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The cumulative effect of carbon and nitrogen sources on Akinete differentiation in the rice-field cyanobacterium *Anabaena* sp.

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Abstract
Cellular differentiation is one of the unique features of filamentous cyanobacteria (Nostocales). *Anabaena* sp. is a filamentous cyanobacterium capable of heterotrophy and cellular differentiation into nitrogen-fixing heterocysts and thick walled desiccating cells, the akinetes. For induction of akinetes in the culture of *Anabaena* sp., combination of sugars and potassium nitrate were added to the basal medium. The results revealed the shortening of period of akinete differentiation. SDS-PAGE experiments indicated that some new proteins appeared while formation of some other proteins was inhibited during the process of akinete differentiation.

Keywords: Cyanobacteria, *Anabaena* sp., carbon, nitrogen, akinetes, heterocysts

Introduction
Cyanobacteria are the ancient and diverse group of Gram-negative eubacteria characterized by higher plant type oxygenic photosynthesis. They occupy a wide range of habitats as free living as well as in symbiosis with other organisms (Adams and Duggan, 1999) [1]. Many filamentous cyanobacteria form trichomes consisting of vegetative cells for photosynthetic growth, heterocysts for fixing nitrogen and akinetes for propagation as seeds (inoculum) in the fields for next crop (Singh *et al.*, 2014) [13]. Akinetes are more resistant to unfavourable growth conditions than vegetative cells and can withstand long period of cold and desiccation (Moore *et al.*, 2005) [7]. All akinetes possess a thickened peptidoglycon cell wall, a multicellular extracellular envelope, large deposits of cyanophycin granules and an increased cell size (Adams and Duggan, 1999) [1].
The akinete differentiation in the cyanobacteria has been known to be affected by many environmental and nutritional factors including phosphorus limitation, low temperature (Li *et al.*, 1997), added carbon and nitrogen sources (Singh *et al.*, 2013a;b) [10], high salinity (Mazur-Marzec *et al.*, 2005) [6] and irradiance (Myers, 2011) [8]. However, akinete formation in cyanobacteria is also affected by a critical C: N ratio in addition to nitrogen sources (Suikkanen *et al.*, 2010) [14]. But the work done on cumulative effect of carbon and nitrogen on akinete differentiation in cyanobacteria is scanty. Hence in the present problem, cellular differentiation in the cyanobacterium *Anabaena* sp. is studied in response to carbon plus nitrogen supplemented media with an aim to identify favourable nutritional conditions which may support 100% akinete differentiation in *Anabaena* sp. in minimum possible time so that this organism can be explored for its application in area like algalization/biofertilizer technology programmes.

Material and Methods
Culture maintenance and experimental conditions
The cyanobacterium *Anabaena* sp. is a rice field isolate. The cyanobacterium was grown in basal (modified Chu-10) medium (Safferman and Morris, 1964) [9]. For induction of akinetes in the culture, combination of 1% sugars and 2 mM nitrate (glucose+ potassium nitrate and sucrose+potssium nitrate) were added to the basal medium.

Growth estimation
The growth of *Anabaena* sp. was estimated by determining chlorophyll and protein contents following the methods of Holm (1964) [2] and Lowry *et al.*, (1951) [5] respectively.

Akinete and Heterocyst Frequency
Akinete and heterocyst frequencies were calculated by counting the number of akinetes/ heterocysts present per hundred vegetative cells.

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Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein contents of the akinetes and vegetative cells were determined by running discontinuous acrylamide gel following the method of Laemmly (1970) [3]. Proteins were visualized by Coomassie Brilliant Blue Staining.

Statistical Analysis of Data

The data were analyzed statistically for Standard Deviation and using "Microsoft Excel".

Results and Discussion

Cellular differentiation in cyanobacteria can be controlled by various environmental as well as nutritional factors. The process of heterocyst formation occurs in response to depletion of fixed nitrogen sources while akinete can be induced by energy limitations (Adams and Duggan, 1999) [1]. In the present investigation, the growth of *Anabaena* sp. in basal medium has been studied (Table 1 and 2). It exhibited an increase in overall growth upto 20-22 days and declined (data not shown) thereafter indicating that there may be depletion of nutrients in the culture medium and possibly accumulation of inhibitory metabolites. The differentiation of akinete in *Anabaena* sp. was started with the appearance of small granules within cells on day 8 but actual akinetes were appeared on day 14 and maturation phase got extended up to 48-50 days after which all the cells matured into akinetes in basal culture medium. The heterocyst frequency of *Anabaena* sp. remained almost constant (6.25%) in control cultures up to 12 days and declined after that. As previous reported that the akinete differentiation in cyanobacteria like *Anabaena cylindrica* and *Nostoc* sp. occurred as the growth of the organism ceased (Singh *et al.*, 2011) [12].

The supplementation of glucose+potassium nitrate and sucrose+potassium nitrate significantly reduced the exponential growth phase. Although heterocyst frequency of the test organism in both these combinations declined to zero per cent, still the akinete differentiation started on day 6 but actual akinetes appeared on day 8 and 100% akinetes formed only on day 16 in both the combinations (Table 1, Fig. 1 B, C).

Table1. Pattern of growth and akinete differentiation in *Anabaena* sp. under basal and glucose+nitrate supplemented media.

Time (in days)	Control Conditions			Glucose+pot. Nitrate		
	Chlorophyll Content	Protein Content	Akinete Frequency (%)	Chlorophyll Content	Protein Content	Akinete Frequency (%)
0	0.66±0.02	15.38±0.57	0	0.66±0.02	15.38±0.57	0
2	1.10±0.02	29.74±0.54	0	0.97±0.02	28.73±0.76	0
4	1.38±0.04	45.36±1.97	0	1.28±0.06	54.21±0.09	0
6	1.98±0.02	66.41±2.03	0	1.82±0.15	88.43±1.67	0
8	2.22±0.04	83.70±1.50	0	2.15±0.05	105.39±0.97	27.35±0.98
10	2.73±0.13	110.34±1.47	0	2.49±0.04	123.40±1.11	38.49±0.92
12	3.21±0.09	130.45±2.54	0	2.83±0.11	144.29±1.05	58.63±0.43
14	3.44±0.15	140.34±2.53	4.56±0.57	2.74±0.08	144.42±0.78	82.47±0.57
16	3.54±0.11	167.28±2.06	5.81±0.42	2.55±0.05	144.19±0.18	100.00±0

Table 2: Pattern of growth and akinete differentiation in *Anabaena* sp. under basal and sucrose+nitrate supplemented media.

Time (in days)	Control Conditions			SUCROSE+POT. NITRATE		
	Chlorophyll Content	Protein Content	Akinete Frequency (%)	Chlorophyll Content	Protein Content	Akinete Frequency (%)
0	0.66±0.02	15.38±0.57	0	0.66±0.02	15.38±0.57	0
2	1.10±0.02	29.74±0.54	0	1.07±0.11	28.43±1.08	0
4	1.38±0.04	45.36±1.97	0	1.32±0.10	54.48±1.22	0
6	1.98±0.02	66.41±2.03	0	1.76±0.04	85.47±1.03	0
8	2.22±0.04	83.70±1.50	0	2.08±0.05	98.75±1.33	35.43±0.85
10	2.73±0.13	110.34±1.47	0	2.45±0.05	119.61±2.07	45.43±0.68
12	3.21±0.09	130.45±2.54	0	2.67±0.12	138.39±1.04	63.15±1.15
14	3.44±0.15	140.34±2.53	4.56±0.57	2.46±0.02	141.34±0.96	85.71±0.40
16	3.54±0.11	167.28±2.06	5.81±0.42	2.45±0.10	141.22±0.10	100.00±0

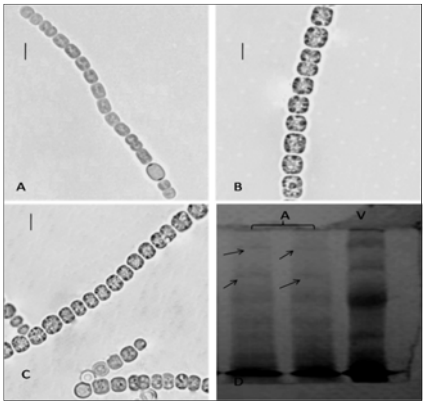

Fig 1: Cellular differentiation and SDS-PAGE protein profiling in *Anabaena* sp.: a filament showing vegetative cells and heterocyst in basal medium (A), akinetes in glucose+nitrate (B) and akinetes in sucrose+nitrate (C) supplemented medium; SDS-PAGE of proteins of vegetative cells {V} and akinetes {A} of *Anabaena* sp. in glucose+nitrate and sucrose+nitrate supplemented medium. (scale bar is equal to 10µm).

There are similar reports where a critical C:N ratio acted as a trigger for akinete formation in cyanobacteria (Adams and Duggan, 1999) [1]. The role of the presence of independent carbon (Singh *et al*., 2013a) [10] and nitrogen (Singh *et al*., 2013b) [11] sources in inducing akinete formation has been also known in *Anabaena naviculoides*. Our investigation confirmed the earlier studies but it is first report in the rice field isolated cyanobacterium *Anabaena* sp. It is breakthrough because this type of cyanobacterial species with 100% sporulation is found to be good members for algalization/biofertilizer technology. As reported that cyanobacteria play a remarkable role in agriculture fields (Singh *et al*., 2014) [13]. The data presented here revealed that supplementation of sugars with nitrate to cyanobacterium speed up the process of akinete initiation and maturation. Hence a nutritional condition supporting better growth or reducing the exponential growth phase, also induced earlier akinetes differentiation is identified.

Running of crude protein extracts of vegetative cells and akinetes of *Anabaena* sp. through acrylamide gel electrophoresis revealed that some new proteins got synthesized while synthesis of some other proteins was inhibited during the differentiation process. These results also confirmed previous reports. The proteins with MW of 20, 43 and 66 kDa were observed only in extracts of akinetes but not in vegetative cells in *Anabaena variabilis* during SDS-PAGE analysis (Zhou and Wolk, 2002) [15].

In summary, the study revealed that glucose+nitrate and sucrose+nitrate combinations triggered the cellular differentiation (especially akinete formation) process in *Anabaena* sp., a rice field, heterocystous N_2 fixing cyanobacterium. All the vegetative cells of this test organism differentiate into akinetes within shorter time period. This may be, therefore, a good member in cyanobacterial biofertilizer technology program. Moreover, this organism would be a good experimental model system to understand mechanism of akinete differentiation since pure population of vegetative cells and akinetes are easily available.

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