ISOLATION AND PURIFICATION OF CYANOBACTERIA

Often initial isolations of cyanobacteria from any environment may give rise to mixed cultures. Therefore, it is essential to purify the individual types of cyanobacteria from the mixture. This could be achieved easily by streak or spread or pour plate techniques. All these are essentially dilution techniques of different types resulting in the physical separation of individual cyanobacterium from the mixture allowing to form district colonies, which can then be picked up to make pure cultures.

A. Isolation Methods:

1. Streak Plate

Principle:

In a streak plate, from the loop containing cyanobacteria, varying numbers of cyanobacteria adhere to the surface of the medium and towards the end of the strake the number gets so much reduced to form separate colonies.

Procedure:

Prepare agar plates containing marine / freshwater medium. Flame the inoculation needle and cool it by jabbing it into the edge of the agar medium. With the drop on the edge away from the body, 'streak' the culture back and forth, edge to edge in parallel line, moving towards the body. When the needle reaches the center of the plate, spin it around 180O and continue streaking, now moving away from the body. This reversal avoids interference with the needle.

2. Spread Plate

Principle:

In spread plate the cyanobacteria in liquid medium are directly spread over the entire surface of the solid medium resulting in separation at many places.

Procedure:

Prepare marine / freshwater agar medium. Sterilize the medium, petriplates and tissue grinder. Grind a small portion of cyanobacteria with the help of tissue grinder homogenously. Take 1 ml of ground mixture and make serial dilutions.

Label the petridishes. Pour the sterilized agar medium into petridishes and allow them to solidify. Inoculate 0.1 ml of diluted cyanobacterial sample mixture onto the surface of agar and 'spread' it thoroughly with 'L' glass rod.

3. Pour Plate

Principle:

In pour plate there is direct dilution of cyanobacteria while being suspended in the pour agar resulting in separation at the time of plating. The addition of solidifying substance to liquid media containing cyanobacterial cells, traps the individual cells in place. In the agar medium instead of floating around when they multiply, as in the liquid medium, they produce a fixed colony of the cells or filaments and grow to form separate colonies.

Procedure:

Prepare a set of sterile 99.9 and 9 ml water blanks in 250 ml conical flasks and 20 ml tubes, respectively. Add 100 mg cyanobacterial sample in 99.9 ml water blank and shake the contents thoroughly. Label it as 10^{-1} dilution.

Transfer 1 ml from 10^{-1} dilution to 9 ml water blank and mix it thoroughly. Label it as 10^{-2} dilution. Transfer 0.1 ml of the diluted suspension to a tube containing molten marine agar medium cooled to 47°C, mix the contents and pour into sterile petridish. Prepare 10^{-3}, 10^{-4}, 10^{-5} dilutions following similar procedure and transfer 0.1 ml suspension from each dilution in separate molten agar medium to prepare pour plates from each dilution. Allow the contents to solidify.

Comments

There is no accurate way of predicting the number of viable cells in a given sample by this method. Therefore, one must always make several dilutions of the sample and pour several plates. However, experience with a certain type of sample will enable to minimize wasteful dilutions. The success of this method depends upon thorough mixing and even distribution of cells.

B. Inoculation Procedure

To grow an organism in a sterilized medium a number of cells or filaments (inoculum) are transferred (inoculated) into the medium with special precautions to maintain the purity of the culture.

In the inoculation procedure the needle or loop, used to transfer cyanobacteria, should be heated to red-hot by flaming immediately, before and after making the transfer. Hold the needle down in the flame, to heat both the whole needle and the lower part of the handle. The flaming destroys any living forms on the surface of the needle or loop. During transfer hold the tube in the left hand and hold the plug between the fingers of the fight hand. Never lay a plug down. Hold the tube as nearly horizontal as possible during transfer and do not leave it open

longer than necessary. The mouths of the tubes from which cultures are taken and into which they are transferred should also be passed through the burner flame immediately before and after the needle is introduced and removed. In addition to destroying any organism on the tip of the tube, flaming tends to create outward convection currents, thus decreasing the chance of contamination.

C. Incubation Conditions

The cultures are normally incubated at 25± 2°C under continuous illumination (2,000-3,000 lux) unless otherwise required. Because of the high concentration of water in agar, condensation of water may form in petridishes during incubation and moisture is likely to drip from the cover onto the surface of the agar and spread out, resulting in a confluent mass of growth and ruining individual colony formation. To avoid this, the plates are incubated bottom side up. The slant cultures should be placed in a slanting position, with the streak towards light source. Always label all petridishes, tubes, flasks and culture bottles with name, date and identification of contents.

D. Culture Room

A culture room (growth room or incubation chamber) for cyanobacteria can be designed depending upon the space and nature of work. In general, a growth room should have a provision of controlled illumination, temperature and aeration facilities for the culture vessels.

E. Isolation Procedures (Rippka et al., 1979)

1. Unicellular Cyanobacteria

a. Transfer samples from primary enrichment tubes to fresh liquid medium and incubate.
b. From the secondary tubes, streak the cyanobacteria, onto the mineral agar plates and incubate.
c. After incubation, isolate colonies with a sterile loop and inoculate into new media and incubate.
d. Successive transfers from liquid to solid media usually result in unicyanobacterial cultures.

2. Filamentous Cyanobacteria

Pure cultures of filamentous forms usually can be obtained simply by repeated liquid transfer of small amounts of material. Phototactic response of the cyanobacteria is also utilized to separated them from other cyanobacteria.

a. Place a small mass of cells on a slide in a liquid medium

b. Cover with coverglass and seal with Vaseline, paraffin, or similar compounds.
c. Incubate slide in unidirectional light for 2-10 hrs. The filaments move from the mass so that single filaments are observed at the outer edge.
d. Isolate with a sterile Pasteur type capillary pipette and transfer it to a tube of liquid medium or on the surface of solidified medium (1%) in a petridish (A low agar concentration less than 1.5% is important for obtaining good growth and the filament rapidly migrates through 1% agar).
e. Incubate in unidirectional light.
f. Isolate filaments that are visibly free of bacterial contamination from the rapidly moving front on the illuminated side of the plate. By several passages through agar axenic filamentous strains can be obtained.

F. Purification Methods

It is often possible to obtain axenic cultures from field material by performing isolation and the subsequent purification steps on plates as described. Sometimes, however, this is not the case, particularly if the natural sample is highly contaminated by bacteria and the cyanobacterial species are immotile and incapable of self-purification by gliding away from their contaminants, or if the single filaments or micro colonies do not grow on solid media. Purification of such cyanobacteria should be attempted after the establishment of unicyanobacterial isolates in liquid cultures.

1. Repeated liquid subculture

This technique has been successfully used when a natural collection is particularly rich in a specific cyanobacterium.

Procedure:

1. Prepare marine liquid medium and distribute it to flask and sterilize
2. Make frequent subcultures to the fresh medium to get rid of the other contaminating bacteria

2. Fragmentation

Procedure:

1. Homogenize filamentous forms with a glass homogenizer for 5-10 min. allowing short filaments of 4-8 cells long, to be obtained.
2. Then follow either streak/spread/pour plate method to obtain individual colonies.

3. Antibiotic treatment:

It may be difficult to remove certain contaminants by repeated and/or ultrasonic treatment. In such instances, use of a chemical method rather than physical method is preferred, but by physical methods such as washing and ultrasonic treatment may proceed. One such chemical method used antibiotics singly or in combination to kill or inhibit the growth of tenaciously attached contaminants. Purification by an antibiotic treatment has been adopted by many workers.

Procedure:

1. Dissolve 100 mg penicillin G (K or Na salt) and 50 mg streptomycin sulphate, together in 10 ml distilled water; add 10 mg chloramphenical dissolved in 1 ml 95 % ethanol to the penicillin-streptomycin solution and mix well.
2. Filter the triple antibiotic solution quickly, using a membrane or seitz filter.
3. Place 1 ml of cyanobacterial suspension to be purified in each of six 125 ml Erlenmeyer flasks, each containing 50 ml culture medium.
4. Add one of the following volumes of antibiotic solution to each of the flasks: 0.125, 0.25, 0.5, 1.0, 2.0, 3.0 ml. This provides penicillin levels ranging from approximately 20-500 mg-1 and corresponding levels of two other antibiotics.
5. Place the culture flasks under suitable conditions for growth.
6. After 24 and 48 hrs, aseptically transfer some cyanobacterial cells from each flask to tubes of sterile, antibiotic free culture medium. Prepare tubes in triplicates at specific intervals and incubate.
7. Check the culture tubes for bacterial contamination after 2-3 days using nutrient agar plates.

Some of the bacteriostatic compounds like potassium tellurite (10 mg^{-1}) has also been used to obtain bacteria free cyanobacterial algal cultures. Purification can also be done by treatment with cycloserine (5 μg^{-1}).

4. Ultraviolet (UV) irradiation

This method has been widely used to obtain pure cultures of cyanobacteria. Place a dilute cyanobacterial suspension in quartz-windowed chamber and irradiate for 20-30 min. at 275 nm. Agitate the suspension by continuous stirring during the irradiation period. Remove the cyanobacterial samples from the irradiation chamber at 5 min. intervals and prepare a large number of dilution cultures from each. Inoculate the diluted cyanobacterial suspension into fresh medium and incubate in a culture room. Usually cultures from long exposure times fail to

grow, while those from shorter exposures are contaminated. However, certain subcultures, particularly from samples with intermediated exposure will be free of bacteria.

G. Test for purity

Presumptively pure cyanobacterial cultures are best grown in liquid medium. Stationary phase cultures are then examined critically under the microscope using phase-contrast objectives and oil immersion. For additional macroscopic tests small drops of the liquid cultures are inoculated onto plates prepared with the appropriate mineral medium supplemented with casamino acids (0.02-0.05% w/v) and glucose (0.5% w/v). More complex media should be avoided since some bacteria will not grow on them.

The plates are then incubated in the dark for several days at a temperature typical for growth of the cyanobacteria to be tested, since any contaminants still present should grow at that temperature. The contaminants will then be easily detected by their superior growth, colour, and other typical appearances (ie. fungal hyphae). If the culture is heavily contaminated the entire area of the test drop will be covered with bacterial or fungal growth; a low degree of contamination will reveal itself in the form of microcolonies on the cyanobacterial lawn. Some contaminants (eg. Gliding bacteria) do not form discrete colonies on the surface of the agar. Their presence can only be confirmed by additional microscopy, after screening small sections from the occupied by the cyanobacterial deposit.

H. Culture Media

To isolate and maintain the marine / freshwater cyanobacteria, appropriate media are used and congenial environmental conditions are maintained. Phosphate, nitrate, magnesium and calcium are the macronutrients generally required by them. The essential micronutrients are iron, zinc, manganese, copper and molybdenum. Since they are mostly adapted to diffused light, good growth is obtained at 1,400 lux light intensity. The optimal temperature required for their growth is 27-35°C. A number of culture media for growing cyanobacteria are available. The most commonly used are ASN-III, ASP-2 and enriched sea water medium (for marine cyanobacteria) and BG11

Composition of media:

1. ASN-III medium: (g l^{-1}) (ATCC: 824)

Nacl	- 25.0
$MgCl_2$ $5H_2O$	- 2.0
KCl	- 0.5
$NaNO_3$	- 0.75
K_2HPO_4 $7H_2O$	- 0.02
$MgSO_4$ $7H_2O$	- 3.5
$CaCl_2$ $2H_2O$	- 0.5
Citric acid	- 0.003
Ferric ammonium citrate	- 0.003
EDTA (disodium salt)	- 0.0005
Na_2CO_3	- 0.02
Trace metal mix A5 + Co *	- 1.0 ml
Distilled water	- 1000.0ml
PH after autoclaving	- 7.5

*Trace metal mix A5 +Co contains (g 1^{-1})

H_3BO_3	- 2.86
$MnCl_2$ $4H_2O$	- 1.81
$ZnSO_4$ $7H_2O$	-0.222
Na_2MoO_4 $2H_2O$	- 0.390
$CuSO_4$ $5H_2O$	- 0.079
$Co(NO_3)_2$ $6H_2O$	- 1.8g
$MgSO_4$ $7H_2O$	- 0.5g
KCl	- 0.06g
$CaCl_2$ H_2O	- 10.0mg
$NaNO_3$	- 5.0mg
K_2HPO_4 $3H_2O$	- 0.5mg
$NaSiO_3$ $9H_2O$	- 15.0mg
Tris	- 0.1g
Vitamin B_{12}	- 0.2μg
Vitamin mix S3*	- 1.0ml
EDTA (disodium salt)	- 3.0mg

$FeCl_3$	- 0.08mg
$ZnCl_2$	- 5.0µg
$MnCl_2$ $4H_2O$	- 0.12mg
$CoCl_2$ $6H_2O$	- 0.3µg
$CuCl_2$ $2H_2O$	- 0.12µg
H_3BO_3	- 0.6mf
Distilled water	- 100.0ml
PH after autoclaving	- 7.6-7.8

1ml of vitamin mix S3 contains

Thiamine HCl	- 0.05mg
Nicotinic acid	- 0.01 mg
Calcium Pantothenate	- 0.01mg
p-aminobenzoic acid	- 1.0µg
Biotin	- 1.0µg
Inositol	- 0.5mg
Folic acid	- 0.2µg
Thymin	- 0.3mg

2. Seawater enrichments

A.	$NaNO_3$	- 10.0mg
	Na_2HPO_4 $2H_2O$	- 2.0mg
	Sea water	- 100.0 ml
B.	KNO_3	- 20.2mg
	K_2HPO_4	- 3.5mg
	$FeCl_3$	- 0.097mg
	$MnCl_2$	- 0.0075mg
	EDTA (disodium salt)	- 1.0mg

Chemicals	**Grams/Litre**
$NaNO_3$	1.5
K_2HPO_4	0.04
$MgSO_4 \cdot 7H_2O$	0.075
$CaCl_2 \cdot 2H_2O$	0.036
Citric acid	0.006
Ferric ammonium citrate	0.006

EDTA (disodium salt)	0.001
$NaCO_3$	0.02
Thiamine HCl	- 1.0µg
Aged sea water	- 75.0 ml
Distilled water	- 25.0ml

3. BG-11medium: (UTEX culture collection of algae)

Trace metal mix:

H_3BO_3	2.86
$MnCl_2 \cdot 4H_2O$	1.81
$ZnSO_4 \cdot 7H_2O$	0.222
$NaMoO_4 \cdot 2H_2O$	0.39
$CuSO_4 \cdot 5H_2O$	0.079
$Co(NO_3)_2 \cdot 6H_2O$	0.049

Trace metal mix	1 ml
Distilled water	1000 ml
pH	7.5

The medium was sterilized by autoclaving at 121°C, 15 1bps for 20 min.

4. ASP 2 Medium (ATCC: 1222)

Na_2EDTA (3.0 g/L)	10 ml
P1 metals	1.0 ml
Iron solution	1.0 ml
KCl (60 g/L)	10 ml
$CaCl_2 \cdot 2H_2O$ (37 g/L)	10 ml
K_2HPO_4 (50 g/L)	1.0 ml
NaCl	18 g
Buffer solution	10 ml
$MgSO_4$	2.44 g
Deionized water to	1.0 L

Iron solution:

$FeCl_3 \cdot 6H_2O$	1.95 g
10 M HCl	5 ml
Deionized water to	500 ml

Filter sterilize.

Buffer solution:

TAPS	100 g
TAPSO	100 g
Deionized water to	1.0 L

pH 7.7

P1 metals:

H_3BO_3	17.15 g
$MnCl_2 \cdot 4H_2O$	2.16 g
$ZnSO_4 \cdot 7H_2O$	0.334 g
$CuSO_4 \cdot 5H_2O$ (0.1 g/5 ml)*	0.075 ml
$CoCl_2 \cdot 6H_2O$ (0.1 g/5 ml)*	0.3 ml
$Na_2MoO_4 \cdot 2H_2O$ (0.11 g/5 ml)*	1.0 ml
Deionized water to	500 ml

5. Bristol Medium (UTEX culture collection of algae)

$NaNO_3$ solution (25 g/L)	10 ml
$CaCl_2 \cdot 2H_2O$ solution (2.5 g/L)	10 ml
$MgSO_4 \cdot 7H_2O$ solution (7.5 g/L)	10 ml
K_2HPO_4 solution (7.5 g/L)	10 ml
KH_2PO_4 solution (17.5 g/L)	10 ml
NaCl solution (2.5 g/L)	10 ml
Deionized water to	1 L

6. Chu's #10 Medium Modified (ATCC medium 341)

$Ca(NO_3)_2 \cdot 4H_2O$	0.232 g
K_2HPO_4	0.01 g
$MgSO_4 \cdot 7H_2O$	0.025 g
Na_2CO_3	0.02 g
$Na_2SiO_3 \cdot 5H_2O$	0.044 g
Ferric citrate	3.5 mg
Citric acid	3.5 mg
Agar	15.0 g

Metal Solution	1.0 ml
Distilled water	1.0 L

Metal Solution:

H_3BO_3	2.4 g
$MnCl_2 \cdot 4H_2O$	1.4 g
$ZnCl_2$	0.4 g
$CoCl_2 \cdot 6H_2O$	0.02 g
$CuCl_2 \cdot 2H_2O$	0.1 mg
Distilled water to	1.0 L

For the isolation and maintenance of heterocystous cyanobacteria the source of combined nitrogen ($NaNO_3$ or KNO_3) in the synthetic medium should be omitted and possibly be replaced by the corresponding chlorides.

3. Solid medium

An easy and common way to make a solid medium is to add a solidifying agent to the liquid medium. The most common solidifying agent is agar whose melting point is 97-100°C. The boiled agar containing medium on cooling solidifies at about 42°C. Agar is a galacton, a complex carbohydrate composed of galactose molecules and cannot be brokendown by cyanobacteria. It is usually used at a concentration of 1.5%, however higher concentration (1.8%) gives a harder medium.

IDENTIFICATION OF CYANOBACTERIA

Key to the orders

- Unicellular or united in colonies of define or indefinite shape, no differentiation into base and apex. Cells spherical, oval or elongate, reproduction by fission, no exospores or endospores; nanocytes present in some genera---------------------------- ---------**Chroococcales**
- Unicellular, showing basal-apical differentiation. Attached forming rather definite layers on submerged rocks and shells, or epiphytic; reproduction by non-motile endospores or exospores--**Chamaesiphonales**
- More or less distinctly filamentous, attached, often forming parenchymatous thalli with prostrate and erect filaments, without differentiation into trichome and filaments, no hormogonia, no heterocysts, endospores in sporangia----------------------------**Pleurocapsales**
- Distinctly filamentous, with trichome and filament organized, hormogones present, often with heterocysts, akinites, exospores or endospores, pseudohormogonia present.
    - Trichome or filaments unbranched or with false branching------------------**Nostocales**
    - With true branching, with a differentiation of prostrate and erect portion (heterotrichous) --**Stigonematales**

Key to the families

Order: Chroococclaes

a. Cells unicellular or forming colonies, not forming filament like growth----**Chroococcaceae**
b. Cells of the thallus arranged in a linear series forming pseudo filamentous growth----------- --**Entophysalidaceae**

Order: Chamaesiphonales

a. Plants attached usually epiphytic, showing a difference between base and apex; With exospores--**Chamaesiphonaceae**

   With endospores--**Dermocarpaceae**
b. Plants consist of free or loosely joined filaments with many rows of cells showing base and apex differentiation: reproduction through lateral or terminal constriction of cells---**Siphononemataceae**

Order: Pleurocapsales

a. Thallus not forming filaments, at the most resembling filaments------------**Pleurocapsaceae**

b. Thallus forming filaments or with a structure which is filament like---------------**Hyellaceae**

Order: Nostocales

a. Trichomes without false branching or with incipient false branching--------------------------**(2)**

b. Trichomes commonly false branched--**(4)**

2. Without heterocysts; spores commonly absent-------------------------------**Oscillatoriaceae**

2. With heterocysts and spores--**(3)**

3. Trichomes differentiated into base and apex--------------------------------**Microchaetaceae**

3. Trichomes not so differentiated---**Nostocaceae**

4. Without an intercalary meristamatic zone and generally without a terminal hair---**Scytonemataceae**

4. With an intercalary meristamatic zone and a terminal hair------------------**Rivulariaceae**

Order: Stigonematales

a. Trichomes and lateral branches uniseriate: heterocysts lateral usually at the ends of short branches--**Nostochopsidaceae**

b. Trichomes and lateral branches multiseriate, or if uniseriate with thick firm sheaths which are not confluent--**Stigonemataceae**

c. Trichomes with reverse 'V' shaped branching, sheath firm or diffluent, with intercalary heterocysts--**Mastigocladaceae**

Key to the genera

Family: Chroococcaceae

a. Cells single, or a few together in a shapeless colony--**(2), (4)**

b. Cells generally many in a single colony--(**5**)

(2) Individual sheath variously coloured, vesicular and broad and formed one in another--**(3)**

(2) Individual sheath colourless, not vesicular, cells spherical--------------------*Chroococcus*

(3) Cells spherical---*Gloeocapsa*

(3) Cells elongate, cell division transverse---*Gloeothece*

(4). Cells spherical, without individual mucilage envelope----------------------*Synechocystis*

(4). Cells elongate, without individual mucilage envelope----------------------*Synechococcus*

(5). Cells without any regular or definite arrangement---**(6)**

(5). Cells with definite arrangement (transverse and longitudinal rows) in distinct flat colonies---*Merismopedia*

(6). Cells compactly arranged into microscopic colonies mostly planktonic-----*Microcystis*

(6). Cells loosely arranged, mostly not planktonic forming macroscopic colonies---------(**7**)

(7). Cells spherical--*Aphanocapsa*

(7). Cells ellipsoidal to cylindrical---*Aphanothece*

Family: Entophysalidaceae

a. Cells disc shaped or spherico-disocoid, in a single row in the sheath forming pseudo filamentous cylindrical thallus---*Johannesbaptistia*

b. Thallus crustaceous, not cylindrical, cells more than a single row, with vesicular sheath, daughter cells of unequal size--*Entophysalis*

c. Cells with unlamellate sheath or without individual sheath in a common mucilage, cells in distinct radial rows--*Chlorogloea*

Family: Chamaesiphonaceae

a. Cells first spherical, later slender, club shaped (or ovate or pyriform), sheathed, attached at the base, in mature cells sheath open at the apex, exospores are cut off successively from the anterior end of the protoplast--*Chamaesiphon*

Family: Dermocarpaceae

a. Endospores formed by division of the protoplast in three directions--------------*Dermocarpa*

b. Endospores formed by transverse division-- *Stichosiphon*

Family: Siphononemataceae

a. Thick filamentous thallus with many tows of cells enclosed by a delicate sheath; distinctly showing base and apex differentiation. Reproduction through three planes of division or lateral or terminal constriction of cells---*Siphononema*

Family: Pleurocapsaceae

a. Cells densely packed, cubical or variously pressed cells in a rounded or cubical colony, cell division in three directions, endospores present-------------------------------***Myxosarcina***

b. Thallus composed of densely packed cells in the prostrate system and parallel erect filaments consist of single series of cells, filaments branched or unbranched, endospores present--***Radaisia***

Family: Hyellaceae

a. Thallus filament like, perforating a calcareous substratum, branched, showing prostrate and erect regions, endospores are formed in large sporangia------------------------------***Hyella***

b. Thallus sessile, short, adherent to substratum, cells spherical, angular by mutual compression, not arranged in vertical rows, envelopes not gelatinizing-----------***Xenococcus***

Family: Oscillatoriaceae

a. Trichomes without sheath--**(2)**

b. Trichomes with sheath---**(5)**

(2) Cells of the trichome not visible or unicellular, spirally coiled---------------------***Spirulina***

(2) Cells of the trichome clearly visible--**(3)**

(3) Trichome regularly spirally coiled---***Arthrospira***

(3) Trichome more or less straight not regularly spirally coiled---------------------------------**(4)**

(4) Trichomes not in bundles---***Oscillatoria***

(4) Trichomes in the form of bundles, free swimming---------------------------***Trichodesmium***

(5) Trichomes typically many in a sheath---**(7)**

(5) Trichomes single within a sheath---**(6)**

(6) Trichomes with prominent firm sheath, not in bundles------------------------------***Lyngbya***

(6) Filaments mostly in erect bundles, with false branches----------------------------***Symploca***

(6) Sheath thick mucilaginous, filament single---------------------------------------***Katagnymene***

(6) Filament forming a thallus with more or less confluent sheath----------------***Phormidium***

(7) Sheath more or less slimy, filaments twisted into rope like bundles---------***Microcoleus***

(7) Filaments branched, sheath soft, more or less diffluent trichomes fewer in each sheath ---*Dasygloea*

(7) Sheath slimy, trichomes many, end cell with a calyptra-----------------------*Hydrocoleum*

Family: Nostocaceae

a. Trichomes without firm sheath--(2)

b. Trichomes with firm sheath---(5)

(2) Trichomes few celled, heterocysts one or at both ends, generally endophytic---*Richelia*

(2) Trichome many celled, not endophytic--(3)

(3) Trichomes with uniform cells, heterocysts absent---------------------------*Pseudanabaena*

(3) Trichomes with intercalary heterocysts--(4)

(4) Filaments single or in a formless gelatinous mass, cells slightly constricted--*Anabaena*

(4) Filaments generally in a definite colony, cells deeply constricted-------------------*Nostoc*

(5) Trichomes in some parts of the thallus more than one in a sheath-------*Hormothamnion*

(5) Trichomes single within a thick sheath---*Nodularia*

Family: Scytonemataceae

a. Filaments false branched, heterocysts absent--(2)

b. Filaments false branched, heterocysts present---(3)

(2) Apices of the trichome distinctly broader or narrower than the rest of the trichome----- --*Ammatoidea*

(2) Apices of the trichome as broad as the rest--*Plectonema*

(3) Single trichome in a sheath, sheath mostly with parallel lamellation, apex not tapering--*Scytonema*

Family: Microchaetaceae

Trichomes single with a distinct sheath,slightly narrower at the tips, hetrocysts basal, seldom intercalary---*Microchaete*

Family: Rivulariaceae

a. Trichomes single in a sheath, simple or false branched, false branches free, heterocysts basal, seldom intercalary--*Calothrix*

b. More than one trichome in a sheath, filaments false branched, branches coalescent, heterocysts basal seldom intercalary---*Dichothrix*

Family: Nostochopsidaceae

a. Filaments with single row of cells, two types of branches; long branches ending in a long hair, other short and cylindrical branches terminated by heterocysts-------------*Mastigocoleus*

Family: Mastigocladaceae

a. *Nostoc* like thallus, trichomes radially arranged ending in a hair, branches true (reverse 'V' shaped) heterocysts intercalary (seldom three pored heterocysts), hormogones formed from the apices of radial branches---*Brachytrichia*

Family: Stigonemataceae

a. Thallus heterotrichous, lateral branches, not much different from the main filament, heterocysts intercalary---*Hapalosiphon*

b. Thallus heterotrichous, trichomes in the young portions or lateral branches are two rowed, in older or main filament with four to many rows of cells; heterocysts intercalary or lateral---*Stigonema*