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COLLECTION, PURIFICATION, AND CULTURE
OF CYANOBACTERIA

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Detailed studies of the structure, physiology, biochemistry, and toxin production of cyanobacteria require a dependable and reproducible supply of selected organisms. Axenic cloned strains of cyanobacteria should be used whenever possible. Several physical and chemical techniques are available to purify cultures to the axenic condition. Cultural techniques are described here which can be used to grow adequate amounts, greater than 100 g fresh weight, of cyanobacteria in about four weeks.

Many practical aspects of the purification and culture of cyanobacteria and phytoplankton are summarized by Castenholz (1970); Stanier et al. (1971); Siegelman and Guillard (1971); Allen (1973); Guillard (1975); Stanier and Cohen-Bazire (1977); Siegelman and Kycia (1979), Rippka et al. (1979); and Rippka et al. (1980). The Handbook of Phycological Methods edited by J. R. Stein (1973a), The Biology of Blue-Green Algae edited by N. G. Carr and B. G. Whitten (1973), and The Blue-Green Algae edited by G. E. Fogg, W. D. P. Stewart, P. Fay, and A. E. Walsby (1973) also contains extensive information.

Methods summarized here for the collection, purification, and culture of cyanobacteria can be used to grow many kinds of freshwater and marine phytoplankton with only minor modifications. Techniques adequately described in the cited literature are not fully detailed in this report. It must be emphasized that there are no definitive rules for purification and culture. Many suggestions are provided here, but in the final analysis there is no substitute for the ingenuity and imagination of the individual investigator.

Collection Cyanobacteria not available from culture collections can be obtained from natural sources. Government public health and water pollution control agencies can often provide information on the occurrence of blooms and particular locations for collection of specific organisms.

A plankton net, about 40 μ m mesh size, can be used to collect and concentrate freshwater samples (available from Wildlife Supply Co., Saginaw, MI, Ernest A. Case., Andover, NJ, or V. W. R. Scientific Inc.). It is towed through the water from shore or from a boat. Lake, pond, or stream bottom mud samples may contain organisms not present in the waters during the collection period. Marine cyanobacteria are obtained from sediments, algal mats, rocks, or plankton tows. A tabulation of collection sites of marine and freshwater cyanobacteria is provided by Rippka et al. (1979).

Collected liquid samples should be transferred into two to four different liquid culture media in flasks at the collection site. The initial cultures are held temporarily at room temperature (~25°C), under diffuse daylight illumination. Mud and rock samples are held in small bottles or vials; a moistened filter paper inserted in the container lid, not in contact with the sample, prevents water loss.

On return to the laboratory it is prudent to transfer portions of the collections into the same two to four different liquid culture media. Observations of the initial cultures can provide suggestions for selection of a suitable culture medium. Samples should be streaked on 0.75-1% agar plates as soon as possible after collection

to initiate purification. The agar plates are prepared with the same two to four liquid culture media. Suspensions of the mud samples are placed in several liquid culture media and streaked on agar plates as for liquid samples. Storage of mud samples is probably satisfactory at 5°C. Laboratory cultures of freshwater cyanobacteria will generally grow at about 25°C. Illumination is provided with 50 to 100 ft. c. of continuous cool white fluorescent light. Temperatures of about 18°C are required for some marine forms and hot above 37°C for hot spring cyanobacteria.

Cyanobacteria are often available from general culture collections and specialist collections. Lists of collections are provided by Stein (1973b) and Komarek (1973). The Indiana University Culture Collection (IUCC) was transferred to the University of Texas (UTEX), Austin, TX. (Starr, 1978).

The American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, holds their collection of cyanobacteria at liquid nitrogen temperature. Storage of cyanobacteria at low temperature, when satisfactory, is an important method for maintaining genetic stability (Holm-Hansen, 1973).

Purification The purification of cyanobacteria is essential to avoid possible complexities associated with contaminant microorganisms. Many forms are easily purified, but others may require prolonged and dedicated effort (Allen, 1973). Physical and chemical methods for cyanobacterial purification are listed by Allen (1973), Carr et al. (1973), and Carmichael and Gorham (1974). Additional useful methods are described by Guillard (1973) and Hoshaw and Rosowski (1973). Streaking on agar plates is probably the most widely used purification method (Stanier et al. (1971); Allen (1973); and Rippka et al. (1979)). Brief treatment, 1 to 2 min, of the inoculum in a 50- to 100-watt ultrasonic bath, blending with glass beads (McCurdy and Hodgson (1974)), or grinding in a glass homogenizer (Allen (1973)) is used to break filamentous forms into short filaments prior to streaking on agar. Washing filamentous forms in sterile spot plates, depression slides (Guillard (1973)), or on 10- μ m-pore-size polycarbonate membranes (Nuclepore Corp. Pleasanton, CA) can reduce contamination (J.B. Waterbury, personal communication).

Alternation of streaking on agar and growth in liquid culture media is continued until the culture is purified. Streaking of the plates is simplified by placing three to four sterile 6-mm glass beads on the agar plate, after the addition of inoculum, and then horizontally shaking the plate. The beads are removed from the plate prior to incubation. Agar plates are held in transparent polystyrene boxes to reduce water loss and contamination (Stanier, et al. (1971)).

Colonies should be selected from agar plates as soon as possible to avoid overgrowth by contaminants. The larger the number of agar plates used and isolates selected, the better the chances of obtaining an axenic isolate.

Purification of phototactically motile forms is assisted by unilateral illumination (Stanier et al. (1971); Allen (1973)). Stock cultures of cyanobacteria are conveniently maintained on agar slants. All sterile transfers are made in a laminar flow hood equipped with a 0.3- μ m air filter. Flaming of flasks and tubes is avoided in laminar flow hoods because of disturbance of the laminar air flow. If fungal contamination of agar plates occurs in the laminar flow hood, a simple still-air hood may be substituted (Guillard, 1973).

Media A large number of liquid mineral media are available for culturing freshwater and marine cyanobacteria. Their compositions are given by Stanier et al. (1971); Siegelman and Guillard (1971); Nichols (1973); McLachlan (1973); Carr et al. (1973); Guillard (1975); and Rippka et al. (1979). Selection of a satisfactory freshwater medium is achieved by trying several media, e.g. the D medium of Kratz and Myers (1955); ASM-1 medium of Gorham et al. (1964); BG-11 or BG-11₀ medium of Allen (1973) and Rippka et al. (1979); and WC medium of Guillard (1975). Marine media are similarly selected. Consultation of the literature provides initial guidance, but there is no substitute for personal experience.

Equipment to prepare high quality water is commercially available. Distilled or reverse-osmosis water are subjected to carbon absorption, mixed-bed ion exchange, and 0.2- μ m filtration to a specific resistance of 18 megohms-cm. Complete purification units are available from The Barnstead Co., Boston, MA, and from Millipore Corp., Bedford, MA, or they can be assembled from commercially available modules. Salts used for media preparation are reagent grade. For large volumes of artificial marine media, noniodized food-grade NaCl and USP grade $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ can be substituted for reagent-grade chemicals after preliminary comparative trials. Large volumes of culture medium are prepared in a 120-l polyethylene drum with a bottom spigot. The drum is mounted on a dolly about 50 cm high to permit filling of media bottles. A heavy duty stirrer is mounted on a column attached to the dolly with its stainless steel shaft and propeller in the drum. The stirrer aids dissolution of the chemicals and pH adjustment. A sealed plastic body combination pH electrode with a protected glass tip is used for pH monitoring. The liquid culture medium is dispensed into 10 l or 20 l autoclavable polypropylene bottles, which are used instead of borosilicate glass bottles for media sterilizing and storage. They are lighter, unbreakable, and have large handles for ease of lifting. A 20-cm² cover of two layers of unbleached muslin with a double thickness of aluminum foil between the muslin layers, is securely fastened over the bottle mouth with a twist tie (available from garden stores).

No problems have been encountered using liquid culture media autoclaved in polypropylene bottles. Possible toxicity for sensitive organisms should be tested (Blankley (1973)).

Silicone sponge closures (Bellco Glassware, Vineland, NJ) are used to cover flasks and small bottles containing culture media. Bare aluminum foil covers should not be used on flasks or bottles prior to autoclaving because they act as heat insulators and barriers to the penetration of steam into the containers. Indicating autoclave tape is placed on all materials prior to sterilization.

Small Cultures The general techniques used follow procedures carefully described by Guillard (1975). The flasks are not aerated but are shaken occasionally.

Large Cultures Borosilicate glass or polypropylene bottle cultures, 8- to 20-l, are aerated at about 350 ml min⁻¹ with 0.07 to 0.1% CO₂ in air. The air-CO₂ mixtures are obtained by mixing 100% CO₂ from a cylinder with carbon-filtered air (Koby Junior Filters, Koby Inc., Melrose, MA), using a back-pressure-compensated gas proportioner (Aalborg Instruments, Munsey, NY).

The bottle cultures are illuminated with continuous cool-white fluorescent light at about 200 ft. c. It may be desirable to reduce the light intensity for three to four days after starting a bottle culture by interposing one or two layers of window screen between the culture and lamps.

The temperature selected for growing large cultures is generally a compromise between specific requirements and available facilities. A large number of cyanobacteria grow satisfactorily at about 25°C. Forms demanding high or low temperatures are grown in illuminated incubators, preferably with glass doors permitting an unobstructed view of the cultures and external illumination if desired. The doors are kept locked during culturing of toxic forms.

Continuous Culture Apparatus Cyanobacteria are cultured in 8- or 20-1 borosilicate glass or polypropylene bottles. The system used is shown in Figure 1. White or neoprene rubber stoppers are used, or silicone rubber stoppers if rubber stoppers prove unsatisfactory. The stoppers and associated tubing and fittings are wrapped in a double thickness of muslin and autoclaved. Nonwoven cellulose sterilization wrap (American Hospital Supply, Evanston, IL) is currently under trial and appears to be a satisfactory reusable substitute for muslin. The stopper assembly and associated tubing are sterile transferred to the bottles in a laminar flow hood. A pair of stainless steel radiator hose clamps attached to the neck of borosilicate glass bottles permits the rubber stopper to be securely fastened with large rubber bands or string. All fittings are attached to 7-mm medium-wall glass tubing with silicone rubber tubing. Aeration inlet and venting outlets are through 30 or 37 mm membrane (0.3 μ m) air vents (Gelman Instruments, Ann Arbor, MI or Schleicher and Schull, Keene, NH) which are reusable. Venting is also accomplished with 25-mm or 47-mm polycarbonate Swin-Lok holders (Nuclepore, Pleasanton, CA) using 1 μ m

microfibre filters (Whatman, Inc., Clifton, NJ). Glass filters are preferred to other types in the venting outlets because they are less subject to blockage on accumulation of the cyanobacteria on their surface. All tubing from the air-CO₂ supply to the aerator tubes, and for all liquid transfers, except the silicone tubing in the peristaltic pumps, are made with 1.2-m extension tubes having luer lock end fittings on each end (Pharmaseal Laboratories, Glendale, CA). All glass tubing utilized for liquid transfer or aeration has a luer lock fitting attached with silicone rubber tubing to allow attachment of the extension tubes. The luer lock fittings are obtained from discarded extension tubes.

Aeration manifolds are constructed of polyvinylchloride aquarium manifold pipe and plastic valves (Kenwill Co., Athens, GA). A luer lock fitting is attached to each valve, adopting a uniform convention for the direction of attachment of the fittings. A pair of peristaltic pump heads, mounted in tandem to maintain synchrony, are driven by a variable speed motor for liquid transfer (Cole Parmer, Chicago, IL). Flow rates of 1 to 10 ml min⁻¹ provide a useful range which may also be varied by changing the size of the pump head.

The continuous culture apparatus simplified the growing of cyanobacteria. The following protocol is used for large scale culture. A sterile borosilicate glass or polypropylene bottle is inoculated with about 1 liter of an actively growing culture and 1 liter of sterile medium is added by pumping. The volume of the

culture was approximately doubled daily by pumping in sterile medium. Sterile medium can also be added by siphoning. When the culture reaches about mid-log phase, a sterile culture harvest bottle is attached to the apparatus and filled to a volume of about 18 l by pumping the cyanobacteria and medium from the culture bottle in two to three days. Concurrently, the culture bottle is maintained at a volume of about 18 l by addition of sterile medium. The newly filled culture harvest bottle is removed from the apparatus, and allowed to reach the desired cell density. The procedure of filling culture harvest bottles can be continued for four to six weeks. Deterioration of the culture is indicated by excessive and persistent foaming. Advantages of the peristaltic pump are: ease of sterilizing, elimination of lifting sterile medium bottles, and control by timers. The availability of inexpensive microprocessor-controlled variable timers (Chrontrol, San Diego, CA) permits the development of a simple, automated, continuous culture system. Batch culturing of a single bottle can also be performed with the same facilities and equipment.

Harvesting Cultures are conveniently harvested by continuous centrifugation. The inlet of a variable speed peristaltic pump is connected with 6.4-mm-o.d. polyethylene tubing to the aeration tube of a culture bottle by a luer lock fitting. The outlet of the pump is connected to the continuous flow centrifuge inlet. The maximum flow rate is a function of rotor speed and ease of sedimentation.

Typically, a flow rate of 400 ml min^{-1} at 12,000 xg will provide efficient harvesting of many cyanobacteria. Following harvest, the

combined cell paste may need to be recentrifuged for 5 min at 18,000 xg to remove additional medium. Alternately, the harvested cells are collected on sharkskin filter paper on a buchner funnel to remove excess medium. Addition of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ to a final concentration of 5 mM in the culture medium may assist the harvesting of freshwater forms.

Special Methods Herdman et al. (1970) synchronized growth of a cyanobacterium for at least three divisions by deprivation of light and CO_2 . Dialysis culture, a technique for examining the physiology and biochemistry of cyanobacteria (see Jensen et al. (1972); and Schultz and Gerhardt (1969)) permits culturing at constant nutrient conditions and studies of the nature of autoinhibition processes. Membranes with a wide range of molecular weight cutoffs are now available as a new investigative tool for dialysis culture.

Comments The purification of cyanobacteria is a critical prerequisite for further studies. Agar plating is generally the procedure used initially, but some recalcitrant cyanobacteria are not freed easily of contaminants and other techniques must be tried. Chemical and physical treatments for purification must be adjusted in concentration and dosage, respectively, for each organism. Success in purification depends on persistence and patience. Treatments which can induce mutations, such as ultraviolet irradiation, should be used with caution.

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Figure Legend

Figure 1 Continuous culture apparatus. The components are not drawn to scale and are as follows: A, sterile medium bottle; B, culture bottle; C, culture harvest bottle; D, synchronous peristaltic pumps; E, glass microfibre filters; and F, membrane air vent and air-CO₂ mixture inlet.