Selective Isolation of Blue-green Algae from Water and Soil

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SUMMARY

For the isolation of blue-green algae from soil and fresh water, temperature is a selective factor of major importance. In a nutritionally non-selective mineral medium, which at 25° supported growth of blue-green algae and of many eucaryotic algae, the development of eucaryotic algae was almost completely suppressed by incubation at 35°. Both the number and variety of blue-green algae recoverable at 35° were greater than those recoverable from parallel cultures incubated at 25°. When a combined-nitrogen source was omitted from the enrichment medium, only blue-green algae developed at both 25° and 35°; the microflora consisted exclusively of heterocyst-forming filamentous types, other groups of blue-green algae being eliminated.

INTRODUCTION

The extension of the techniques of microbiology to the study of blue-green algae has been slow, and relatively few representatives of the group have so far been obtained in pure culture (Koch, 1964). The isolation of these organisms from natural sources would be facilitated if cultures could be enriched in them by methods which effectively counter-selected eucaryotic algae. One such method was discovered by Beijerinck (1902), who showed that blue-green algae of the *Nostoc* type can be selected by using a mineral medium devoid of a combined-nitrogen source. Subsequent studies (Allen, 1952; Fogg, 1956) suggest that the ability to fix nitrogen is largely restricted to blue-green algae belonging to the Nostocaceae and is by no means universal in the members of this class. Consequently, Beijerinck's enrichment method, although completely effective in eliminating competition from eucaryotic algae, yields an extremely restricted fraction of the total blue-green algal microflora. Apart from the nitrogen-fixers, the blue-green algae seem to have nutritional requirements very similar to those of other algae, so that the possibility of devising other enrichment methods based on the principle of nutritional selection does not appear to be promising. Among algae, thermophily is virtually confined to blue-green algae; and cultures can be effectively enriched in the thermophilic members of the group at temperatures in the range from 60 to 75° (Peary & Castenholz, 1964). However, this physiological property is also a relatively rare one within the group.

A physiological study of a few pure strains (Kratz & Myers, 1955) suggested that even mesophilic blue-green algae may have a temperature range significantly higher than that characteristic of most other algae: the temperature optima were 32·5° for *Nostoc muscorum*; 35° for *Anabaena cylindrica*; and 41° for *Anacystis nidulans*. In commenting on these findings, Fogg (1956) remarked: 'it should be noted that the

temperatures which they [the strains studied by Kratz & Myers] are able to tolerate are much higher than those for algae of other groups obtained from similar environments. Thus, for most species of *Chlorella*, 25° is the optimum for growth and temperatures above 30° are lethal.'

We have recently examined the temperature ranges of many more pure strains of mesophilic blue-green algae, including members of the Chroococcaceae, Nostocaceae and Oscillatoriaceae. Seventeen of these strains were isolated by us from soil and water, at temperatures never in excess of 28°. Twelve other strains were received from other laboratories; although we do not have information about the temperatures used for primary isolation, it is probable that they did not exceed 30°. Every strain in this collection can grow at 35°; eleven can grow at 40°; and two (the strain of *Anacystis nidulans* studied by Kratz & Myers, 1955, and a similar strain isolated by us) can grow at 45°. These results suggested that selection for the ability to grow at a temperature of 35° might provide an effective means for the enrichment of many types of blue-green algae in a nutritionally non-selective medium which was able to support the growth of other kinds of algae at lower temperatures. The experiments described below were undertaken to test this hypothesis.

METHODS

In all the experiments to be described, the mineral base of Hughes, Gorham & Zehnder (1958) was used. It contained (g./l.): K_2HPO_4 , 0.039; $MgSO_4 \cdot 7H_2O$, 0.075; Na_2CO_3 , 0.020; $CaCl_2 \cdot 2H_2O$, 0.027; $Na_2SiO_3 \cdot 9H_2O$, 0.058; ethylenediamine-tetraacetic acid, 0.001; citric acid, 0.006; Fe citrate, 0.006. To this was added 1 ml./l. of microelement mixture A5 (composition, g./l.: H_3BO_3 , 2.86; $MnCl_2 \cdot 4H_2O$, 181; $ZnSO_4 \cdot 7H_2O$, 0.222; $Na_2MoO_4 \cdot 2H_2O$, 0.391; $CuSO_4 \cdot 5H_2O$, 0.079; $Co(NO_3)_2 \cdot 6H_2O$, 0.0494). Preliminary experiments showed that when supplemented with $NaNO_3$ at 1.5 g./l., this medium supported good growth of all the blue-green algal strains tested. It is also suitable for the growth of many unicellular green algae, diatoms and uelenoids.

For enrichment experiments, 5 ml. samples of this medium, either nitrogen-free or supplemented with $NaNO_3$, were dispensed into test-tubes, which were plugged and sterilized. After inoculation, the tubes were placed in plastic racks in illuminated water-baths, maintained at a temperature of either 25° or 35°, and incubated without gassing or agitation. The illumination was provided laterally by banks of cool white fluorescent tubes, which gave an intensity at the entrance window of approximately 600 ft.-candles.

The sources of inocula for enrichment experiments are listed in Table 1. Water specimens were homogenized by agitation for 30 min. on a rotary shaker before sampling. Soil specimens (10 g.) were mixed with the medium to give a total volume of 50 ml. and agitated for 30 min. on a rotary shaker before sampling.

Serial 10-fold dilutions were prepared from each water sample or soil suspension, and used to inoculate three series of tubes: one of the nitrogen-free medium, and two of the medium supplemented with $NaNO_3$. The nitrogen-free series was incubated at 35°; one of the nitrate-containing series was incubated at 35°, and one at 25°. Algal growth became visible after incubation for 10–14 days; experience showed that the microflora did not change significantly upon longer incubation. Microscopic analysis of the dilution series were, accordingly, undertaken routinely after 14 days of incubation.

Analyses were performed as follows. Each dilution series was initially examined microscopically for the presence of visible algal growth; and the tube furnished with the smallest inoculum which showed growth was first subjected to microscopic examination. When the growth was uniform, a single wet mount was prepared; when dispersed patches of growth were evident, a wet mount was prepared from each patch. The wet mounts were examined with phase-contrast illumination, and the organisms observed were scored in the following categories:

(1) Blue-green algae: (a) unicellular; (b) filamentous, with heterocysts; (c) filamentous, without heterocysts; (d) branching.

(2) Eucaryotic algae: (a) green algae; (b) diatoms; (c) euglenoids.

Table 1. *Source materials used in enrichment experiments*

Water specimens		Soil and mud specimens	
W 1	Shallow courtyard pond, Berkeley, January sampling	S 1	Fresh moist soil under tree, Berkeley
W 1 a	Same, May sampling	S 2	Fresh muddy soil, Berkeley
W 2	Shallow courtyard pond, contiguous to 1; January sampling	S 3	Fresh muddy soil, Berkeley
W 2 a	Same, March sampling	S 4	Dried soil from rice paddy, Central Valley of California
W 2 b	Same, May sampling	S 5	Mud from stagnant ditch, Berkeley
W 3	Small open pond, Healdsburg	S 6	Greenhouse soil, Berkeley
W 4	Small open pond, Berkeley	S 7-S 9	Three different dried tropical soil samples, obtained from Ghana
W 5	Freshwater lake, Berkeley		

Within each category, types which could be recognized as distinctly different from one another by virtue of size, form or pigmentation were separately scored. Multiplication by the dilution factor for the tube in question then yielded, for each algal type recorded, the minimal number present in the inoculum.

The tubes in the same series which had received larger inocula were subjected in turn to microscopic analysis, and only those algal types not previously observed were recorded; in each case, the minimal number in the inoculum was calculated. The cumulative data for the tubes in each dilution series were then summed and compared.

It should be specifically noted that the use of 10-fold dilution steps, as well as the fact that only one tube in a series was inoculated from each dilution, make the quantitative aspects of this study relatively imprecise; the true numbers of algae present in the materials examined were, no doubt, often considerably greater than the numbers recorded. However, our primary intention was not to make a precise ecological analysis, but rather to determine whether a satisfactory method for the differential enumeration and selective enrichment of blue-green algae could be developed.

RESULTS

Table 2 shows the outcome of a series of enrichment experiments made with water samples, in most of which no algae could be detected initially by direct microscopic examination. As shown by the data for the dilution series incubated at 25°, all contained appreciable numbers of eucaryotic algae, ranging from 200 to over 20,000/ml. Blue-green algae were also detected in most enrichments at 25°, the numbers ranging from 10 to 2200/ml. Elevation of the temperature of incubation to 35° produced a major change in the outcome of the enrichments. Eucaryotic algae were virtually

eliminated, a very small number of types (all unicellular green algae) developing in the tubes which received the largest inocula; in nearly every case the total number of blue-green algae recovered was appreciably higher than at 25°. The results obtained with sample W 2b are particularly noteworthy. This sample contained a large initial population of eucaryotic algae (over 12,000/ml.), and only 10 blue-green algae/ml. were detected in the dilution series incubated at 25°. Incubation at 35° decreased the count of eucaryotic algae to 1/ml., and revealed a minimal blue-green algal population of 710/ml., 70 times that detected at 25°. Clearly therefore the development of many blue-green algae had been prevented, under non-selective growth conditions, as a result of overgrowth by the large eucaryotic algal population also present in the material analysed.

Table 2. *The influence of temperature of incubation and nitrogen source on the recovery of blue-green and eucaryotic algae from samples of fresh water*

The figures represent minimal numbers/ml. in the sample, as inferred from microscopic analyses of enrichment cultures inoculated with serial dilutions of the material.

Temperature of incubation ... Nitrogen source ... Type of algae ... Sample	25°		35°		35°	
	NO ₃ ⁻		NO ₃ ⁻		N ₂	
	Blue-green	Eucaryotic	Blue-green	Eucaryotic	Blue-green	Eucaryotic
W 1	1,220	3,300	1,340	10	1,000	0
W 1 a	2,210	7,460	2,310	30	2,030	0
W 2	100	240	110	10	10	0
W 2 a	40	220	600	2	132	0
W 2 b	10	12,300	710	1	220	0
W 3	30	590	45	5	0	0
W 4	100	200	100	0	100	0
W 5	0	20,000	1	0	1	0

The further selection obtained by using incubation at 35° with a nitrogen-free medium is also shown in Table 2. Eucaryotic algae were totally eliminated; and the number of blue-green algae was typically decreased, though often only to a slight extent.

Quantitative data on enrichments with a series of soil specimens (Table 3) showed that cultivation at 35° was again highly effective in preventing the growth of eucaryotic algae, and in improving the recovery of blue-green algae from materials which contained a large initial eucaryotic algal population (e.g. samples S 7 and S 9).

Table 4 summarizes some qualitative aspects of these experiments. For several source materials, we have listed the total number of different types of blue-green and eucaryotic algae recovered in the parallel enrichment series. It is evident that, as a rule, enrichment at 35° increased the variety (as well as the absolute numbers) of blue-green algae which were detected in any given soil or water sample, and at the same time greatly decreased the variety of eucaryotic algae.

Table 5 shows the qualitative and quantitative effects of omitting a combined-nitrogen source on the outcome of some enrichments for blue-green algae at 35°. The omission of nitrate effectively abolished the development both of unicellular blue-green algae and of filamentous types which do not form heterocysts, and specifically favoured

the growth of some heterocyst-forming filamentous types. These experiments provide additional support for the conclusion that nitrogen fixation is characteristic of certain members of the Nostocaceae, but with other classes of blue-green algae is uncommon or absent.

Table 3. *Influence of temperature of incubation and nitrogen source on recovery of blue-green and eucaryotic algae from soil and mud samples*

The figures represent minimal numbers per gram in the sample, as inferred from microscopic analysis of enrichment cultures inoculated with serial dilutions of the material.

Temperature of incubation ... Nitrogen source ...	25° NO ₃ ⁻		35° NO ₃ ⁻		35° N ₂	
	Blue-green	Eucary- otic	Blue-green	Eucary- otic	Blue-green	Eucary- otic
Type of algae ...						
Samples						
California soils						
S 1	0	10 ⁵	0	0	0	0
S 2	0	10 ⁴	0	0	0	0
S 3	0	10 ⁵	0	0	0	0
S 4	2,000	4,000	3,000	0	3,000	0
S 5	1,100	10 ⁵	1,100	0	1,000	0
S 6	1,000	4,100	2,000	0	100	0
Tropical soils						
S 7	1,000	31,400	11,500	2,000	13,300	0
S 8	2,100	320	5,090	3	370	0
S 9	3,000	4 × 10 ⁴	14,200	0	2,000	0

Table 4. *The influence of the temperature of incubation and the nitrogen source on the number of types of blue-green and eucaryotic algae which can be detected by enrichment of water and soil samples*

Temperature of incubation ... Nitrogen source ...	25° NO ₃ ⁻		35° NO ₃ ⁻		35° N ₂	
	Blue-green	Eucaryotic	Blue-green	Eucaryotic	Blue-green	Eucaryotic
Type of algae ...						
Samples						
W 1	5	6	9	1	1	0
W 1 a	6	21	7	4	7	0
W 2	2	4	2	1	1	0
W 2 a	6	6	7	2	6	0
W 2 b	1	15	9	1	4	0
W 3	1	25	2	1	4	0
W 4	2	1	1	0	1	0
W 5	0	2	1	0	1	0
S 6	1	5	2	0	1	0
S 7	2	9	7	2	7	0
S 8	5	5	14	3	5	0
S 9	3	4	7	0	2	0
Cumulative totals	34	103	68	15	40	0

We have experimented to a limited extent with other variations on these basal enrichment procedures. From a few mud and water samples, dilution series were prepared at 45°, as well as at 35° and 25°. No development of blue-green algae was ob-

served at 45°, a finding which concurred with our initial observation that very few strains isolated at temperatures below 30° are able to grow at 45°. Several parallel series of nitrogen-free enrichments were incubated at 25° and at 35°; the results, both qualitative and quantitative, were closely similar, except that growth was more rapid at the higher temperature.

Table 5. *The influence of the nitrogen source on blue-green algal development in enrichments at 35°*

Each figure represents the minimal number of cells of a given type of blue-green alga in the source material, as inferred from the examination of enrichment cultures inoculated with serial dilutions.

Sample	Nitrogen source	Type of blue-green algae		
		Unicellular	Filamentous with heterocysts	Filamentous without heterocysts
W 1	NO ₃ ⁻	10	1000, 100, 100, 10	100, 10, 10, 1
	N ₂	0	1000	0
W 2a	NO ₃ ⁻	100, 100, 1	100, 100	100, 100
	N ₂	0	100, 10, 10, 10, 1, 1	0
S 9	NO ₃ ⁻	1000	10,000, 1000	1000, 1000, 100, 10
	N ₂	0	1000, 1000	0
S 8	NO ₃ ⁻	1000	10, 10, 10, 10, 10, 10	1000, 1000, 100, 10, 10, 10
	N ₂	0	100, 100, 100, 10	10
S 4	NO ₃ ⁻	1000	1000	1000
	N ₂	0	1000, 1000, 1000	0

DISCUSSION

A mineral medium which permits the enrichment of many different kinds of algae from soil and water when incubated at 25° becomes highly selective for blue-green algae when incubated at 35°. This fact shows that the temperature maxima of mesophilic blue-green algae are in general higher than those of most eucaryotic algae. There are undoubtedly exceptions to this generalization; for example, a temperature of 35° is too high to permit the growth of certain planktonic blue-green algae which contain gas vacuoles and which can be readily enriched at 25° (G. Cohen-Bazire, personal communication). Nevertheless, temperature selection offers a means for primary isolation of a wide range of blue-green algae, particularly from samples where they constitute a small minority of the total algal population. The utility of this technique might well be increased by varying other parameters, such as light intensity, pH value and nutritional factors.

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