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# Effect of Nutrients and Aeration on $O_2$ Evolution and Photosynthetic Pigments of *Anabœna torulosa* during Akinete Differentiation

T.A. SARMA, G. AHUJA, J.I.S. KHATTAR

*Department of Botany, Punjabi University, Patiala – 147 002, India*

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**ABSTRACT.** The addition of a nitrogen (nitrate) and carbon sources (acetate, citrate and fructose) and phosphate deficiency (nitrate medium deficient in phosphate) under unaerated conditions induced akinete differentiation in *Anabœna torulosa*. Aerated cultures of this organism in these nutrients did not differentiate akinetes. Oxygen evolution by aerated cultures was higher when compared to unaerated cultures, which concurred with high chlorophyll content of aerated cultures. Nitrate nitrogen supported high phycocyanin content in unaerated cultures; phycocyanin and allophycocyanin contents were low under aerated conditions. The contents of phycocyanin, allophycocyanin, phycoerythrin and carotenoids gradually decreased at the mature akinete phase. Under aerated conditions, chlorophyll content rose and the content of all the pigments increased with the growth rate of the organism.

Of various nutritional and environmental factors, nitrogen sources (Sarma and Malhotra 1989), carbon sources (Sarma and Khattar 1993) and phosphate deficiency (Sarma and Khattar 1992; van Dok and Hart 1996) were shown to regulate akinete differentiation in cyanobacteria. Of the physical factors, light, temperature and water stress were implicated to control akinete differentiation (Wolk 1965; Sutherland *et al.* 1979; Sarma and Swarn Kanta 1980; Li *et al.* 1997; Sarma and Ghai 1998; Agrawal and Singh 1999*a*,*b*).

It is a general practice to bubble the cultures of cyanobacteria with a mixture of air and $CO_2$ to obtain increased biomass. Recent studies on *Anabœna torulosa* identified aeration as another factor regulating akinete differentiation (Sarma *et al.* 1998). Although variation of pigments such as phycobiliproteins, in relation to nutrients and environmental conditions leads to chromatic adaptation (Bennet and Bogorad 1973; Tandeàu de Marsac 1977; Gingrich *et al.* 1982), relative proportion of these pigments in relation to carbon and nitrogen sources during akinete differentiation, and the influence of aeration on these pigments has not yet been studied. We therefore studied the effects of aeration on the rate of $O_2$ evolution and on the contents of photosynthetic pigments during akinete differentiation.

## MATERIALS AND METHODS

*Organism and culture conditions.* The cyanobacterium *Anabœna torulosa* (CARM) LAGERH. ex BORN et FLAH, an isolate of this laboratory (Sarma and Swarn Kanta 1979) was propagated in Allen and Arnon's (1955) nitrate-free medium (without any addition of N) in a culture room at 28 ± 2 °C and illuminated with cool day-light fluorescent lamps (*Philips*) for 14 h per d giving a radiant flux of 9.5 W/m$^2$. Potassium nitrate (10 mmol/L) was used as nitrogen source. Acetate (10 mmol/L), citrate (1 mmol/L) and fructose (50 mmol/L) were used as carbon sources. Phosphate deficiency in nitrate medium was obtained by omitting phosphate from this medium. Obviously, all media contained dissolved $N_2$. To study the effect of aeration, filter-sterilized moist air was bubbled through the cultures continuously with the help of an aerator at a flow rate of 10 mL/h.

*Growth and akinete frequency.* The growth of the organism was measured as $A_{600}$ of the cell suspensions and also as protein content according to Lowry at intervals of 2 d. Microscopic observations were simultaneously made to determine the phase of akinete differentiation. The frequency of akinetes is expressed as percentage of akinetes per 1000 cells.

*Photosynthetic oxygen evolution.* Cultures of *A. torulosa* grown in respective nutrients (Table I) were condensed (4–8 g/L of chlorophyll *a*; Chl *a*) and a photosynthetic efficiency was estimated by measuring the amount of $O_2$ evolved in light (irradiance 19 W/m$^2$) at 28 ± 2 °C by using an oxygen electrode fitted with a reaction vessel of 10-mL capacity (Oximeter OXI-191).

*Photosynthetic pigments.* For the extraction and quantification of pigments, known volumes of the suspension of organism were centrifuged (5000 ***g***, 5 min). The pellet was resuspended in the same volumes of methanol (for Chl *a*) or acetone (for carotenoids) or phosphate buffer (pH 6.7; for phycobiliproteins). For extraction of Chl *a*, methanol extract was shaken thoroughly, kept in a hot water bath (60 °C, 1 h) and the

amount of Chl *a* was measured (MacKinney 1941). For carotenoids, the acetone extract was shaken thoroughly and kept for 1 h at room temperature and the pigment was estimated according to Webber and Wetten (1981). Phycobiliproteins were extracted by subjecting the cells to mild sonication in phosphate buffer and repeated freezing and thawing; the amounts of phycocyanin (PC), allophycocyanin (AC) and phycoerythrin (PE) were quantified (Bennet and Bogorad 1973).

The cultures were homogenized gently to get 2–3-celled trichomes and the cell concentration was estimated by counting in a hæmocytometer. The amounts of the photosynthetic pigments are expressed in fg per cell.

## RESULTS AND DISCUSSION

Table I shows that the addition of nitrogen and carbon sources under unaerated conditions enhanced the growth of the organism almost to the same extent when compared to nitrate-free control. Aeration of the cultures resulted in increased growth even in nitrate-free medium, which is significantly higher than that formed on nitrate and carbon sources under unaerated conditions. Thus aerated cultures exhibited better growth performance in all the nutrients. This is also reflected by the protein content of the cultures. All the cells in the trichomes from unaerated cultures in nitrate-free medium started accumulating granules after 8 d (initiation) followed by a 12-d maturation period after which mature akinetes with a frequency of 15 ± 1 % appeared (after 20 d). In the cultures with nitrate, nitrate medium without phosphate, acetate, citrate and fructose, akinete differentiation occurred already after 4 d and mature akinetes differentiated after 9–11 d with variable higher frequencies. Maximum percentage of akinetes was noted in a nitrate medium without phosphate (45 ± 5 %) followed by acetate (39 ± 4 %) and citrate (28 ± 4 %). These results are consistent with the findings of Sarma and Malhotra (1989) and Sarma and Khattar (1992) on the same organism. On the other hand, trichomes in aerated cultures in the presence of all the nutrients showed slightly enlarged cells with few granules which further did not differentiate into akinetes even after 30 d. Likewise, cultures of *Nostoc* strain PCC 7524 gassed with air or a mixture of $O_2$–$N_2$ (21 : 79) did not produce akinetes while cultures bubbled with the mixture air–$CO_2$ (95 : 5) produced akinetes (Sutherland *et al.* 1979).

**Table I.** Growth ($A_{660}$), protein content (g/L), akinete frequency[a] and $O_2$ evolution[b] (mmol/g Chl *a* per h) in cultures of *A. torulosa*

| Medium | Unaerated cultures | | | | Aerated cultures | | |
|---|---|---|---|---|---|---|---|
| | $A_{660}$ | protein | akinete frequency | $O_2$ evolution | $A_{660}$ | protein | $O_2$ evolution |
| Nitrate-free | 0.5 ± 0.03 | 34 ± 0.7 | 15 ± 1.0 | 90 ± 11.5 | 0.8 ± 0.03 | 95 ± 7.1 | 124 ± 23.1 |
| Nitrate | 0.6 ± 0.02 | 63 ± 0.7 | 21 ± 1.0 | 56 ± 0.1 | 1.3 ± 0 | 160 ± 14.1 | 88 ± 0.1 |
| Nitrate–phosphate | 0.7 ± 0.03 | 60 ± 1.4 | 45 ± 5.0 | 54 ± 0.3 | 1.3 ± 0.10 | 150 ± 7.0 | 83 ± 0.2 |
| Acetate | 0.6 ± 0.01 | 48 ± 2.8 | 39 ± 4.0 | 58 ± 5.8 | 1.8 ± 0.08 | 163 ± 11.3 | 86 ± 5.2 |
| Citrate | 0.7 ± 0.03 | 41 ± 2.1 | 28 ± 4.0 | 57 ± 4.7 | 1.8 ± 0.04 | 150 ± 14.2 | 96 ± 21.7 |
| Fructose | 0.6 ± 0.06 | 50 ± 4.2 | 13 ± 0.9 | 85 ± 20.0 | 0.6 ± 0.03 | 315 ± 38.9 | 113 ± 20.8 |

[a]Estimated in nitrate-free cultures after 20 d, in other nutrients after 12 d; in aerated cultures akinetes were not formed.
[b]Estimated after 3-d growth in all nutrients.

The rate of $O_2$ evolution was lower in the presence of all the nutrients (54–58 mmol/g Chl *a* per h) as compared to nitrate-free cultures (90) except in fructose (85). The rate of $O_2$ evolution by aerated cultures was significantly higher than that in unaerated cultures. The lower rate of $O_2$ evolution by the unaerated cultures might be due to a cessation in photosynthetic activity as all the cells in the nutrients tested were undergoing akinete differentiation. This might be the reason for the lower $^{14}CO_2$ incorporation by the isolated akinetes of *Anabæna cylindrica* (Fay 1969) and *Nostoc* strain PCC 7524 (Sutherland *et al.* 1979). Further, lower rates of photosynthetic $O_2$ evolution have been reported in the isolated akinetes of the strain PCC 7524 (Chauvat *et al.* 1982), *Nostoc spongiæforme* (Thiel and Wolk 1983), *Anabæna doliolum* (Rao *et al.* 1984) and *Fischerella muscicola* (Singh and Kashyap 1988).

Under aerated conditions, photosynthetic $O_2$ evolution was found to be higher, thus indicating greater photosynthetic capacity of the aerated cultures. Michael *et al.* (1993) observed that, in 5 % $CO_2$ in air, carboxysomes were randomly distributed; in standing cultures they were near the periphery suggesting that

the peripheral arrangement of carboxysomes may provide more efficient utilization of the internal carbon pool from cultures where carbon sources were limiting.

The variation of pigment contents in different nutrients under unaerated and aerated conditions is given in Table II. In nitrate-supplemented aerated cultures, Chl *a* content increased up to 12 d (from 180 after 2 d to 500 fg per cell on day 12); under unaerated conditions, the content was lower after 12 d, *i.e.* the free akinete day (250 fg per cell). Aerated cultures in nitrate medium deficient in phosphate, acetate and fructose exhibited a similar content of Chl *a* (290–300 fg per cell) after 12 d; with the addition of citrate, the Chl *a* content was slightly lower (230 fg per cell). The carotenoid content either remained the same or slightly decreased as in unaerated cultures. However, in aerated cultures the carotenoid content increased.

**Table II.** Contents[a] of chlorophyll *a* (Chl *a*) and carotenoids (CA), phycocyanin (PC), allophycocyanin (AC) and phycoerythrin (PE) in unaerated and aerated cultures of *A. torulosa* after a 2- and 12-d cultivation

| Medium | Pigment | Unaerated | | Aerated | |
|---|---|---|---|---|---|
| | | 2 | 12 | 2 | 12 |
| Nitrate-free[b] | Chl *a* | 170 | 180 | 140 | 170 |
| | CA | 130 | 120 | 80 | 120 |
| | PC | 1840 | 1630 | 1950 | 880 |
| | AC | 1260 | 930 | 1200 | 720 |
| | PE | 340 | 310 | 310 | 240 |
| Nitrate | Chl *a* | 160 | 250 | 180 | 500 |
| | CA | 180 | 90 | 110 | 280 |
| | PC | 740 | 840 | 910 | 2950 |
| | AC | 1950 | 960 | 1720 | 1520 |
| | PE | 2230 | 410 | 1750 | 340 |
| Nitrate–phosphate | Chl *a* | 100 | 300 | 100 | 300 |
| | CA | 100 | 110 | 100 | 110 |
| | PC | 2010 | 2970 | 1740 | 1740 |
| | AC | 2010 | 1060 | 1740 | 1530 |
| | PE | 3010 | 1290 | 2060 | 1140 |
| Acetate | Chl *a* | 170 | 200 | 90 | 320 |
| | CA | 130 | 90 | 100 | 110 |
| | PC | 1450 | 850 | 1110 | 2480 |
| | AC | 1910 | 590 | 990 | 1910 |
| | PE | 510 | 310 | 200 | 770 |
| Citrate | Chl *a* | 210 | 170 | 110 | 230 |
| | CA | 160 | 90 | 70 | 130 |
| | PC | 1890 | 1140 | 1150 | 1440 |
| | AC | 2280 | 780 | 980 | 1010 |
| | PE | 540 | 320 | 230 | 550 |
| Fructose | Chl *a* | 340 | 190 | 320 | 290 |
| | CA | 70 | 80 | 50 | 110 |
| | PC | 1550 | 1120 | 870 | 1700 |
| | AC | 1340 | 420 | 820 | 630 |
| | PE | 540 | 140 | 240 | 220 |

[a]The variation in the values (fg per cell) of pigments was not more than 5 %.
[b]Nitrate-free cultures were subjected to pigment estimation after 4 d, which represents the vegetative cell stage and after 20 d for the free akinete phase.

In nitrate-free-medium grown unaerated cultures, AC and PE contents doubled – from 1260 on day 4 to 2570 fg per cell after 8 d (AC) and from 340 on 4 d to 620 fg per cell after 8 d (PE) – on initiation day and decreased thereafter. After addition of nitrate under unaerated conditions, the PC content significantly increased (from 740 on day 2 to 4570 fg per cell after 8 d) especially at the maturation stage. Afterwards it declined (to 844 fg per cell after 12 d) on the free akinete day. Unaerated cultures grown in a nitrate medium deficient in phosphate also showed consistent increase in PC content (from 201 after 2 d to 297 fg per cell after 12 d). In aerated nitrate-grown cultures, the contents of PC and AC increased gradually up to day 12. In the aerated cultures grown in nitrate medium without phosphate, PC, AC and PE contents were lower (1740, 1530 and 1140 fg per cell, respectively of PC, AC and PE) than in unaerated cultures. The aerated cultures

after addition of acetate, citrate or fructose exhibited a gradual increase in the contents of PC, AC and PE. On the contrary, Los (1995) reported that carbon compounds, such as glucose, caused a general decrease in phycobiliprotein content while nitrogen sources (*i.e.* nitrate or nitrite) supported and maintained synthesis of these pigments during aging of *A. cylindrica* and *Nostoc punctiforme* cultures. In general, the content of PC, AC and PE decreased at the time of mature akinete formation in unaerated cultures, whereas in aerated cultures, the amount of these pigments progressively increased.

Since all the cells are simultaneously committed to undergoing initiation of akinete differentiation in all the nutrients, the cultures represent a mixture of cells in the process of akinete formation, maturing akinetes and fully mature akinetes. Correlation of the pigment content in unaerated and aerated cultures with reference to akinete differentiation reveals that PC content either remains unchanged or is slightly increased at the time of akinete formation in unaerated cultures in all nutrients. However, the contents of PC, AC, PE and carotenoids gradually decreased with the differentiation of akinetes. Similarly, changed amounts of phycobiliproteins were reported in the akinetes of *Anabæna fertilissima* (Reddy 1983); on the contrary, PE has not been detected in the akinetes of *Nodularia spumigena* (Pandey and Talpasayi 1981). Variations in pigment concentrations in akinetes of cyanobacteria have also been reported. For example, in the akinetes of *A. cylindrica* PC was absent, β-carotene was reduced, xanthophyll concentration increased and Chl *a* was replaced by phæophytin (Fay 1969). The akinetes of *Nostoc* strain PCC 7524 doubled the amount of Chl *a* and unchanged the PC content (Sutherland *et al.* 1979). The akinete-producing cultures of *A. torulosa* (unaerated) exhibited an unchanged or a doubled amount of Chl *a* while the PC content increased up to the maturation period.

In aerated cultures, where akinetes were not formed, all the pigments increased with the growth of the organism. Concomitantly with increased Chl *a* concentration in aerated cultures the evolution of $O_2$ by these cultures also increased.

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