^6 colony-forming units (CFU) per ml, whereas replicate tubes incubated at 1,013 bars (1 bar = 10^5 N/m^2 = 0.98692 atmosphere) indicated 5.0×10^6 CFU/ml. The standard errors are 0.9×10^6 and 1×10^6 CFU/ml, respectively. Those incubated at 1,013 bars did not form any colonies. The cultures incubated at 1,013 bars did form colonies after subsequent depressurization and incubation at atmospheric pressure, but these indicated only 1.1×10^5 CFU/ml, with a standard error of 0.1×10^5 CFU/ml. The paucity of colonies in the 1,013-bar culture was expected, as this pressure is sufficient to inhibit growth of most organisms originating from atmospheric pressure environments.

There have been discussions previously on methods and approaches to the preparation of silica gel media for microbiological uses (1, 3, 5-11). Some methods result in media that can be streaked (1), whereas others allow preparation of pour tube cultures (10). The temperature, pH, and salinity of silica gel media can be regulated according to the expected tolerances of the organisms being cultured (5). The particular nutrient conditions selected need to be investigated regarding their effects on gelation time, syneresis, and precipitation effects. The gelled medium as prepared here is fragile compared with agar.

Two final precautions should be noted in the use of this medium for culturing bacteria at high pressures. First, consideration should be given to the effects of temperature and pressure on the pH of the medium (4). Second, measures should be taken to minimize or obviate the effects of adiabatic compressions which can lead to undesirable temperature increases (2, 12).

Silica gel media should thus be useful in the cultivation of marine psychrophiles, autotrophs, and chemolithotrophs. And, as in our application, it is useful in the isolation and cultivation of marine barotolerants and barophiles.

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