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REVIEWS

Modern Methods for Isolation, Purification, and Cultivation of Soil Cyanobacteria

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Abstract—Up-to-date methods for isolation of cyanobacteria from soil samples, removal of accompanying microflora, obtaining axenic strains, and conditions and media for subsequent cultivation are reviewed. Characterization of soil as a specific habitat for cyanobacteria is provided. Comparative analysis of pH and elemental composition of the liquid phase of most soil types with the media for cultivating cyanobacteria is carried out. The functional role of the major components required for the cultivation of cyanobacteria is described. The problems associated with isolation, purification, and cultivation of soil cyanobacteria, as well as the relevant solutions, are discussed.

Keywords: soil cyanobacteria, isolation techniques, contaminant removal, cultivation media, mixed cultures, axenic strains

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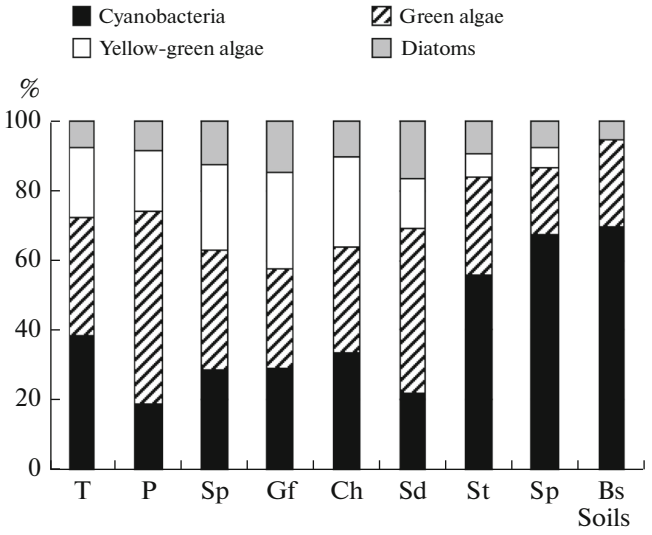
Cyanobacteria (*Cyanobacteria/Cyanophytes/Cyanoprokaryotes*) are a diverse group of oxygenic photosynthetic prokaryotes with unique physiology, broad ecological valence and plasticity. It allows them to dominate in diverse ecological habitats, including extreme ones: deserts, hot springs, hypersaline and alkaline basins, volcanic substrates, etc. (Seckbach, 2007). Emergence of cyanobacteria in the early Precambrian was one of the most important evolutionary events (Schopf, 1996). Further development of the plant kingdom was based on numerous intracellular symbioses between cyanobacteria and eukaryotic heterotrophs. The functional role of cyanobacteria in ecosystems is associated with their ability to perform oxygenic photosynthesis and nitrogen fixation and to produce extracellular organic compounds. They are involved in the formation of travertine and stromatolites; a considerable portion of limestone on the Earth emerged as a result of their metabolic activity (Komárek, 2006).

In the context of identification and classification, cyanobacteria are a complex group (Komárek et al., 2014), because the morphological characteristics conventionally used for their order- and family-level identification, as well as the type of thallus organization, trichome polarity, the presence and type of branching, the presence of sheaths, heterocytes, or akinetes, cell and trichome size, etc., seemed to appear and disappear several times during their long-term and intricate evolutionary history (Gugger and Hoffmann, 2004; Schirrmeister et al., 2011; Komárek, 2013; Shih et al., 2013). It is doubtless that assessment of many morphological characteristics of cyanobacteria should be revised. It is also probable that frequent convergence events were also characteristic of the group of cyanobacteria, resulting in the high hidden diversity and the absence of morphological variability of some taxa (Dvořák et al., 2015). However, description of both the known and novel cyanobacteria, in its turn, is impossible without obtaining their strains, i.e., without their isolation, purification, and cultivation.

SOIL AS A SPECIFIC HABITAT FOR CYANOBACTERIA

According to modern concepts, soil is a complex of simultaneously existing but absolutely different microenvironments (Zvyagintsev et al., 2005) that maintains the vast diversity and biomass of microorganisms: by some estimates, up to 2.6×10^{29} cells worldwide or 5% of all the prokaryotic cells on Earth (Voroney and Heck, 2015). Compared to other environments, soil has the richest microbial pool. The kilometers of fungal hyphae and over 10^9 cells of bacteria and archaea belonging to tens of thousands of different species are contained in 1 g of soil on average (Voroney and Heck, 2015); the total DNA length in 1 g of soil is more than 1500 km (Trevors, 2010). Such "overstock" of microorganisms, in the absence of conditions for their active growth and reproduction, is caused by the difficulty of rapid horizontal and vertical transport of microorganisms in soil for assimilation of all substances taken up



Diversity of culturable species of cyanobacteria and eukaryotic algae in the soils of Russia (according to our data and the data of Shtina et al., 1998). Designations: T, tundra; P, podzol; Sp, sod-podzol; Gf, gray forest; Ch, chernozem; Sd, solod; St, solonetz; Sp, saline playa; Bs, brown semidesert soil.

(Zvyagintsev et al., 2005). Cyanobacteria are an integral component of diversity of the soil microflora. Thus, 295 cyanobacterial species have been found in the soils of Russia (Shtina, 1996). It was shown that different soil types are characterized by different complexes of cyanobacterial taxa; therefore, their composition can be one of the soil diagnostic criteria (Shtina et al., 1998). Cyanobacteria are influenced by the following soil factors: hydrological regime, pH, and salt and granulometric compositions. For example, the indicators of soil moisture content below or equivalent to the wilting point are xerophilic cyanobacteria of the order *Oscillatorialles* (Shtina et al., 1998). The granulometric composition of soil has an indirect effect on the development of cyanobacteria, because light sandy soils have a low moisture-holding capacity and are characterized by drier conditions. Saline soils are characterized by the presence of halophilic cyanobacteria of the genera *Oscillatoria*, *Microcoleus*, *Schizothrix*, etc. Alkaline soils are characterized by predominance of cyanobacteria of the genera *Nostoc*, *Anabaena*, *Tolypothrix*, etc. The maximum dominance of cyanobacteria in the structure of algal groups is observed in solonetz, saline playa, and brown semidesert soil (see figure), i.e., under the conditions of moisture deficit, sparse plant cover, enhanced insolation, salinity, and alkalinity. However, cyanobacteria are commonly found also under milder soil conditions due to soil heterogeneity and microzonality.

ISOLATION OF CYANOBACTERIA FROM SOILS; CONDITIONS AND MEDIA FOR THE CULTIVATION OF CYANOBACTERIA

In the presence of visible growth of cyanobacteria on soil as crusts, films, or stains, they are sampled and cultivated to obtain mixed and pure cultures. However, the ability to grow on soil is typical of mainly filamentous cyanobacteria of the genera *Cylindrospermum*, *Nostoc*, *Anabaena*, *Oscillatoria*, *Phormidium*, *Microcoleus,* etc. (Domracheva, 2005), which make up only a small percentage of the total cyanobacterial pool in soil. In most cases, investigating the diversity of soil cyanobacteria requires collection of individual or mixed soil samples, usually with metal instruments (a knife, a blade, a trowel), into a plastic or glass container under sterile conditions. The sampling depth is usually 0–5 cm for virgin soils and 0–10 cm for cultivated soils (Khaziev and Kabirov, 1986). The soil type and geographical positions of the sampling points are registered for future reconstruction of the cultivation conditions maximally similar to the natural ones in nutrient medium composition, temperature, illumination, and pH.

The following conventional techniques for stimulation of the growth and reproduction of microorganisms can be used to isolate cyanobacteria from soil samples: cultivation of the initial sample (plate cultures with in situ glass slides, aqueous and soil/aqueous cultures) and inoculation of nutrient media with a small portion of the soil sample (suspension inoculation method, the method of soil clumps, “accretion glass” washouts) (Kostikov et al., 2001). These manipulations eventually result in obtaining mixed cultures of cyanobacteria and their further purification and reduction to monocultures growing in liquid and on solid (1–2% agar) nutrient media. The most widespread media for the cultivation of mixed and algologically pure cyanobacterial cultures are BG-11, Z8, Cyanophycean, ASN III, and Gromov no. 6.

The BG-11 medium originally proposed by Hughes et al. (1958) and later modified by Allen (1968), Rippka et al. (1979), and other researchers is extensively used for the isolation and cultivation of most cyanobacteria. The BG-11_0 medium is also used in the nitrogen-free variant and in the variant with 1.0 g/L NaCl (Waterbury, 2006). The Z8 medium described by Kotai (1972) is used for the cultivation of freshwater planktonic cyanobacteria (Skulberg, 1983). The nutrient medium Gromov no. 6 was developed by Soviet algologists (Gromov and Titova, 1983) and may be used in two variants: with and without nitrogen (for heterocyteous nitrogen-fixing cyanobacteria). The Cyanophycean medium is the simplest one in this list of nutrient media; it is characterized by the minimum number of components and can be successfully used for the isolation and cultivation of oligotrophic cyanobacteria. Unlike the above media suitable for cultiva-

Table 1. Chemical composition of the culture media for cyanobacteria

Component	Culture medium, g/L				
	BG-11	ASN III	Z8	Cyanophycean	Gromov no. 6
$NaNO_3$	1.5	0.75	0.467	–	–
KNO_3	–	–	–	5.0	1.0
$Ca(NO_3)_2 \cdot 4H_2O$	–	–	0.059	–	–
K_2HPO_4	0.04	0.02	0.031	0.05	0.2
$MgSO_4 \cdot 7H_2O$	0.075	3.5	0.25	0.05	0.2
$MgCl_2 \cdot 6H_2O$	–	2.0	–	–	–
$CaCl_2 \cdot 2H_2O$	0.036	0.5		–	0.15
NaCl	–	25.0	–	–	–
KCl	–	0.5	–	–	–
Na_2CO_3	0.02	0.04	0.021	–	–
$NaHCO_3$	–	–	–	–	0.2
Citric acid	0.006	0.003	–	–	–
$FeNH_3$-citrate	0.006	0.003		–	–
Microelement solution	1 mL				

tion of freshwater cyanobacteria, ASN III is recommended for halophiles (Rippka et al., 1981, 1988). The chemical composition of macro- and microelement solutions of the culture media is listed in Tables 1 and 2, respectively. Let us briefly consider the functional roles of essential elements for the cultivation of cyanobacteria.

Macroelements are present in the nutrient media in sufficiently large quantities, since they are involved in the energy and plastic metabolism of cyanobacteria. The presence of inorganic carbon species, carbonates (BG-11, ASN III and Z8) or hydrocarbonates (Gromov no. 6), is necessary for the high rates of cyanobacterial growth. Nitrogen, sulfur, and phosphorus are usually added to the media as nitrates, sulfates, and hydrophosphates and comprise proteins, nucleic acids, vitamins, and lipids of cyanobacteria. Potassium is responsible for osmosis in the cells; sodium, calcium and magnesium are the components of enzymes, chlorophyll *a*, membranes, and cell walls. The trace elements of primary importance for cyanobacteria are such metals as iron, manganese, cobalt, copper, molybdenum, zinc, nickel, vanadium, and tungsten; in their absence, the activity of many enzymes (nitrogenase, urease, cytochrome oxidase, etc.) is impossible. Boron, which is involved in the formation of cyanobacterial heterocytes and akinetes (Overmann, 2006), is present only in the BG-11, ASN III, and Z8 media. The commonly used chelating agents are EDTA, sometimes citric acid or citrate (BG-11 and ASN III).

It should be noted that the above media have been accepted from the practice of cultivation of aquatic cyanobacteria and are actually the analogs of fresh- or seawater. They therefore cannot imitate the diversity of soil conditions. In the ideal case, the medium for the cultivation of soil cyanobacteria must be similar to the liquid phase of soil under study, since the soil liquid phase reproduces all characteristic features of soil in its composition, closely contacts other phases, transfers nutrients, and provides microorganisms with water (Zvyagintsev et al., 2005; Trofimov and Karavanova, 2009). Comparison of the elemental compositions of soil solutions and lysimetric waters of some soil types with the nutrient media presented above (Table 3) leads to a conclusion that the latter may not reflect the specificity of particular soil conditions. Since specific media for soil cyanobacteria have not yet been developed, the following guidelines should be followed, which were developed based on the analysis of a number of algological works and personal experience in the culturing practices.

(1) Transfer of cyanobacteria from natural soil to artificial laboratory conditions must be successive and gentle, since this transition is a stress factor for microorganisms (Hagemann, 2002). For example, we use the following combination of techniques: obtaining mixed cultures by cultivation of the initial substrate (plate cultures with in situ glass slides and soil-water cultures) and soil inoculation into liquid nutrient media (BG-11 with and without nitrogen, Cyanophycean) in 96-well culture plates. Then the mixed cultures are transferred and purified on solid nutrient

Table 2. Composition of trace element solutions of the culture media for cyanobacteria

Component	Microelements for culture media, g/L			
	BG-11, ASN III	Z8	Cyanophycean	Gromov no. 6
H_3BO_3	2.86	3.1	–	–
$NaBO_3 \cdot 4H_2O$	–	–	–	2.63
$MnCl_2 \cdot 4H_2O$	1.81	–	0.246	–
$ZnSO_4 \cdot 7H_2O$	0.222	0.287	–	0.22
$Na_2MoO_4 \cdot 2H_2O$	0.39	–	0.024	–
$CuSO_4 \cdot 5H_2O$	0.079	0.125	–	0.079
$Co(NO_3)_2 \cdot 6H_2O$	0.0494	0.146	–	0.02
$Na_2EDTA \cdot 2H_2O$	1.0	0.0037	4.5	10.0
$K_2MgEDTA \cdot 2H_2O$	0.5	–	–	–
$FeCl_3 \cdot 6H_2O$	–	0.0028	0.582	–
$ZnCl_2$	–	–	0.03	–
$CoCl_2 \cdot 6H_2O$	–	–	0.012	–
$MnSO_4 \cdot 4H_2O$	–	2.23	–	1.81
$Na_2WO_4 \cdot 2H_2O$	–	0.033	–	–
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	–	0.088	–	1.0
KBr	–	0.119	–	–
KI	–	0.083	–	–
$Cd(NO_3)_2 \cdot 4H_2O$	–	0.154	–	–
$NiSO_4(NH_4)_2SO_4 \cdot 6H_2O$	–	0.198	–	–
$Cr(NO_3)_3 \cdot 9H_2O$	–	0.041	–	–
$V_2O_4(SO_4)_3 \cdot 16H_2O$	–	0.035	–	–
$Al_2(SO_4)_3K_2SO_4 \cdot 24H_2O$	–	0.474	–	–
$FeSO_4 \cdot 7H_2O$	–	–	–	9.3

media, with axenization of the strains whenever necessary.

(2) For realization of biotic potential of different groups of cyanobacteria, including weakly competitive and slowly growing organisms, it is recommended not to limit the cultivation periods to several weeks or months but to increase the time of observation of mixed cultures to 6–12 months. In this case, the groups of cyanobacteria will replace each other in the laboratory microcosm according to their ecological and physiological requirements, and researchers will be able to describe the species with different types of life strategies varying in growth rates, competitiveness, and resistance to environmental factors.

(3) Table 3 shows that some nutrient media have a weakly acidic reaction, while most cyanobacteria are neutrophiles and alkaliphiles (Rippka et al., 1981). Therefore, HEPES should be used to obtain the neutral and alkaline media (pH 7–8) (Rippka, 1988).

(4) Due to the highly dynamic and microzonal distribution of micro- and macroelements within the soil liquid phase, it is not advisable to fully reproduce its chemical composition and to use it as a culture medium. It would be sufficient to use several nutrient media of different compositions and to regulate pH value whenever necessary. In our studies of cyanobacteria from a variety of zonal soils (solonetz, brown semidesert, chestnut, and gray forest soils), BG-11 with or without a nitrogen source was the optimal medium providing for the maximum taxonomic diversity, which was also characterized by simplicity of preparation and the low cost of its components.

(5) Inoculation of a solid nutrient medium with cyanobacteria should occur not only by spreading over

Table 3. The content of some macroelements and pH of the liquid phase in different types of soils and culture media for cyanobacteria

Media	Soil type*						
	pH	Element conteny, mg/L				Method for obtaining the soil liquid phase	Reference
		Na	K	Ca	Mg		
Sod-strongly podzolic soil (A_0)	4.1	2.6	13.0	26.4	8.4	Lysimetric water	Ponomareva and Sotnikova, 1972
Podzol (A_0)	4.3	1.7	2.2	5.5	1.0	Lysimetric water	Ponomareva and Sotnikova, 1972
Brown forest soil (H)	4.6	1.3	3.7	3.1	0.7	Vacuum filtration	Karavanova and Belyanina, 2007
Gray forest soil (A 0–10 cm)	5.3	2.7	4.8	25.0	5.8	Lysimetric water	Zolotareva et al., unpublished
Podzolic soil (AE + H)	5.9	2.7	2.1	5.2	1.0	Vacuum filtration	Karavanova and Belyanina, 2007
Humus-gley soil (A_s)	6.1	1.8	2.0	6.0	1.2	Vacuum filtration	Karavanova and Belyanina, 2007
Tundra gley soil (OA 0–10 cm)	6.4	10.0	10.0	24.0	10.0	–	Belitsina et al., 1988
Leached chernozem (A 0–10 cm)	6.4	1.1	3.4	5.5	5.6	Lysimetric water	Zolotareva et al., unpublished
Solod (A 0–10 cm)	6.4	46.0	5.0	72.0	38.0	–	Belitsina et al., 1988
Meadow solonets (AE 0–6 cm)	7.8	299.0	31.0	52.0	25.0	–	Belitsina et al., 1988
Crust saline playa (A 0–5 cm)	7.9	47430.0	3660.0	840.0	3790.0	–	Belitsina et al., 1988
Regular chernozem (A 0–10 cm)	8.1	6.7	33.6	170.0	38.4	Displacement with ethanol	Volkova, 1975
Z8	6.0–7.0	135.4	14.0	10.0	24.7		Scandinavian Culture ..., 2015
Cyanophycean	6.3–6.6	0.6	1956.2	0	4.9		Our data
BG-11	6.9–7.4	414.6	18.0	9.8	7.4		Rippka et al., 1981; Waterbury, 2006
ASN III	7.1–7.5	10055.7	271.3	136.3	584.3		Rippka et al., 1981
Gromov no. 6	7.7–7.9	56.4	476.5	54.2	19.7		Our data

* The soil layer and, in some cases, thickness are given in parentheses.
Designations: n-dash means “no data”. A, humus horizon; A_s, sod horizon; AE, humus-eluvial podzolized horizon; A_0, forest litter; H, humus accumulation horizon; OA, ground litter.

ager surface, but also by stab inoculation, because some cyanobacterial species grow poorly on agar surface but develop well inside the agar (Watanabe et al., 1998).

(6) It should be taken into consideration that medium composition can influence the morphological parameters of cyanobacterial cells. For example, cultivation of *Spirirestis rafaelensis* on the BBM medium (the standard medium for green microalgae) was accompanied by considerable morphological changes in the cells of this cyanobacterium compared to the nitrogen-free Z8 medium (the width of trichomes and filaments increased, the color changed from olive green to blue-green, and the filaments became less twisted). The authors supposed the Z8 medium to be more suitable for the cultivation of this cyanobacterium (Flechtner et al., 2002). Thus, at the stage of isolation of cyanobacteria, a wide variety of nutrient media and cultivation techniques should be used to reveal their maximum diversity. However, the isolated strains should be transferred into conventional growth media for cyanobacteria for their subsequent identification (BG-11 with and without nitrogen, Z8, ASN III, etc.).

The temperature and light regimes for cultivating cyanobacterial strains should also be close to those of their natural habits. As a rule, the illumination range of 10–75 μmol photons m^{-2} s^{-1}, the temperature range of 20–25°C, and the day/night lighting conditions (12 : 12, 14 : 10, or 16 : 8 h) are suitable for a wide variety of cyanobacteria (Waterbury, 2006).

OBTAINING MONOCULTURES AND AXENIC STRAINS OF CYANOBACTERIA

Purification of mixed cultures and obtaining of the algologically pure and axenic strains is a prerequisite for accurate identification of soil cyanobacteria. The key steps are both direct microscopic examination of grown cultures and assessment of the colony size, shape, and color, of the pattern of growth on agar, and of the presence or absence of mucus. Attempts at taxonomic research of cyanobacteria in mixed cultures are associated with an increased risk of erroneous identification of different stages of development of a species as several different species. For example, cyanobacteria of the genus *Gloeocapsa* have a complex life cycle: the *Aphanocapsa*-like stage is characterized by the presence of homogeneous mucus instead of individual mucous envelopes; the resting stages with well-developed sheaths are common under unfavorable conditions (Komárek and Anagnostidis, 1998). Therefore, representatives of this genus in a mixed culture can be easily confused with representatives of other cyanobacterial genera such as *Aphanocapsa*, *Chroococcus*, *Gloeocapsopsis*, etc. (Shalygin, 2012). Furthermore, one of the important diagnostic features of cyanobacteria of the genus *Phormidium* used for species identification is the shape of terminal cells (Komárek and Anagnostidis, 2005). This feature should be observed in mature filaments and, hence, the cyanobacterial strain must be cultivable. Such observations of culture morphology at different life stages are required for correct identification of many cyanobacteria and are, in their turn, impossible without pure cultures (monocultures). Cyanobacterial strains grow in the same liquid media (or on the same solid media) and under the same conditions as mixed cultures. Pure cultures of cyanobacteria not only prevent the possibility of "double counting" of separate stages of their development but are also necessary for further morphological description and molecular genetic analysis.

The simplest and most widespread methods for obtaining cyanobacterial monocultures are isolation from colonies obtained by streak inoculation or isolation of a single cell/filament with a micropipette under a microscope followed by its transfer into/onto a fresh liquid/solid medium. Other frequently used techniques include serial dilution or centrifugation of a mixed culture with preliminary homogenization of the colonies by grinding or sonication; filtration of a mixed culture through the membranes with a known pore size; and application of phototaxis of motile cyanobacteria. Increased pH of the medium (Sena et al., 2011) and an increase in the cultivation temperature to 35°C (Allen and Stanier, 1968) inhibit the growth of satellites, algae, and protozoa. Different methods or their combinations should be used depending on the type of organization of the cyanobacterial thallus (coccoid or filamentous), cell size, and the degree of motility. As a rule, an algologically pure culture suitable for determining its taxonomic affiliation is obtained after several purification procedures. However, it is not always possible. For example, it was shown that the marine cyanobacterium UCYN-A, which has not been cultured under laboratory conditions, lacks some genes that are responsible for important metabolic pathways. Therefore, these cyanobacteria live in symbiosis with microscopic unicellular algae which, in exchange for organic nitrogen, supply cyanobacteria with the nutrients formed as a result of photosynthesis (Thompson et al., 2012). It should not be ruled out that soil cyanobacteria may have such relationships with other microorganisms.

In some cases, axenic strains of cyanobacteria are needed to perform additional physiological tests or biochemical analyses for taxonomic identification. It should be emphasized that soil cyanobacteria are a rather complex object in the context of achieving their axenicity. First, soil contains numerous and diverse potential contaminants of cyanobacterial cultures: bacteria, fungi, protozoa, etc., which are difficult to eliminate. Second, many cyanobacteria form extensive mucous envelopes inhabited by closely associated heterotrophs. Nevertheless, if the stage of axenization is crucial, it can be achieved by the following methods used all together in a series or selectively depending on

the features of purified cyanobacteria and their contaminants.

(1) The first stage in the purification of colonial and filamentous cyanobacteria is homogenization of their colonies and long filaments by centrifugation with glass beads, glass-wool filtration, and ultrasonic treatment (Andreyuk et al., 1990; Vázquez-Martínez et al., 2004; Han et al., 2014).

(2) In the case of mucus-forming cyanobacteria, it is necessary to remove mucous sheaths, capsules, or amorphous slime around the cells and filaments, because many contaminants may be attached to the mucous envelopes or live directly inside them. The mucus can be removed by filtering the culture through asbestos filters (Pankratova et al., 2008) or by adding surfactants, e.g., cetylpyridinium bromide and sodium oleate (Zhubanova, 2013).

(3) The single-cell technique consists in mechanical sampling of a cell/filament free from contaminants with a micropipette or a micromanipulator under a microscope (Shiraishi, 2015). The method is simple but requires numerous repeats.

(4) UV irradiation of the isolates (Pankratova et al., 2008; Zhubanova, 2013). Axenic cyanobacterial cultures can be obtained only when the target objects are UV-resistant and other contaminants are sensitive to UV radiation.

(5) Serial dilution of a culture, usually 1 : 1000, 1 : 10000, and 1 : 100 000 (Sena et al., 2011), is efficient for unicellular cyanobacteria or after the stage of homogenization of the colonies and long filaments.

(6) Density gradient centrifugation (Bolch and Blackburn, 1996) can be successfully used if there is a substantial difference in cell density between cyanobacteria and their contaminants.

(7) Treatment with antibiotics and antimycotics. Their selection should be individual in each case depending on the nature and degree of contamination.

Table 4 presents the examples of antibiotics successfully used to obtain axenic cultures of some cyanobacteria. Generally, there are some common techniques that should be used in antibacterial treatment of cyanobacterial isolates:

—The culture is treated with antibiotics under continuous agitation;

—An organic substrate (e.g., 0.1% glucose) is added to the medium and the cultures are stored in the dark for 1–2 days for stimulation of the growth of heterotrophic contaminants and subsequent inhibition of the biosynthesis of their cell walls (or other targets) in the presence of antibiotics;

—The cells of cyanobacteria are thoroughly washed after antibacterial treatment; and

—The inhibitors of cell wall synthesis and cell growth inhibitors are not used simultaneously (Guillard, 2005).

With the application of only one of the above methods, it is usually possible to reduce only the initial contamination of mixed culture; hence, researchers have to use different combinations of the manipulations described above, depending on concomitant microorganisms, to obtain the axenic strains of cyanobacteria. Apart from these techniques, rather original protocols are presented. Thus, Dubinin et al. (1992) successfully used 0.001 M potassium cyanide to remove bacterial satellites from the enrichment culture of the cyanobacterium *Microcoleus chthonoplastes* (=*Coleofasciculus chthonoplastes*). Elango et al. (2008) proposed a simple technique for purification of the cyanobacterium *Westulopesis* sp. from heterotrophic bacteria and fungi: to use double-distilled water instead of the culture medium. However, we failed to purify a nonaxenic strain *Leptolyngbya* sp. by using this technique. Thus, the procedure of axenization of cyanobacterial strains seems to be rather labor- and time-consuming, requires an individual approach in any particular case, and is not always justified. For example, continuous application of antibiotics for many years may result in serious problems with the strains. The study of Barile (1983) showed that 72% of the cultures permanently growing on antibiotic-containing media were contaminated with mycoplasma, while only 7% of them were infected in the absence of antibiotics. In turn, the vast majority of mycoplasmas are resistant to antibiotics: 80% to gentamycin, 98% to erythromycin, and 73% to kanamycin (Lundin and Lincoln, 1994). Moreover, the use of some antibiotics, particularly penicillin and ampicillin, may cause more intensive growth of resistant bacteria in cyanobacterial mixed cultures (Ferris and Hirsch, 1991). Some cyanobacteria in axenic cultures are characterized by impaired cellular morphology, instability, or complete absence of growth (Dubinin et al., 1992). Therefore, it would be better only to minimize the contamination so that the stock cultures of cyanobacteria could be maintained in a collection. For long-term storage, the strains are usually cultivated at 10–15°C, with reinoculation 1–2 times per year (Acreman, 1994; Lee and Shen, 2004), or with the application of modern cryopreservation techniques (Harding et al., 2004; Rastoll et al., 2013).

CONCLUSIONS

At present, the estimated levels of diversity of cyanobacteria from different habitats vary from 2000 (Sant'Anna et al., 2006) to 8000 species (Guiry, 2012). The realism of these assumptions depends on the range of up-to-date methods for the isolation, purification, and cultivation of cyanobacteria used by algologists, and then upon the identification techniques. The species diversity of soil cyanobacteria seems to be underestimated primarily due to methodological problems of their isolation and cultivation.

Table 4. Axenic cultures of cyanobacteria obtained using antibiotic treatment

Antibiotic	Dose, μg/mL	Supplementary conditions	Cyanobacteria	Reference
Lysozyme Tienam	20 100	Light incubation, 7 days	Filamentous cyanobacteria	Sarchizian and Ardelean, 2010
Cyclohexamide	20	Light incubation, 7 days	Cyanobacteria	Sarchizian and Ardelean, 2010
Ampicillin	1000	Addition of 0.02–0.1% casamino acids and 0.5% glucose; incubation in the dark, 24–48 h	Cyanobacteria	Rippka, 1988
Ampicillin + Penicillin + Cefoxitin + Meropenem	61.6 + 85.8 + 76.9 + 38.9	Dark incubation, 48 h	*Arthrospira* sp.	Sena et al., 2011
Imipenem + Cyclohexamide	100 + 20	Dark incubation, 24 h	*Nodularia spumigena*	Hong et al., 2010
Kanamycin	100			
Lyzozyme	0.001	Stirring for 60–90 min, washing	*Anabaena flos-aquae*, *Aphanotheca nidulans*	Kim et al., 1999
Cyclohexamide	50–100	Light incubation, 7–10 days		
Imipenem + Ampicillin + Penicillin + Cefoxitin + Cycloheximide	100	Addition of 2.5% sucrose, 0.5% yeast extract, and 0.5% bacto peptone; dark incubation, 18–24 h	*Oscillatoria* sp.	Ferris and Hirsch, 1991
Imipenem	100	Addition of 2.5% sucrose, 0.5% yeast extract, and 0.5% peptone; dark incubation, 18–24 h	*Microcystis aeruginosa*	Bolch and Blackburn, 1996
Nystatin + Cycloheximide	100 + 100	Light incubation, 3–4 weeks	*Microcystis aeruginosa*	Bolch and Blackburn, 1996
Imipenem + Cycloheximide	100 + 20	Addition of 0.1% glucose; dark incubation, 24 h	*Arthrospira platensis*	Choi et al., 2008
Neomycin + Cycloheximide	100 + 20	Addition of 0.1% glucose; light incubation, 24 h	*Arthrospira platensis*	Choi et al., 2008
Carbenicillin	100	Dark incubation, 24 h	*Phormidium animalis*	Vázquez-Martínez et al., 2004
Streptomycin	100			
Chloramphenicol	100			
Kanamycin	150			

In spite of the prevailing opinion that there is a considerable percentage of unculturable bacteria and that only 1–20% of soil microorganisms are successfully converted into a culture (Manucharova, 2009), and despite the rapid advances in metagenomics, a living culture is presently the only unquestionable evidence of the existence of an object in space and time. The optimal choice of methods for the isolation, purification, and cultivation of cyanobacteria will ensure the success of any investigation of a given object: from taxonomy, floristics, and phylogeny to bioengineering, biotechnology, and biomonitoring.

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