

Effect of nutritional factors on the culture of *Nostoc* sp. as a source of phycobiliproteins

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Summary. The effect of nutritional factors in a new culture medium (BW₃) is described for the cyanobacterium *Nostoc* sp. The growth of *Nostoc* sp. was higher in BW₃ than in control media currently used for cyanobacteria. With medium BW₃ the content of the pigment c-phycocyanin depended on the culture conditions employed, particularly the nature of the nitrogen and carbon sources. Higher amounts of c-phycocyanin amounting to 20.1% on a dry-weight basis were accumulated when both sources were supplied in the gas phase of the culture. The phycobiliproteins of *Nostoc* sp. were resolved into two components: c-phycocyanin ($\lambda_{\max} = 614$ nm) and allophycocyanin ($\lambda_{\max} = 652$ nm). The phycobiliprotein composition was 30% allophycocyanin and 70% c-phycocyanin. The culture of *Nostoc* sp. in BW₃ medium seems promising as a source of biomass for the production of natural dyes.

Introduction

For the production of mass cultures of cyanobacteria, the use of reliable culture media which can sustain good growth of the selected strain is a necessary prerequisite. The literature contains many media suitable for cultivating pure species of cyanobacteria. In some cases a small number of media are effective in the maintenance of a large and diverse collection of cyanobacteria (Rippka et al. 1979). However, they are usually not optimized

for the production of mass cultures, especially when a new strain is isolated.

The most striking characteristic of cyanobacteria is the presence of brilliantly coloured accessory pigments, the phycobiliproteins. The high levels of phycobiliproteins that cyanobacteria can accumulate under favourable environmental conditions (Myers and Kratz 1955) make them attractive as a source of natural pigments for food products such as beverages and dry beverage mixtures (Langston and Maing 1983). These proteins are also widely used as fluorescent tags in a variety of diagnostic and research applications (Glazer and Stryer 1984; Kronick 1986).

This paper describes the results obtained with a culture of a strain of *Nostoc*, a filamentous heterocystous cyanobacterium, using a culture medium particularly designed for the strain. Growth and pigment content of the alga in batch cultures were analysed under different nutritional conditions. The purification and initial characterization of the phycobiliproteins of *Nostoc* are also presented.

Materials and methods

Strain isolation. A slimy algal material taken from brackish waters of the Province of San Luis, Argentina, was inoculated in media G II (Hughes et al. 1958) and BW (see Culture conditions) under continuous illumination. After 10 days a profuse growth of a heterocystous filamentous species of cyanobacterium was observed in BW₃. Trichomes from the surface were inoculated first into fresh BW₃ liquid medium and then onto BW₃ agar medium. After 4–5 transfers of the outer trichomes to BW₃ agar-slant medium, a *Nostoc* sp. strain free from other algae was obtained. The strain identification (performed by Dr. S. Guarrera, Museum of Natural Sciences, Phycology Division, UNLP, La Plata, Argentina) was based on structural properties determined by light microscopy. The strain was kept in liquid and agar BW₃ medium.

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Cultivation conditions. Several culture media (referred to as BW) were tested for their ability to support growth of the *Nostoc* sp. They were obtained by varying the concentration of salts of a nutrient solution used for the culture of *Chlorella* (Silva and Pirt 1984). The BW₃ medium finally used contained, per litre: KH₂PO₄, 0.25 g; NaHCO₃, 0.5 g; MgSO₄·7H₂O, 0.37 g; CaCl₂·2H₂O, 40.0 mg; FeCl₃·6H₂O, 24.0 mg; Na₂ ethylenediaminetetraacetic acid (EDTA)·2H₂O, 85.4 mg; ZnSO₄·7H₂O, 4.5 mg; Na₂MoO₄·2H₂O, 2.5 mg; CuSO₄·5H₂O, 2.0 mg; MnSO₄·4H₂O, 4.0 mg; Na₂B₄O₇·7H₂O, 8.7 mg; NaVO₃, 0.12 mg; CoCl₂·6H₂O, 1.25 mg; NiSO₄·7H₂O, 0.20 mg. The BW₃ medium was made up in the following five solutions, which were autoclaved separately at 1 atmosphere for 15 min and then mixed after cooling: (a) KH₂PO₄ and NaHCO₃ adjusted to pH 7.5 with NaOH, (b) CaCl₂ and MgSO₄, (c) FeCl₃+EDTA (2 mol per mol Fe), (d) other salts+EDTA (1 mol per mol metal) adjusted to pH 7 with NaOH, (e) NaCl. Nitrogen-free media GII (Hughes et al. 1958) and C (Kratz and Myers 1955) were used as controls; NaCl (2 g/l) was added to all the media employed.

The biomass was determined by dry weight measurements. The cells were centrifuged, washed twice with distilled water and dried at 100°C for 16 h. The specific growth rate was calculated by the expression: $\ln x/x_0 \cdot t^{-1}$ in the logarithmic phase of growth (Pirt 1975), where x_0 =initial biomass concentration (g dry weight. l⁻¹) and x =biomass concentration at time t .

Serum bottles of 500 ml containing 250 ml culture medium were used for growth. The serum bottles were injected with gas every 24 h and stoppered with rubber bungs. The pH was manually controlled at 7.5. The cultures were agitated on a rotary shaker at 100 rpm and incubated at 30° ± 0.5°C in a Psycro-Therm Controlled Environment Shaker (New Brunswick Scientific, Edison, NJ, USA) under continuous illumination of 12.2 J·cm⁻¹ per hour (fluorescent tubes).

Analytical methods. Chlorophyll a and total carotenoids were determined in 80% v/v acetone extracts of cells incubated for 30 min at 30°C and then for 16 h at 4°C. Chlorophyll a and total carotenoid concentrations were calculated according to McKinney (1941) in the supernatants at 663 nm and 460 nm respectively after the acetone extracts were centrifuged at 15000 rpm for 10 min. For the extraction of pheycobiliproteins, wet cells were suspended in 30 mM Na-phosphate buffer, pH 7. The water-soluble proteins were released by two freezing and thawing steps followed by incubation at 4°C for 96 h in the dark. The slurry was centrifuged at 10000 rpm for 1 h, and the supernatant was spared for the purification step. The concentration of c-pheycyanin was determined spectrophotometrically by using the specific extinction coefficient $E_{1\text{cm}}^{1\%} = 67.5$ at 614 nm, the principal absorption maximum (see Spectroscopic measurements).

Purification of pheycobiliproteins. Lyophilised water-soluble extract (0.5 g) was suspended in 30 ml of 30 mM Na-phosphate buffer, pH 7, containing 2 mM sodium azide, and the solution was fractionated stepwise with ammonium sulphate. The precipitates of the 0%–20%, 20%–45%, 45%–65% and 65%–75% saturated (NH₄)₂SO₄ solutions were resuspended in 25 ml of 30 mM Na-phosphate buffer, pH 7, containing 0.15 M NaCl and dialysed against 2 l of the same buffer overnight. Each fraction was chromatographed on a DEAE-Sephadex A 50 column (Pharmacia Fine Chemicals, Uppsala, Sweden). The column (1 × 12 active bed, pre-equilibrated with 0.15 M NaCl, 30 mM Na-phosphate buffer, pH 7) was developed with a linear gradient of 0.15–0.45 M NaCl in 30 mM Na-phosphate buffer, pH 7, using a total volume of 60 ml. Fractions of 3 ml

were collected and their absorption recorded at 280, 570, 614 and 652 nm. The fractions with the highest A₆₁₄/A₂₈₀ (c-pheycyanin) and A₆₅₂/A₂₈₀ (allopheycyanin) ratios were pooled and the absorption spectra recorded.

Spectroscopic measurements. Room temperature spectra were obtained with a Beckman 25 recording spectrophotometer (Beckman Instruments, Palo Alto, Calif, USA). The c-pheycyanin extinction coefficient was determined according to O'Carra (1965) using the purified fractions with the highest A₆₁₄/A₂₈₀.

Polyacrylamide gel electrophoresis (PAGE). Sodium dodecyl sulphate (SDS)-PAGE of the fractions precipitated with (NH₄)₂SO₄ and dialysed against 30 mM Na-phosphate buffer, pH 7, was performed by the method of Laemmli (1970). The polyacrylamide slab gels (1.5 mm thickness) were prepared with 30% acrylamide-0.8% methylene bisacrylamide in TRIS/HCl buffer, pH 8.8. The electrophoresis was carried out at 25°C. The gels were stained with Coomassie Brilliant Blue R-250. Protein determination was performed according to Lowry et al. (1951).

Results and discussion

Influence of nutritional factors on growth

In initial experiments the growth of *Nostoc* sp. using different media was compared. Best growth occurred in BW₃ medium (0.75 g/l) compared to that obtained in media C and G II (0.22 g/l and 0.11 g/l, respectively) at 192 h of culture.

The major differences between the media used were the absence of any salt containing combined nitrogen in BW₃ medium and the concentrations of iron, zinc, copper and cobalt. In addition, the salts of vanadium and nickel present in BW₃ were absent in media C and G II. The level of iron in BW₃ was 4.8 ppm, almost five times the optimum of 1 ppm reported for the growth of *Nostoc muscorum* on N₂, but half the amount needed for growth on nitrate (Eyster 1972). The concentration of molybdenum in BW₃ medium (1 × 10⁻⁶ M) was ten times higher than the optimum reported for the growth of cyanobacteria on N₂ (Eyster 1972).

The BW₃ medium was used as basal medium for further experiments, which included variations in the concentration of NaCl, the source of carbon and nitrogen and the composition of the gas phase. The values of the biomass, specific growth rate and pigment composition of the cells were determined (Table 1). *Nostoc* sp. has specific requirements for NaCl although it showed little tolerance to it. Cultures grown in the presence of 4.5 g NaCl/l and 9 g NaCl/l showed higher values of specific growth rate and biomass respectively, while cultures grown in the presence of 18 g NaCl/l were completely inhibited.

Table 1. Growth of *Nostoc* sp. in BW₃ medium with various added nutrients under different gas phases^a

| Addition ^b (g/l) | Gas phase | Biomass ^c (g/l) | Specific growth rate (h ⁻¹) | Pigment concentration (% dry-weight) | | |
|--|--|-------------------------------|---|--------------------------------------|---------------------------|--------------------|
| | | | | Chloro- phyll a | Total caro- tenoids | c-Phyco- cyanin |
| NaCl (2) | 3% CO ₂ :97% N ₂ | 0.368 | 0.009 | 1.47 | 0.27 | 20.10 |
| NaCl (4.5) | 3% CO ₂ :97% N ₂ | 0.403 | 0.014 | 1.48 | 0.28 | 20.10 |
| NaCl (9) | 3% CO ₂ :97% N ₂ | 0.485 | 0.001 | 1.46 | 0.31 | 20.18 |
| NaCl (18) | 3% CO ₂ :97% N ₂ | No growth | | | | |
| KNO ₃ (1); NaHCO ₃ (4.5); NaCl (4.5) | Air | 0.515 | 0.014 | 2.04 | 0.26 | 13.47 |
| Urea (0.3); NaHCO ₃ (4.5); NaCl (4.5) | Air | 0.367 | 0.009 | 1.73 | 0.27 | 9.75 |
| NH ₄ Cl (0.5); NaHCO ₃ (4.5); NaCl (4.5) | Air | 0.128 | 0.007 | 1.80 | 0.25 | 11.50 |
| KNO ₃ (1); NaCl (4.5) | 3% CO ₂ :97% air | 0.211 | 0.011 | 1.70 | 0.26 | 9.40 |
| Urea (0.3); NaCl (4.5) | 3% CO ₂ :97% air | 0.461 | 0.014 | 1.72 | 0.32 | 12.37 |
| NaHCO ₃ (4.5); NaCl (4.5) | N ₂ | 0.332 | 0.014 | 1.98 | 0.33 | 10.46 |

^a *Nostoc* sp. was cultured in 500-ml serum bottles containing 250 ml BW₃ medium, stoppered with rubber bungs, at 30° ± 0.5° C under warm white fluorescent illumination and agitated in a rotary shaker at 100 rpm. The pH was manually controlled to 7.5 every 24 h at which time the cultures were bubbled with the gas phase indicated for 5 min

^b Additions were made to BW₃ while keeping the other components constant

^c Biomass dry-weight at 144 h of culture

It was observed that the BW₃ medium supported good growth of *Nostoc* sp. if the sources of carbon and nitrogen were either supplied in the gas phase or included as mineral salts in the culture medium (Table 1). The same specific growth rate, 0.014 h⁻¹, was observed both in the presence of sodium bicarbonate and nitrogen from nitrate and with a mixture of 3% CO₂:97% N₂. However, when the nitrogen and carbon sources were included as salts in the culture media, the results obtained depended mainly on the nature of the nitrogen source. Growth was higher using KNO₃, decreased with urea and was poor in the presence of NH₄Cl. As BW₃ medium did not have sufficient buffer capacity, the pH drifted upwards with increasing cell density. These drifts in pH values to 8.5–10.5 could have been particularly harmful in the case of using NH₄Cl as the nitrogen source, because of the production of toxic levels of free ammonia. According to Azov and Goldman (1982) free ammonia can produce a 98% reduction in the rate of photoassimilation of ¹⁴C in *Scenedesmus obliquus* growing with 10 mM NH₄Cl and 2 mM NaHCO₃ at pH 8.4. If only the nitrogen source was included in the medium, growth was higher in the presence of urea than with KNO₃. Sodium bicarbonate sustained good growth of *Nostoc* sp. using N₂ in the gas phase.

The fact that pH was manually controlled to 7.5 in a discontinuous way imposed a restriction on the potential levels of biomass that could be achieved with *Nostoc* sp. in BW₃ medium. At increasing cell concentrations, besides high values

of pH, light limitation can occur, resulting in the establishment of environmental conditions under which exponential growth is not produced. In fact, with the culture system employed, logarithmic growth was observed only in the first stages of the cultures. After 96 h there was a failure to maintain constant exponential growth in all the conditions analysed. Similar results were reported by Ward (1985) in batch cultures of *Synechococcus leopoliensis*.

Influence of nutritional factors on pigment content

The pigment content of *Nostoc* sp. cultivated in BW₃ medium varied with the nutrients employed. The major variations were observed in the content of c-phyco-cyanin (Table 1). The highest content of c-phyco-cyanin was obtained with CO₂ as carbon source and N₂ as nitrogen source, with a mean value of 20.1% of the biomass dry weight in comparison with 11.5% when both sources were added as mineral salts to the culture medium. The content of c-phyco-cyanin remained fairly constant in the presence of 2.0, 4.5 and 9.0 g NaCl/l, but varied markedly using NaHCO₃ as carbon source, and KNO₃, urea and NH₄Cl as nitrogen sources. As c-phyco-cyanin not only acts as an accessory pigment in the transfer of light energy to chlorophyll a (Bogorad 1975), but also as a nitrogen storage compound in cyanobacteria (Bous-siba and Richmond 1980), it is likely that its concentration within the cell will depend on various

environmental conditions. This has been shown for the effect of light quality, light intensity, high temperature, CO₂ concentration and nitrogen starvation (Tandeau de Marsac 1977; Goedheer 1976; Oquist 1974; Eley 1971; Allen and Smith 1969; Boussiba and Richmond 1980). The results reported in this work show that for the strain of *Nostoc* isolated the content of c-phycoerythrin depends in part on nutritional factors, particularly the nature of the carbon and nitrogen sources. A study on the effect of other environmental conditions is planned.

Purification and initial characterization of phycobiliproteins

The absorption spectrum in the visible region of a crude water-soluble extract of *Nostoc* sp. is shown in Fig. 1A. The absorption maximum was observed at approximately 615 nm with a slight shoulder at 650 nm. Ion exchange chromatography on DEAE-Sephadex A 50 of the 20%–45% (NH₄)₂SO₄ fraction resulted in the separation of c-phycoerythrin and allophycocyanin. The absorption maxima of c-phycoerythrin and allophycocyanin were 614 nm and 652 nm, respectively (Fig. 1B, C). The specific absorption coefficient of c-phycoerythrin was $E_{1\text{cm}}^{1\%} = 67.5$. The phycobiliprotein composition was 30% allophycocyanin and 70% c-phyco-

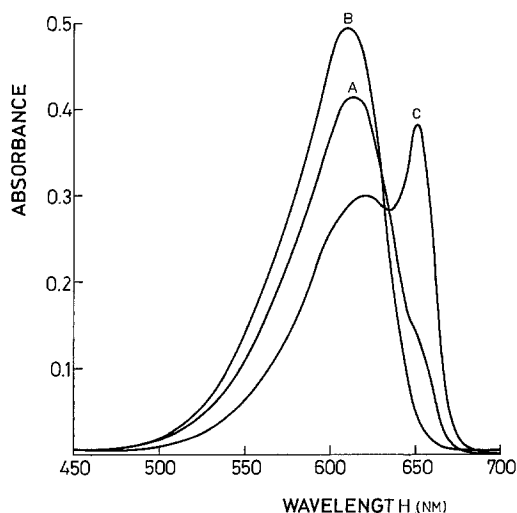


Fig. 1. Absorption spectra of *Nostoc* sp.: A, crude water-soluble extract; B, c-phycoerythrin; C, allophycocyanin. c-Phycocyanin and allophycocyanin were purified by ion exchange chromatography of the 20%–45% ammonium sulphate fraction from a column of DEAE-Sephadex A 50

cyanin was similar to that of the endophytic cyanobacterium *Anabaena azollae* (Tyagi et al. 1980).

Phycobiliproteins are the main light-harvesting pigments of photosynthesis in cyanobacteria. Whereas the phycobiliproteins c-phycoerythrin and allophycocyanin are always present (Yamanaka and Glazer 1981) more variation is found in the occurrence of additional phycobiliproteins such as c-phycoerythrin (λ_{max} at 565 nm) and phycoerythrocyanin (λ_{max} at 568 nm) (Stanier and Cohen-Bazire 1977; Eder et al. 1978). The c-phycoerythrin absorption maximum at 614 nm of *Nostoc* sp. is the same as that reported in *Anabaena* sp. 6411 and *A. variabilis* (Bryant et al. 1976). The absorption maximum of allophycocyanin (λ_{max} at 652 nm) is similar to allophycocyanins from other unicellular and filamentous species (Cohen-Bazire et al. 1977; Bryant et al. 1981; Boussiba and Richmond 1979). Unlike the majority of other strains of *Nostoc* from the Pasteur Culture Collection (Rippka et al. 1979) the strain isolated does not produce either c-phycoerythrin or phycoerythrocyanin. Allophycocyanin B (λ_{max} at 670 nm) which is reported to be present in almost all cyanobacteria (Glazer and Bryant 1975) was also not detected. Similar results were found in the characterization of the phycobiliproteins of *Gloeobacter violaceus* (Bryant et al. 1981).

Polyacrylamide gel electrophoresis in the presence of SDS of the different fractions precipitated with (NH₄)₂SO₄ showed that there was no precipitation of proteins other than c-phycoerythrin and allophycocyanin until the 45%–60% fraction was reached (data not shown). From 45%–60% onwards, in addition to the bands of c-phycoerythrin and allophycocyanin, a number of bands appeared which corresponded to unidentified proteins and polypeptides. Protein determinations according to Lowry et al. (1951) on the different (NH₄)₂SO₄ fractions showed that, with the addition of (NH₄)₂SO₄ to 45% saturation, approximately 80% of the total phycobiliproteins present in the soluble extract was precipitated free from other proteins and polypeptides. If we also consider that the content of c-phycoerythrin may be up to 20.1% on dry weight basis, and can be easily released from the cells, the culture of *Nostoc* sp. in BW₃ medium seems promising as a source of biomass for the production of natural dyes.

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