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Control of sporulation in the filamentous cyanobacterium *A nabaena torulosa*

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Abstract. In the cyanobacterium *Anabaena torulosa,* sporulation occurred even during the logarithmic growth phase. Sporulation was initiated by differentiation of the vegetative cell on one side, adjoining the heterocyst followed by differentiation of the vegetative cell on the other side. Subsequently, spores were differentiated alternately on either side to form spore strings. The sequence of sporulation supports the previous notion that a gradient of spore maturation exists in cyanobacteria and also indicates that the gradient is manifested unequally on either side of heterocysts. Sporulation was absent or negligible in a minerally enriched medium but ocurred readily in a minimal medium. The extent of sporulation was inversely related to phosphate concentration. Sporulation was enhanced at higher temperature. Incandescent light, but not fluorescent light, greatly stimulated sporulation suggesting possible involvement of red light in spore differentiation. Addition of filtrate, from 5 to 8 day old cultures, to freshly inoculated *A. torulosa* greatly enhanced sporulation indicating the influence of extracellular products in spore formation.

Keywords. *Anabaena torulosa;* akinete sporulation; cyanobacterium; blue-green alga.

Introduction

Many filamentous cyanobacteria are characterized by the presence of a third cell type called spore or akinete in addition to the vegetative cell and the heterocyst. The three cell types occur in patterned sequence which differ from species to species. For instance, in *Anabaena cylindrica,* spores are differentiated adjacent to heterocysts (WoIk, 1965), whereas in *Anabaena doliolum* (Tyagi, 1974) and *Nostoc* PCC 7524 (Sutherland *et al.,* 1979) spores start to differentiate midway between two heterocysts. Heterocysts have been implicated in the regulation of spore formation and a gradient of spore maturation has been proposed (WoIk, 1965; Tyagi, 1974; Simon, 1977). However, it has been reported (Sinha and Kumar, 1973) that *A. doliolum* is able to sporulate without first forming heterocysts and in *Nostoc linckia* heterocysts seem to inhibit sporulation (Singh *et al.,* 1972). The normal pattern of spore formation in *A. cylindrica* is drastically altered by provision of the arginine analogue canavanine and spores develop randomly in the filament (Nichols *et al.,* 1980).

The conditions of culture and physiological factors favouring spore formation have not been adequately documented, although some of these features are known

(WoIk, 1975; Nichols and Carr, 1978). Phosphate deficiency stimulates sporulation in *Cylindrospermum* (Glade, 1914; Fisher and WoIk, 1976), *Anabaena cylindrica* (WoIk, 1965) and *Nodularia spumigena* (Pandey and Talpasayi, 1980), High light intensity, high concentration of phosphate (WoIk, 1965), potassium nitrate and ammonium chloride (Tyagi, 1974) have been shown to inhibit spore formation in cyanobacteria whereas certain amino acids, calcium glucuronate and hydrogen enhance sporulation in *C. licheniforme* (Hirosawa and WoIk, 1979a). The presence of an extracellular positive effector which stimulates sporulation was demonstrated in *Cylindrospermum licheniforme* (Fisher and WoIk, 1976) and a low molecular weight (M_r 151) active substance which appeared to be the major single effector has since been purified to homogeneity from this organism (Hirosawa and WoIk, 1979b). In this paper, the factors favouring sporulation in *Anabaena torulosa* are described. Unlike other cyanobacteria examined so far, where sporulation occurs in stationary phase cultures, *A. torulosa* initiates sporulation even during logarithmic growth. Sporulation could therefore be studied independently of the effects of decreased growth rates.

Materials and methods

Organism

Anabaena torulosa Lagerh. (see Desikachary, 1959) isolated in axenic culture in this laboratory (Fernandes, 1978) was the experimental material.

Culture conditions

Stock cultures were maintained on agar slants (1%, w/v Difco agar) enriched with two fold diluted cyanophycean medium (David and Thomas, 1979). Cultures for experimental purpose were grown as required in the diluted cyanophycean medium, or in Glade's medium (Glade 1914) which has the following composition (mg/litre): $Ca(NO_3)_2.4H_2O$,1000;$MgSO_4.7H_2O$, 200; K_2HPO_4,200; $FeSO_4.7H_2O$, 50. Other conditions for routine growth of culutres were as described before (Thomas 1972). Erlenmeyer flasks (500 ml or 1 litre) were used and 4000 lux light intensity was provided by a bank of fluorescent lamps. Temperature was 28 ± 1° C. The cultures, 5 cm deep, were aerated at the rate of 2 litre per min.

Cell counts and growth determinations

Counts of vegetative cells, heterocysts and spores in the algal filaments were made as described earlier (Thomas and David, 1971). Those cells which were having thick walls and were at least twice as long and distinctly wider than the average vegetative cell (Simon, 1977) were considered as spores. Total protein content remains unchanged in sporulating cyanobacteria, whereas dry weight and chlorophyll content increase in a linear manner (Simon 1977). The problem of dehydrated mucilage rapidly absorbing moisture results in erratic dry weight measurements. Growth was therefore assessed in this study by determining chlorophyll content (Mackinney, 1941).

Sporulation in liquid and solid media and the effect of temperature

One ml suspension of a six-day old aerated culture of *A . torulosa* was inoculated into

Erlenmeyer (150 ml) flasks containing either the diluted cyanophycean medium (liquid) or containing 1% (w/v) agar (solid) prepared in diluted cyanophycean medium. Sets of flasks were incubated at 29, 32 and 37°C as still cultures. Temperature was adjusted by using a water bath. Incandescent light (1000 lux) was provided by tungsten filament lamps. The effect of temperature was also examined in aerated cultures grown in diluted (1:3) Glade's medium at 32 and 40° C in the presence of incandescent light (3000 lux).

Effect of light source and intensity of sporulation

A. torulosa was inoculated in diluted (1:3) Glade's medium. Sets of flasks were incubated in fluorescent light (3000 lux) or incandescent light (3000 lux) at 29°C. Effect of light intensity on sporulation was examined using incandescent light at 1000, 3000 and 5000 lux.

Effect of phosphate on sporulation

Six-day old cultures grown in cyanophycean medium, centrifuged, washed and resuspended in sterile distilled water, served as inoculum. Sporulation was then examined during growth in diluted (1:3) Glade's medium containing 0.3, 0.7, 1.0 and 1.4 mM phosphate. The culture flasks were incubated at an intensity of 3000 lux incandescent light.

Stimulation of sporulation by culture filtrate

A. torulosa was grown in diluted (1:3) Glade's medium 1 litre conical flasks at 40°C with incandescent light (3000 lux). After 8 days, the cultures were centrifuged and the supernatant was passed through Millipore filter (0.45 μm). The culture filtrate so obtained was mixed with fresh Glade's medium in various proportions. Five day old *A. torulosa* grown in cyanophycean medium was harvested, washed in distilled water and then inoculated into the media containing the various proportions of the culture filtrate.

Sequence of sporulation

The time course and sequence in which spores appear on either side adjacent to heterocysts were examined in cultures grown in Glade's diluted medium with incandescent light (3000 lux) at 32°C.

Results

Sporulation in liquid and solid media and the effect of temperature

Anabaena torulosa grew well in both Glade's and cyanophycean (figure 1) media. Sporulation occurred readily in Glade's liquid medium (figure 2c, 4c). Temperatures upto 40°C did not affect growth (figure 2a), but higher temperature (40°C) induced early sporulation (figure 2c), compared to relatively low temperatures (32°C, figure 2; 29°C, figure 4). Heterocyst frequency was also relatively higher at 40°C (figure 2b).

Although Glade's liquid medium was very effective in favouring sporulation (figure 2c; 4c) *A. torulosa* did not sporulate in liquid cyanophycean medium at

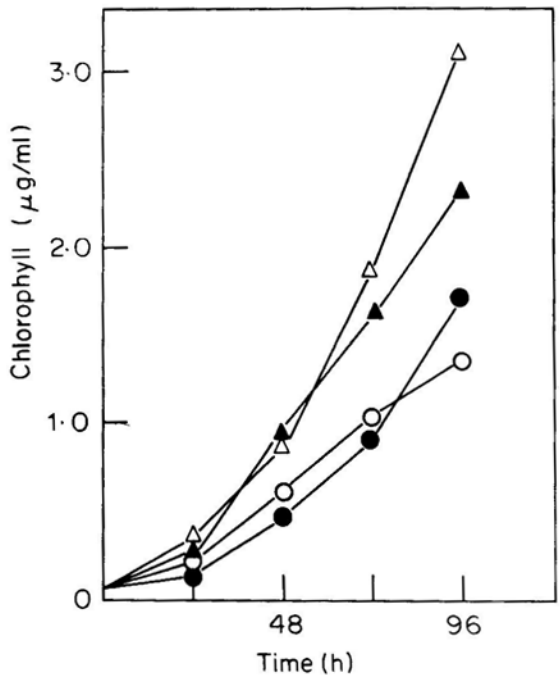

Figure 1. Growth and sporulation of *Anabaena torulosa* at 29°C in a minimal (Glade's) medium and a minerally enriched (cyanophycean) medium, in the presence of either incandescent (3000 lux) or fluorescent (3000 lux) light. Growth in Glade's meidum: incandescent (▲) and fluorescent (O) light. Growth in cyanophycean medium: incandescent (Δ) and fluorescent (●) light.

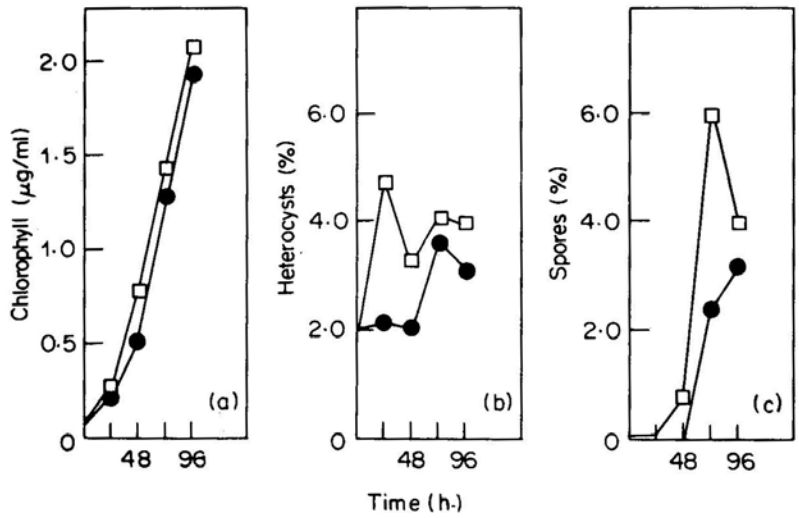

Figure 2. Effect of temperature on (a) growth, (b) heterocyst production and (c) sporulation of *Anabaena torulosa* in Glade's medium in the presence of incandescent light (3000 lux). 32°C (●); 40°C (□). The enhancement in sporulation at 40°C over that at 32°C was statistically significant with L.S.D. values at p=0.05 level being 1.05, 1.35 and 0.78 for 48,72 and 96 h old cultures respectively.

lower (29 and 32°C) temperatures (figure 3b). Only at a higher temperature (37^0C) there was indication of sporulation. However, when the alga was inoculated on solid (agar 1%, w/v) cyanophycean medium sporulation occurred readily (figure 3a) and was influenced greatly by temperature. On solid medium, sporulation at 37°C was several times that at 29°C and about two to three times as much as at 32°C.

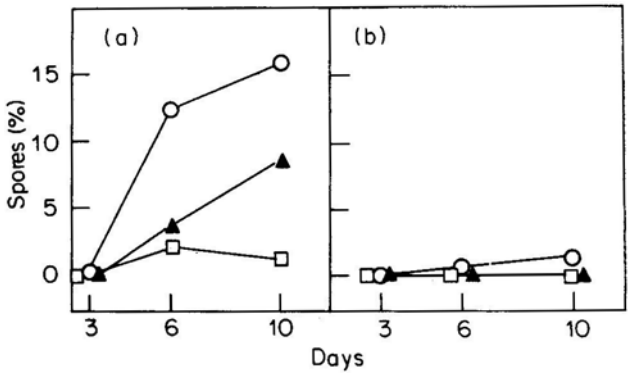

Figure 3. Sporulation of *Anabaena torulosa* in (a) solid (l% agar) and (b) in liquid cyanophycean medium at 29°C (□), 32°C (▲), and 37°C (O) in the presence of incandescent light (1000 lux).

Effect of light source and intensity on sporulation

Heterocyst frequency was not affected by the quality of light except in the late linear phase of growth, when more heterocysts occurred in incandescent light (figure 4b). The effect of light quality was most remarkable on the differentiation of spores. Whereas sporulation occurred readily after 3 days of growth at 29°C and reached nearly 7% on day 5 in incandescent light, there was no sporulation in fluorescent light (figure 4c).

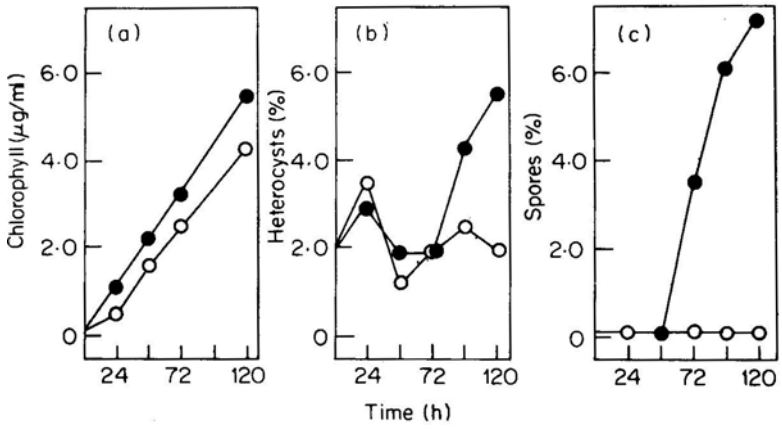

Figure 4. Effect of light source (3000 lux) on (a) growth, (b) heterocyst production and (c) sporulation.of *Anabaena torulosa* in Glade's medium at 29°C. Incandescent light (●); fluorescent light (O).

Under incandescent light intensities of 1000, 3000 and 5000 lux heterocyst percentage was maintained near 4 during the growth period, but sporulation was enhanced nearly in proportion to the light intensity used (table 1).

Table 1. Effect of light intensity on sporulation in *Anabaena torulosa.*

Light Intensity (lux)	Age of culture								
	48 h			72 h			96 h		
	spores %	L S D p=0.05	L S D p=0.01	spores %	L S D p=0.05	L S D p=0.01	spores %	L S D p=0.05	L S D p=0.01
1000	0.20			1.83			1.46		
3000	1.74	1.15	2.55	5.09	1.30	2.60	4.46	0.47	1.05
5000	4.49	0.47	1.10	6.00	0.21	0.65	7.40	0.70	1.45

Effect of phosphate on sporulation

Low phosphate concentration markedly enhanced sporulation (figure 5), the incidence of sporulation being inversely related to phosphate concentration. Even in the minerally enriched cyanophycean medium, which did not generally favour sporulation, lack of phosphate induced spore formation. Low phosphate concentration had no apparent effect on growth and heterocyst differentiation (figure 5).

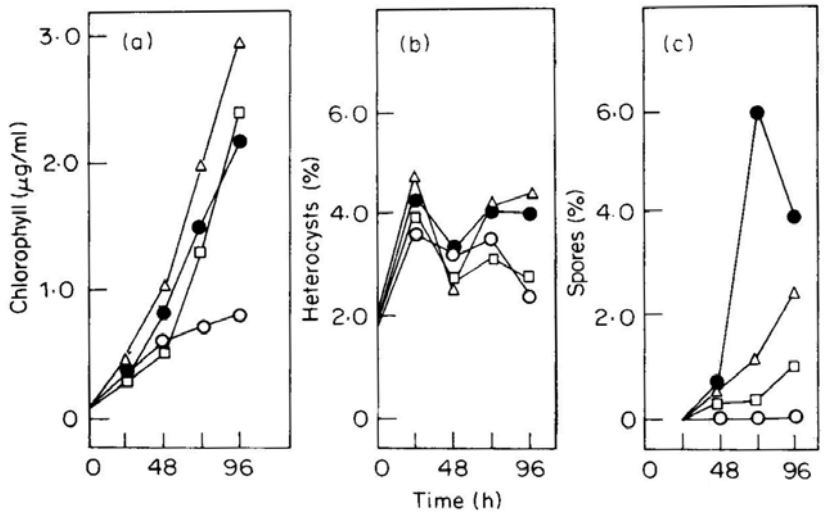

Figure 5. Effect of phosphate on (a) growth, (b) heterocyst production and (c) sporulation of *Anabaena torulosa* at 3000 lux incandescent light and at 29°C in Glade's medium containing 0.3 (●), 0.7 (Δ), 1.0 (❑) and 1.4 (O) mM K_2HPO_4.

Extracellular spore-stimulating substance

When *Anabaena torulosa* was grown in the filtrate obtained from 8 day old cultures (see Methods), sporulation was greatly enhanced (table 2). Culture filtrate and fresh Glade's medium in the ratios 1:1,1:3 and 1:5 also stimulated sporulation when

Table 2. Effect of culture filtrate on sporulation in *Anabaena torulosa.*

Ratio of culture filtrate to fresh medium[a] (v/v)	Age of culture (h)								
	48 h			72 h			96 h		
	spores %	LSD p=0.05	LSD p=0.01	spores %	LSD p=0.05	LSD p=0.01	spores %	LSD p=0.05	LSD p=0.01
0 : 1	0.00			4.70			6.96		
1 : 0	5.80	0.33	0.57	12.19	0.74	1.30	13.21	1.15	2.00
1 : 1	3.30	0.32	0.56	9.42	0.65	1.15	10.80	0.98	1.75
1 : 3	0.99	0.12	0.21	6.60	0.72	1.25	9.06	1.20	2.25
1 : 5	0.50	0.05	0.08	5.80	0.98	1.70	7.20	1.15	2.05

[a] Filtrate of 8 day old cultures were diluted with fresh Glade's medium (see Methods).

compared to the efficiency in Glade's medium alone. Heterocyst frequency was not distinctly affected by the presence of culture filtrate. Although growth was clearly inhibited in the experiments with culture filtrate alone, dilution with fresh medium prevented the inhibition without significantly decreasing the stimulatory effect on sporulation.

Sequence of sporulation

When 6-day old A *torulosa* grown in cyanophycean medium (non-sporulating) was inoculated into Glade's medium and grown at 32°C, spores differentiated normally within 48 h (table 3). In a typical experiment, after 41 h of growth 65% of the

Table 3. Sequence and pattern of sporulation in *Anabaena torulosa.*

Hours	Number of spore strings				Total cell number	
	S-H[a]	S-H-S[a]	S-S-H-S[a]	S-S-H-S-S[a]		
24	0	0	0	0	4948	(111)[b]
31	0	0	0	0	3576	(102)
41	104 (65.0)[c]	32 (20.0)[c]	0	0	18,042	(233)
48	125 (68.6)[c]	54 (29.6)[c]	0	0	16,310	(264)
72	155 (34.6)[c]	267 (59.6)[c]	26 (5.8)[c]	0	19,090	(268)
96	79 (16.0)[c]	332 (69.7)[c]	58 (12.2)[c]	7 (1.4)[c]	11,621	(148)

S-H,S-H-S etc. denote the pattern of spore string formation contiguous to heterocysts. S and H stand for spore and heterocyst respectively. Spores away from heterocysts were not found in the filaments examined in this experiment.

[b] Values in parenthesis are number of filaments examined.
[c] Values in parenthesis indicate per cent heterocysts having the specific type of spore string.

heterocysts had only one adjacent spore (figure 6a; spore –heterocysts—vegetative cell or S-H type, table 3) and 20% had a spore on either side of the heterocysts (figure 6b S-H-S type). The remaining heterocysts had no attached spore. By 48 h, almost all heterocysts had attached spores and the percentages of S-H and S-H-S types were 69 and 30, respectively. At 72 h, the respective % values changed drastically to about 35 and 60 (table 3). In addition, nearly six % of the heterocysts had two spores on one side and a single spore on the other adjacent side (S-S-H-S type; figure 6c). By the end of the fourth day (96 h), the % of S-H type decreased further with concomitant increase in S-H-S and S-S-H-S types. Moreover, some heterocysts had also two spores on either side (S-S-H-S-S; figure 6d). These data indicate that the sequence of sporulation pattern development was: S-H→ S-H-S→ S-S-H-S→S-S-H-S-S.

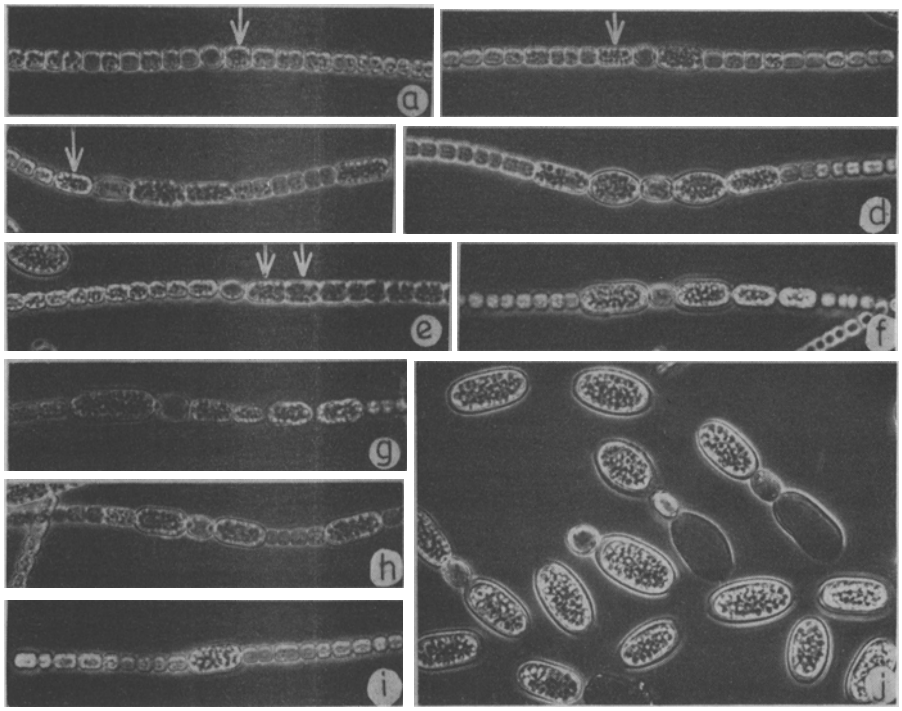

Figure 6. Phase contrast photomicrographs of *Anabaena torulosa* showing the sequence of formation of spore chains. Magnification: X-830. (a) Initiation of a spore adjacent to one side of a heterocyst (S-H type pattern) is followed by (b) spore formation on the other side (S-H-S type). Subsequently spore strings form in (c) S-S-H-S and (d) S-S-H-S-S pattern. Occasionally the pattern is more unilateral resulting in the formation of (e) S-S-H, (f) S-S-S-H-S,or (g) S-S-S-S-H-S spore strings. Rarely spores appear (h, i) several cells away from heterocysts. Filaments of old cultures lyse leaving behind large mature spores (j) often with their attachments to heterocysts intact. Arrows indicate spores at various stages of differentiation.

Rarely, the sequence occurred more unilaterally with spore strings forming only on one side of the heterocyst (S-S-H; figure 6e) resulting later in S-S-S-H-S (figure 6f) or S-S-S-S-H-S (figure 6g) patterns. Very rarely, spores were also initiated few or many cells away from heterocysts (figure 6h, i). After about five days' growth in G lade's medium, the cyanobacterial filaments broke up, releasing spores (figure 6j) often along with the heterocysts to which they were attached.

Discussion

In *A. cylindrica,* sporulation has been shown to occur mainly during the late logarithmic and stationary phases of growth (Simon, 1977). In *A. torulosa* under conditions favourable for sporulation, such as growth in a minimal (Glade's) medium and provision of incandescent light, spores are initiated even during the exponential phase. During this period, growth is not diminished in Glade's medium and is comparable to that in the minerally enriched cyanophycean medium. This indicates that the occurrence of sporulation is not an effect resulting from decreased growth rate.

Relatively high temperature is known (WoIk, 1965) to facilitate sporulation in *A. cylindrica,* although temperature above 30°C was not considered suitable because of deleterious effects on growth. In *A. torulosa* higher temperatures are found to favour sporulation and even a temperature of 40°C does not hamper growth and enhances sporulation.

The present results with *A. torulosa* showing enhanced sporulation in high light intensity are in contrast to the results with *A. cylindrica* (Wolk, 1965) where low light intensity has been shown to favour sporulation. However, it appears that spectral quality of the light is more important in sporulation than its intensity. In *Nostoc spumigena* red and blue light have been reported to stimulate sporulation (Pandey and Talpasayi, 1980). Our finding that incandescent light stimulates sporultion whereas no sporulation occurs in fluorescent light, suggests the possible involvement of red light in the differentiation of spores. The involvement of light quality on spore differentiation needs to be critically evaluated by using mono-chromatic light

The spore stimulating activity under low phosphate concentrations confirms similar conclusions made earlier (Glade, 1914; Fisher and WoIk, 1976). Even in the minerally enriched cyanophycean medium, which does not normally favour sporulation, lack of phosphate induces spore formation. However, the role of phosphate does not seem pivotal, because sporulation of *A. torulosa* occurs readily in solid (agar) cyanophycean medium containing phosphate. It may be that unlike the situation in liquid cultures, extracellular substances may not get diluted in solid medium, and would therefore be present near algal filaments in amounts sufficient to induce sporulation. The involvement of extracellular products in sporulation has been demonstrated in *C. licheniforme* (Fisher and WoIk, 1976; Hirosawa and WoIk, 1979b) and the enhancement in sporulation observed by us in *A. torulosa* on addition of culture filtrates indicates that this involvement may be of general occurrence in cyanobacteria.

It has been suggested (Wolk, 1965) that a gradient of spore maturation exists in *Anabaena cylindrica* and an assessment of the rate of spore formation in this alga (Simon, 1977) supports this notion. Our results on the course of sporulation in *A. torulosa* also agree with the above conclusion. However, the pattern of sporulation in *A. torulosa* not only suggests a bilateral sequence emanating from the heterocyst, but also indicates that the impulse for sporulation is unequally manifested in that, spores are mostly initiated on one side first and not on both sides simultaneously. This is evident from the time course of initiation of spores on either side of the heterocysts and also from the unequal size of the spores on either side immediately contiguous to heterocysts.

The spatial relationship between heterocysts and spores in *A. cylindrica* is broken in the presence of canavanine suggesting that the physiological state of the vegetative cell, and not dependence on heterocysts is the major determining factor in sporulation (Nichols *et al.,* 1980). This is also suggested by the occasional appearance of spores away from heterocysts in *A. torulosa.* However, under normal conditions heterocysts seems to have a role in regulating spore formation, possibly by the production of a substance which abates the inhibition apparently resident in vegetative cells (Wolk, 1965; Nichols *et al.,* 1980). Although there is no clear evidence as yet to confirm this possibility, the present results and those reported earlier (Wolk, 1965; Tyagi, 1974; Simon, 1977) favour the concept that gradients of spore maturation exist in cyanobacteria.

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