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A sporulation medium for strict anaerobes

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The production of spores, and spore position and shape, are taxonomic criteria in a number of genera of bacteria. The heat resistance of spores is also of interest taxonomically and with respect to the survival of bacteria, particularly pathogens, in a number of habitats. The demonstration of spore formation by growth of bacteria in various standard media and under different conditions (e.g. at sub-optimum temperature) is often not easy. Thus, a number of media designed to encourage spore formation and to produce spores with maximum heat resistance have been introduced, for instance for *Clostridium perfringens* (Ellner 1956; Angelotti *et al.* 1962; Kim *et al.* 1967; Duncan & Strong 1968) and *Sporolactobacillus inulinus* (Kitahara & Lai 1967).

In an investigation of some 400 isolates of cellulolytic bacteria from a cattle-waste, mesophilic, anaerobic digester it became evident that many of the isolates were sporing bacteria. Some were identified with species of known genera, including *Clostridium* and *Sporolactobacillus*, others were suggested as new genera or species of sporing rods, and cocci including sarcinae (Sharma 1983; Sharma & Hobson 1985).

Sporulation was often difficult to induce or detect by growth in various liquid or solid media such as those used for isolation and fermentation tests, by growth at non-optimum temperature or pH, or by starvation or prolonged storage. Media designed to induce sporulation were therefore tested.

Results and Discussion

The bacteria were all isolated from dilution cultures of digester contents using strictly anaerobic techniques (Sharma & Hobson 1985; Hungate 1950).

Ellner's (1956) medium, modified for strict anaerobes by addition of 0·1 g neutralized cysteine HCl per 100 ml and preparation and incubation under oxygen-free CO_2, and with Bacto-Peptone in place of the original BBL 'Polypeptone', did not induce sporulation, or good sporulation, even in some isolates presumptively identified as clostridia by other tests. Spores formed did not survive temperatures higher than 70°C.

The medium of Patel *et al.* (1980) modified for better growth of the digester anaerobes and used for isolation and testing of the bacteria (Sharma & Hobson 1985) supported better sporulation and some cultures survived 80°C. Spores, however, appeared to stick to the cellulose particles in the medium and were difficult to detect and subculture.

After tests with variations of these two media, medium 3 was developed. This contained (g or ml/l): peptone, 10; glucose, 10; $NaHCO_3$, 4; NH_4Cl, 1; $(NH_4)_2SO_4$, 0·150; K_2HPO_4, 0·296; KH_2PO_4, 0·180; $MgSO_4.7H_2O$, 0·120; $CaCl_2.2H_2O$, 0·200; $FeSO_4.7H_2O$, 0·020; NaCl, 0·010; $MnSO_4$, 0·030; $CoCl_2.6H_2O$, $ZnSO_4.7H_2O$, 0·001; $CuSO_4.5H_2O$, $AlK(SO_4)12H_2O$, H_3BO_3, $Na_2MoO_4.2H_2O$, 0·0001; glycerol, 15; gelatin, 60; cysteine HCl, 1. Glucose, cysteine and $NaHCO_3$ were filter-sterilized in solution; the basal medium was autoclaved at 121°C for

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15 min. Final pH was 7·2; preparation, dispensation and incubation were under oxygen-free CO_2.

This medium, incubated at 39°C, which is near the optimum temperature for all isolates, rather than below (to 4°C), gave best results. Spores were easy to differentiate, either unstained or stained by Schaeffer and Fulton's method, and were also identified by a qualitative test for dipicolinic acid (Janssen *et al.* 1958). Spores were obtained with all isolates presumptively identified by other tests with sporing genera, including coccal forms, and some spores survived 80°C for 10 min. Heat testing was done by inoculating 10 ml of PYG medium (used for fermentation and other tests; The Anaerobe Manual, 1977) with 0·1 ml of a culture in medium 3, holding the tube at 70°, 75° or 80°C for 10 to 30 min and incubating the culture at 39°C.

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