

**ENRICHMENT OF HETEROCYST FREQUENCY IN A  
NEW ISOLATE OF *NOSTOC* SP SAG 2306 BY  
2,4-DICHLOROPHENOXYACETIC ACID AND ITS  
SUBSEQUENT IMPACT ON MODULATING  
PHOTOSYNTHESIS/RESPIRATION RATIO AND  
HYDROGENASES ACTIVITIES**

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***Abstract***

Heterocysts are microaerobic specialized nitrogen fixing cells formed in some filamentous cyanobacteria and having the structural and physiological capacity to create and install internal anaerobic environment. The findings presented herein suggest that the auxin herbicide 2,4-D (2,4-dichlorophenoxyacetic acid) might be used for decreasing the culture and/or cellular oxic environment in *Nostoc* sp SAG 2306 that is favorable for enhancing the hydrogenases activities. This is achieved via induction and enrichment of heterocyst frequency. Treatment of the filamentous heterocyst-forming cyanobacterium *Nostoc* sp. SAG 2306 grown in nitrogen-deprived medium with different concentrations of 2,4-D has increased heterocyst frequency up to 19%. Furthermore, 2,4-D induced heterocyst formation in the presence of combined nitrogen (nitrate) up to 90  $\mu$ M. An increase in respiratory oxygen uptake and lowered photosynthesis/respiration (P/R) ratio, observed in 2,4-D treated *Nostoc* cultures, was associated with an increase in hydrogenases activities. 2,4-D caused a decrease in phycocyanin/chlorophyll *a* ratio which

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was associated with a decrease in photosynthetic oxygen evolution suggesting that the decrease in phycocyanins which are a major component of phycobilisomes, the light-harvesting complex of photosystem II in cyanobacteria, due to 2,4-D treatment has a negative impact in oxygen evolution and a positive impact on hydrogenases activities.

Lowered values of phycocyanin/chlorophyll *a* ratio, decreased oxygen evolution, photosynthesis/respiration (P/R) ratio with enhanced respiration and heterocyst formation all have been induced by 2,4-D cooperated to install anaerobiosis for hydrogen metabolism.

**Key words:** cyanobacterium; heterocyst; hydrogenase; *Nostoc*; 2,4-D

### ***Introduction***

Heterocysts are specialized nitrogen fixing cells formed in some filamentous cyanobacteria under nitrogen starvation where some vegetative cells differentiate into heterocysts (Wolk, 1996; Zhang *et al.*, 2006). Once a heterocyst has formed, it cannot revert to a vegetative cell (Kulasooriya and Fay, 1974). In cyanobacteria whose heterocysts are intercalary such as *Nostoc* and *Anabaena*, the differentiated heterocysts are evenly spaced along the filament to form a semi-regular pattern (Yoon and Golden, 1998; 2001). As the filament grows and the number of vegetative cells between heterocysts increases, single vegetative cell midway between two heterocysts develop into a new one, thus preserving the semi-regular pattern of differentiated cells (Wolk, 1996; Golden and Yoon, 2003). The differentiation of cyanobacterial vegetative cells into heterocysts include global change in gene expression (Wolk *et al.*, 1994; Adams, 2000; Wolk, 2000) including that of nitrogenase and other proteins involved in nitrogen fixation (Böhme, 1998; Herrero *et al.*, 2001; Meeks and Elhai, 2002; Golden and Yoon, 2003). Heterocysts do not carry out photosynthesis since they lack RuBP carboxylase activity as well as PSII and phycobilisomes, the light harvesting complex of cyanobacterial vegetative cells (Donze *et al.*, 1971; Codd *et al.*, 1980; Fay, 1992; Wolk *et al.*, 1994). In addition, they are protected from the external oxygen by a glycolipid layer in its cell wall which reduces the permeation of oxygen into the cell (Nicolaisen *et al.*, 2009). Furthermore,

heterocysts produce myoglobin for scavenging any remaining oxygen inside the cell (**Potts *et al.*, 1992**). Consequently, heterocysts form a microaerobic environment that is compulsory for nitrogenases and hydrogenases activity. The ammonia formed by nitrogen fixation is then incorporated into glutamate to form glutamine which is transported to the vegetative cells from heterocysts, and in return, carbohydrates, believed to be sucrose (**Curatti *et al.*, 2002**) is transported from the vegetative cells to heterocysts.

As a byproduct of nitrogen fixation in heterocysts, hydrogen gas is produced. One molecule of hydrogen gas is produced for each fixed molecule of nitrogen. An uptake hydrogenase is particularly expressed in heterocysts (**Houchins and Burris, 1981; Houchins, 1984; Papen *et al.*, 1986; Carrasco *et al.*, 1995**) that catalyzes the consumption of molecular hydrogen through its oxidation into protons and electrons (**Lambert and Smith, 1981; Houchins, 1984; Tamagnini *et al.*, 2000, Tamagnini *et al.*, 2002**) and ATP to be reinvested in cellular energetics and proton pools. Uptake hydrogenase is localized in the thylakoid membranes of heterocysts, where it transfers electrons from hydrogen for the reduction of oxygen via the respiratory chain in a reaction known as oxyhydrogenation or Knallgas reaction (**Dutta *et al.*, 2005**). A knockout mutation of the uptake hydrogenase has increased the hydrogen productivity in heterocysts (**Yoshino *et al.*, 2007**). A bidirectional hydrogenase with the capacity to both taking up and producing hydrogen is also involved in hydrogen metabolism of cyanobacteria (**Houchins, 1984; Flores and Herrero, 1994; Bergman *et al.*, 1997; Appel and Schulz, 1998; Hansel and Lindblad, 1998; Lindblad, 1999; Appel *et al.*, 2000; Tamagnini *et al.*, 2002; Tsygankov, 2007**). The bidirectional hydrogenase is associated with the cytoplasmic membrane and likely functions as an electron acceptor from both NADH and H<sub>2</sub> (**Dutta *et al.*, 2005**) but its biological role is poorly understood (**Boison *et al.*, 1999**). It may release excess reducing power in anaerobic conditions thus acting as an electron valve for the disposal of low potential electrons generated at the onset of illumination (**Stal and Moezelaar, 1997**) or during periods of adaptation to high light intensities (**Appel *et al.*, 2000**) although it is not a universal enzyme. Nevertheless, the physiological

implications of hydrogenases are altogether debatable. Unlike uptake hydrogenase, the bidirectional hydrogenase is helpful in hydrogen production (Dutta *et al.*, 2005) and its function suggested to be independent of nitrogen fixation (Tamagnini *et al.*, 2002) because the biosynthesis of nitrogenase was not a prerequisite for the bidirectional hydrogenase biosynthesis and hydrogen evolution in several unicellular cyanobacterial strains (Howarth and Codd, 1985).

Oxygen is a powerful inhibitor to hydrogenases activities. The enrichment of heterocysts increases the number of the microaerobic cells on the one side and decreases net oxygen productivity in the culture as a whole on the other side that can be a route for increasing the hydrogen gas productivity in cyanobacteria. In this study, both uptake and bidirectional hydrogenases have been characterized in the heterocyst enriched *Nostoc* sp. Since the role of 2,4-D in enhancing heterocyst formation has been recorded (Tiwari *et al.*, 1981), serial concentrations of 2,4-D were added to nitrate deprived medium (BG11<sub>0</sub>). The impact of 2,4-D on heterocyst frequency and its consequences on photosynthesis and hydrogenases activities in a new isolate of *Nostoc* sp are studied.

## ***Materials and Methods***

### **Growth conditions and treatments of *Nostoc***

The filamentous cyanobacterium *Nostoc* sp SAG 2306 (newly deposited strain in Sammlung von Algenkulturen der Universität Göttingen) was locally isolated from a clay soil sample from the agriculture farm, Assiut University, Egypt and identified by SAG in a collaborative work (submitted). *Nostoc* was grown as batch culture in BG11 nutritive media (Rippka and Herdman, 1992) at ambient temperature of 25°C ±1 in a growth chamber (Forma Scientific, USA) in which continuous illumination preformed by 2 fluorescent tubes (day light) giving irradiance of about 13.5 μE.m<sup>-2</sup>.s<sup>-1</sup> at the surface of the culture vessels. Since it is widely known that combined nitrogen inhibits heterocyst formation, *Nostoc* was grown in BG11<sub>0</sub> medium that is a regular BG11 but deprived of combined

nitrogen. In concert with the aim of this work, the BG11<sub>0</sub> cells has been treated with 2,4-D in concentrations (0, 2.5, 5, 10, 20, 30, 50 and 100  $\mu\text{g}\cdot\text{ml}^{-1}$ ) to induce and enrich heterocyst frequency of *Nostoc*. However, undifferentiated filaments were used as inoculum in all experiments.

#### **Growth and analytical assessments:**

Growth was daily assessed by three parameters: optical density at 750 nm, chlorophyll *a* (chl *a*) contents and dry mass. Chl *a* was dark-extracted under cooling in 100% methanol for 24 h, clarified by centrifugation under cooling (4°C) at 7000  $\times g$  for 15 min and quantified spectrophotometrically according to the equations of **Holden (1976)**. For dry mass assessment, aliquots of algal suspension (e.g. 25 ml) were filtered above a pre-weighed glass fiber filter, washed three times with distilled water, oven-dried overnight at 80°C and reweighed periodically up to constancy. For heterocyst frequency determination, the cyanobacterium was photographed, hetrocysts and vegetative cells were counted and heterocyst frequency was calculated according to **Troshina et al. (1996)**. Phycocyanin was determined as described by **Tchernov et al. (1999)**. Protein concentration was determined by CBB G-250 dye binding method described by **Bradford (1976)** using bovine serum albumin (BSA) as a standard protein.

#### **Photosynthesis and respiration:**

Photosynthetic oxygen evolution was measured using a Clark type oxygen electrode computerized to an oxygen monitoring system (OMS, Hansatech Instruments Inc., donation from Alexander von Humboldt foundation to R. Abdel-Basset). O<sub>2</sub> evolution was measured in actinic light (44.5  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  at the surface of the cell) passed through a red optical filter. All measurements were performed at  $25 \pm 1^\circ\text{C}$  stabilized by a circulating water bath. Respiratory O<sub>2</sub> uptake was measured using the same above-mentioned Clark electrode and oxygen monitoring system at identical conditions but in the dark. photosystem I (PSI) activity was followed as described earlier (**Abdel-Basset and Bader, 2008**).

### **Hydrogenases assays:**

Uptake hydrogenase was assayed by the hydrogen dependent reduction of methylene blue according to **Colbeau *et al.* (1980)**. Intact cyanobacterial cells containing 3–4 µg chlorophyll (varied according to the treatment) were centrifuged, concentrated to 0.2 ml and used in assays. The reaction assay mixtures were flushed with hydrogen gas for one minute and the decrease in absorbance of methylene blue at 570 nm was followed periodically using a spectrophotometer (Spectronic 20D, Milton Roy company) at ambient temperature. The hydrogen evolution activity of the bidirectional hydrogenase was monitored using assay mixtures similar to those used by **Yu and Wolin (1969)**. The assay depends on dithionite reduced methyl viologen as the electron donor after nitrogen flushing. The decrease in absorbance at 578 nm was followed periodically using a spectrophotometer at ambient temperature.

### **Characterization of hydrogenases:**

Characterization of the hydrogenases enzymes was carried out using cells grown in nitrate deprived medium (BG11<sub>0</sub>) which is necessary to induce heterocyst formation.

### **Enzyme Kinetics:**

$K_m$  value of hydrogenases for methyl viologen in the hydrogen evolution activity and for methylene blue in the hydrogen uptake activity were determined using Lineweaver–Burk plots of various concentrations of the respective dyes against hydrogenases activities. Characterization included also factors affecting enzymes activity. Temperature ranging from 20 to 50 °C was applied. The pH range from 5.4 to 9.4 using respective buffers (50 mM citrate-phosphate buffer for the pH range from 5.4 to 6.6, 50 mM tris-HCl buffer for the pH range from 7 to 9 and 50 mM CHES-NaOH buffer for the pH range from 9 to 9.4). Characterization included, in addition, photosynthetic effectors DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea] and CCCP [carbonyl cyanide m-chlorophenylhydrazone].

## Results

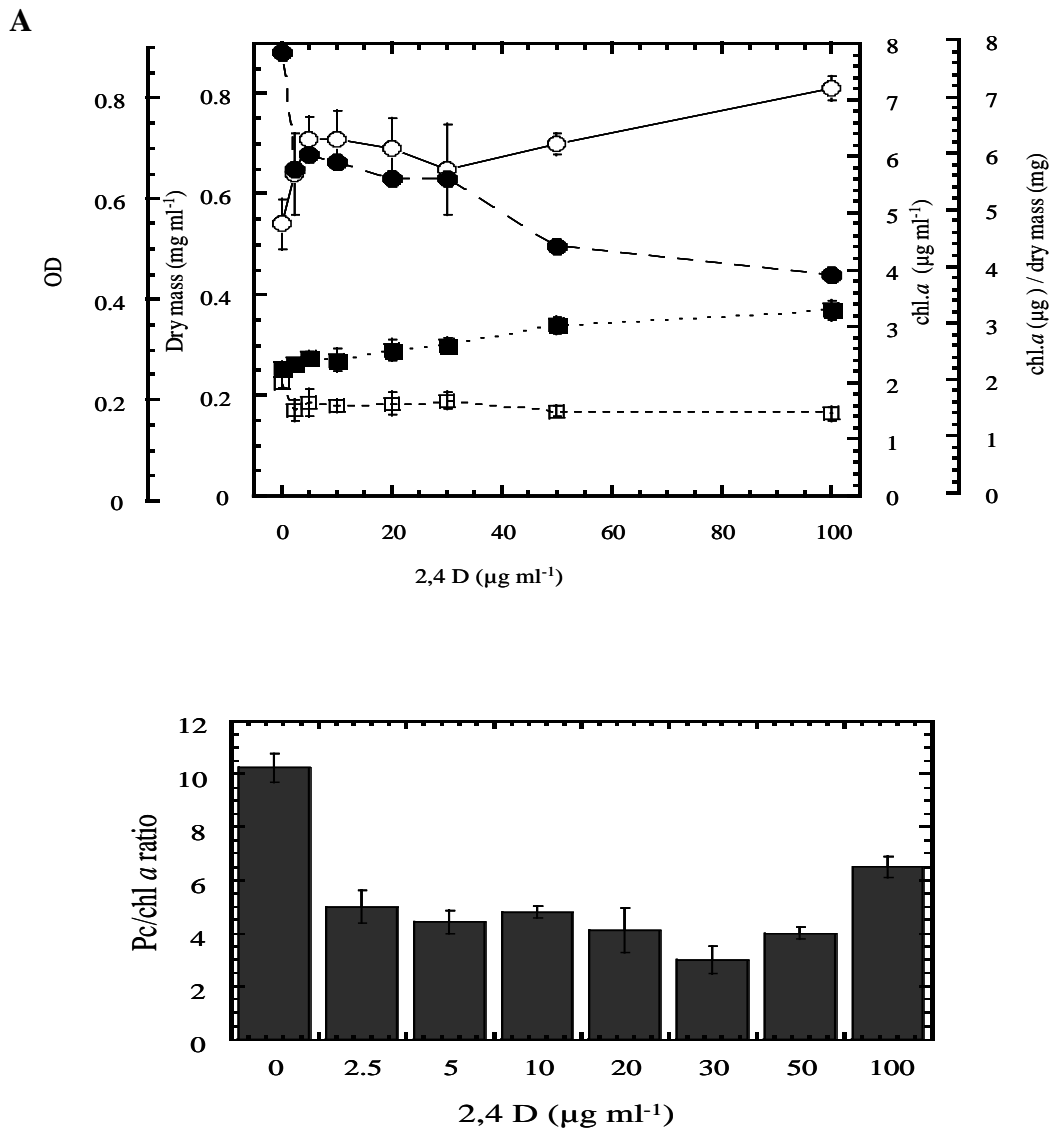
### Effect of 2,4-D treatment on growth of *Nostoc* sp:

Under nitrogen starvation, the cyanobacterium *Nostoc* sp SAG 2306 showed high fragmentation (data not shown) and lower chl *a*/ dry mass (Fig 1A) as heterocysts were formed and enriched at the expense of chl *a* contained in vegetative cells. In control cultures (BG11), chl *a* content of *Nostoc* sp. was 12  $\mu\text{g}\cdot\text{mg}^{-1}$  dry weight whilst in BG11<sub>0</sub> it exhibited only 7.8  $\mu\text{g}\cdot\text{mg}^{-1}$  dry weight. 2,4-D slightly enhanced the growth of *Nostoc* sp. as revealed by optical density and dry mass (Fig. 1A). In contrast, 2,4-D did not significantly affect chl *a* content per culture volume (Fig 1A). However, the chl *a*/dry mass decreased with increasing 2,4-D concentration in the culture medium (Fig. 1A). 2,4-D treatment decreased the phycocyanin/ chl *a* ratio in *Nostoc* sp. grown in nitrogen deprived medium (Fig 1B).

### Effect of 2,4-D treatment on heterocyst frequency of *Nostoc*:

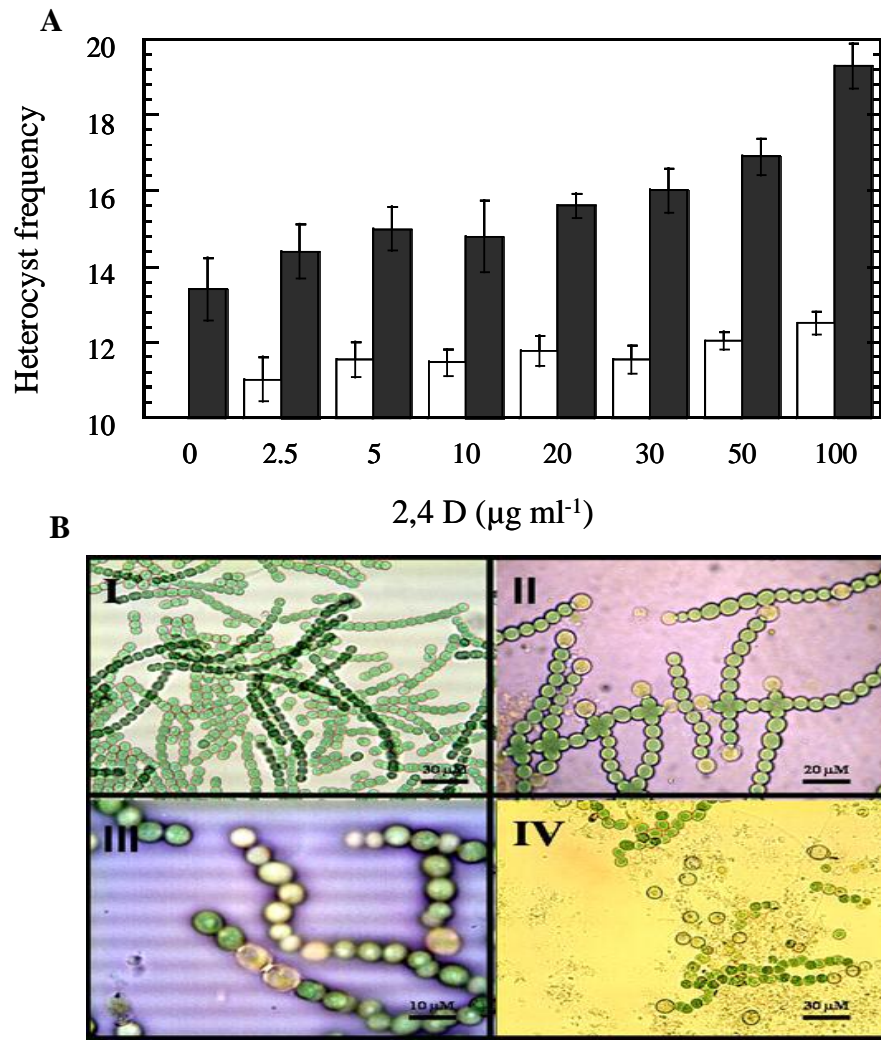
2,4-D induced and enriched heterocysts in *Nostoc* sp. (Fig. 2A). Figure (2 B) shows heterocyst free filaments in BG11 (BI), 2,4-D heterocyst containing fragments (BII), two or more adjacent heterocysts appear occasionally at 100  $\mu\text{g}\cdot\text{ml}^{-1}$  2,4-D (BIII) and detached (BIV). Table (1) presents a detailed follow up of heterocyst formation in *Nostoc*. Heterocysts started to appear at the 6<sup>th</sup> day after cells grown in nitrate replete (BG11) medium were transferred into nitrate-deprived medium (BG11<sub>R</sub>). Transferring the inoculum cells from BG11 simultaneously transfers into the new culture of BG11<sub>0</sub> residual nitrate accounting to only 2% that in BG11, referred to as BG11<sub>R</sub>. Upon removing this residue by washing the inoculum cells with BG11<sub>0</sub> before inoculation and the medium become completely free from any combined nitrogen (BG11<sub>0</sub> culture), heterocysts appeared promptly earlier after only 24 h of cultivation (Table 1).

Collectively, then, full nitrogen strength (BG11 medium) absolutely inhibited heterocyst formation; BG11<sub>R</sub> (deprived of nitrate but containing residual combined nitrogen) induced the appearance of heterocysts at the 6<sup>th</sup> day, BG11<sub>0</sub>



**Figure (1):** Effect of 2,4-D on growth, chl *a*/ dry mass ratio and PC/chl *a* ratio of *Nostoc* sp. SAG 2306 grown in BG11<sub>0</sub> medium. Growth of *Nostoc* (panel A) was assessed with optical density (open circles), dry mass (closed squares) and chl *a* (open squares). The chl *a*/dry mass ratio (closed circles) was calculated using the mean values of both parameters. Phycocyanin content was measured as described in Materials and Methods and the PC/chl *a* ratio (panel B) were calculated at each concentration of 2,4-D-treated *Nostoc* sp. culture. Experiments were repeated three times and mean values and standard errors are shown.





**Figure (2):** Effect of 2,4-D on heterocyst frequency of *Nostoc* sp. SAG 2306 grown in BG11<sub>0</sub> medium (panel A). Heterocyst frequency was followed daily and the data shown for the 3<sup>rd</sup> day (white columns) and the 7<sup>th</sup> day (black columns) of *Nostoc* sp. grown on BG11<sub>0</sub> treated with different concentration of 2,4-D. Experiments were repeated three times and mean values and standard errors are shown. Light microscope photographs of *Nostoc* sp. (panel B). A photograph of the cyanobacterium grown in nitrogen enriched medium (BG11) showing no heterocysts (photo I). The photograph II and III show *Nostoc* sp. grown in nitrogen deprived medium (BG11<sub>0</sub>) and that grown in BG11<sub>0</sub> treated with 100 µM 2,4-D at the 7<sup>th</sup> day of growth respectively. *Nostoc* culture grown in BG11<sub>0</sub> treated or not with 2,4-D showed detached heterocysts at late growth phase (Photo IV).

culture (completely deprived of nitrate and residuals) accelerated heterocyst formation promptly at day 1. The addition of a definite concentration of 60  $\mu\text{M}$  nitrogen (in the nitrate form) to the BG11<sub>0</sub> culture simulated an effect similar to that of BG11<sub>R</sub> since it delayed the appearance of heterocysts to day 6. The same timing of heterocysts appearance might indicate both concentrations are similar. i.e. residual combined nitrogen is equivalent to 60  $\mu\text{M}$  nitrogen at least in effect if not in concentration. The inclusion of the 2,4-D accelerated the formation of heterocysts and increased its frequency in BG11<sub>R</sub> medium. In BG11<sub>0</sub> culture, however, 100  $\mu\text{g.ml}^{-1}$  of 2,4-D did not exert a remarkably enhancing effect on heterocyst formation and/or frequency because any inhibitory or delaying effect ascribed to combined nitrogen was completely not existing (heterocysts actually appeared by the first day).

In another approach trying to find out the concentration of nitrate that 2,4-D can overcome its inhibitory effect on heterocyst formation, concentrations of 15, 30, 60, 90 or 120  $\mu\text{M}$  nitrogen was combined with 100  $\mu\text{g.ml}^{-1}$  2,4-D. In this case, 15  $\mu\text{M}$  nitrogen did not affect heterocyst formation and was similar to BG11<sub>0</sub> culture while 30  $\mu\text{M}$  nitrogen delayed it to the 3<sup>rd</sup> day that has been overcome by 2,4-D at day 2. At a concentration of 60  $\mu\text{M}$  nitrogen heterocyst formation was delayed to day 6 but enhanced by 2,4-D at day 2. Therefore, as the concentrations of combined nitrogen increased its delaying effect was more pronounced and the stimulatory ability of 2,4-D becomes more pronounced. However, the concentration of 120  $\mu\text{M}$  nitrogen inhibited heterocyst formations and the addition of 2,4-D failed to overcome this inhibition (Table 1) i.e. the stimulatory ability on heterocyst formation of 2,4-D to overcome the inhibitory effect is up to certain limits (below 120  $\mu\text{M}$  nitrogen in this case).

#### **Characterization and Kinetics of Uptake and Bidirectional Hydrogenases in *Nostoc* sp. grown in BG11<sub>0</sub>:**

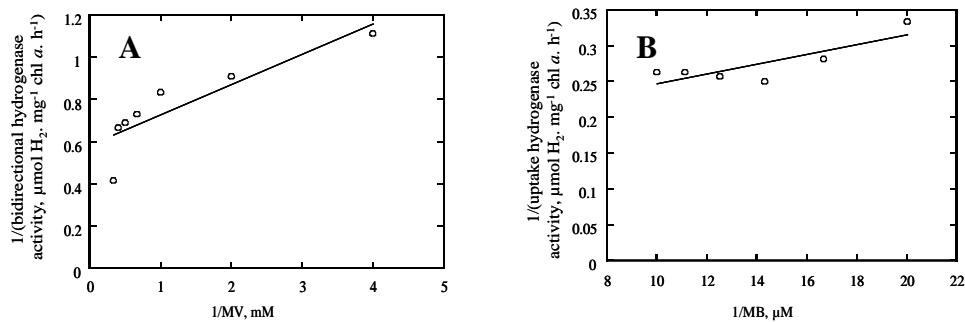
Under ambient culture conditions of the cyanobacterium in BG11<sub>0</sub>, the uptake hydrogenases activity of *Nostoc* sp. was higher than its evolution hydrogenase activity with all enzyme (intact cyanobacterium) concentrations used (data not shown).

**Table (1): Induction and enhancement of heterocyst formation of *Nostoc* sp SAG 2306 by 100 µg.ml<sup>-1</sup> 2,4-D, in the presence or absence of various levels of combined nitrogen (Nitrate form).**

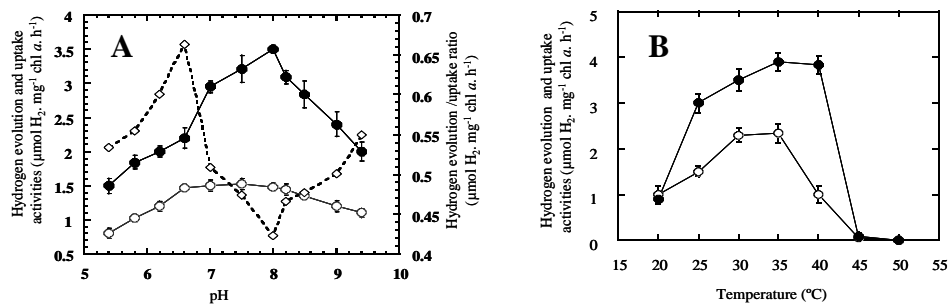
Treatment	Days											
	0	1	2	3	4	5	6	7	8	9	10	
BG11												
BG11 <sub>R</sub>							13	13	13.5	11	9	
BG11 <sub>0</sub>	11	11	9	6	6	5	6	5	4	D		
BG11 <sub>0</sub> <sup>+</sup>	13	14	11	9	7	7	7	↓	↓	D		
BG11 <sub>15</sub>	9	10	6	5	5	5	6	↓	↓	D		
BG11 <sub>15</sub> <sup>+</sup>	12	12	13	10	6	6	5	↓	↓	D		
BG11 <sub>30</sub>				6	7	6	5	4	↓	↓	D	
BG11 <sub>30</sub> <sup>+</sup>			10	10	9	9	6	6	↓	↓	D	
BG11 <sub>60</sub>							13	13.7	15	15	13	
BG11 <sub>60</sub> <sup>+</sup>				13	15	17.3	18.8	20	19.7	19	15	14
BG11 <sub>90</sub>												
BG11 <sub>90</sub> <sup>+</sup>									9	10	10	
BG11 <sub>120</sub>												
BG11 <sub>120</sub> <sup>+</sup>												

\* Heterocyst frequency was calculated as percentage relative to vegetative cells and the average of two experiments are presented. BG11 is the control medium containing 17.6 mM nitrate (3 mM nitrogen), BG11<sub>R</sub> is nitrate deprived medium but still contains residual of combined nitrogen representing only 2% that of BG11 transferred with the inoculum cells, BG11<sub>0</sub> is absolutely free from any combined nitrogen; nitrate or residual. The digits subscripted to BG11 represent the concentration of combined nitrogen (µM) added to zero culture in the form of nitrate, (+) indicates inclusion of 100 µg.ml<sup>-1</sup> 2,4-D in culture medium, ↓ refers to declined heterocyst frequency to trivial numbers due to detachment of which from the filaments, D is detached leaving the filaments heterocyst free.

The uptake hydrogenase activity of *Nostoc* sp. was almost double that of the hydrogen evolution activity of the bidirectional enzyme. The  $K_m$  value of bidirectional hydrogenase for methylviologen was 0.24 mM MV (Fig. 3A) while that of uptake hydrogenase for methylene blue was 28.9  $\mu$ M MB (Fig. 3B). The optimum pH value (Fig. 4A) for hydrogenases evolution/uptake ratio by the cyanobacterium is slightly acidic (pH 6.6). The optimum temperature range (Fig. 4B) for the uptake hydrogenase was 35 - 40 °C while that for bidirectional hydrogenase was 30 - 35 °C.

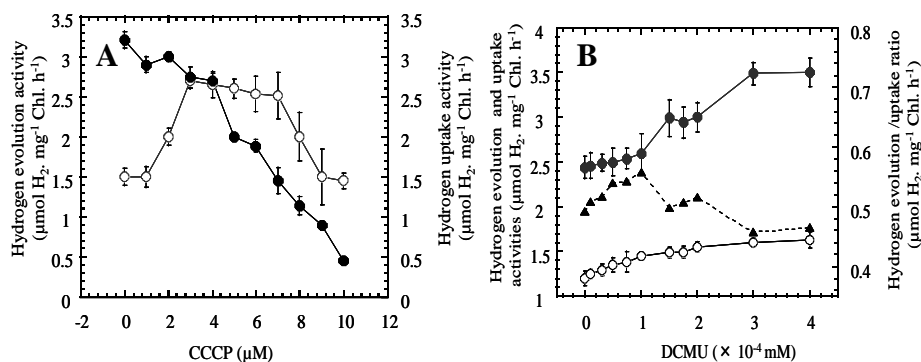


**Fig. 3: Lineweaver–Burk plots for determination of the  $K_m$  values of Bidirectional (panel A) and uptake (panel B) hydrogenases.**



**Figure (4): Effect of pH and temperature on hydrogen evolution and uptake activities of *Nostoc*. Effect of pH (panel A) on the hydrogen evolution (open circles) and uptake (closed circles) activities of 7 day old culture of *Nostoc* grown in BG11<sub>0</sub> medium were measured at various pH values at ambient temperature. The hydrogen evolution/uptake ratios (open cubic) were calculated using the mean values of both activities at each pH. Effect of temperature (panel B) on hydrogen evolution (open circles) and uptake (closed circles) activities of *Nostoc* was measured on potassium phosphate buffer (pH 7 at ambient temperature). Experiments were repeated three times and mean values and standard errors are shown.**

A very sharp decrease in uptake and bidirectional hydrogenase activities was observed at 45 °C (close to zero value) and at 50 °C both uptake and bidirectional hydrogenase activities were zero. The hydrogenase evolution activity of bidirectional hydrogenase increased upon treatments with uncoupler CCCP (Fig. 5A) while the uptake hydrogenase activity decreased. DCMU (specific inhibitor of the electron flow from PSII) increased both hydrogenase evolution and uptake activities (Fig. 5B).

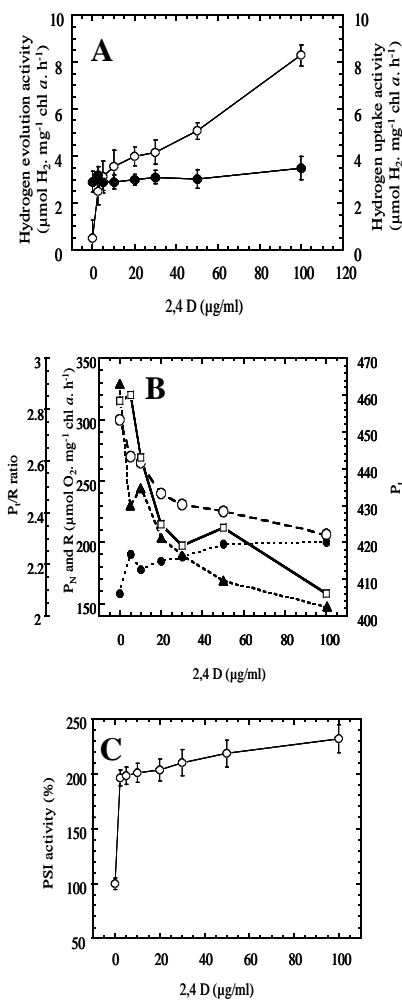


**Figure (5):** Effect of uncoupler CCCP and the PSII inhibitor DCMU on hydrogen evolution and uptake activities of *Nostoc* sp. The effect of CCCP on hydrogen evolution and uptake activities (panel A) was conducted on 7 day old cells of *Nostoc* sp. grown in BG11<sub>0</sub> medium. The hydrogen evolution (open circles) and uptake (closed circles) activities were measured as described in Materials and Methods in presence of various concentrations of (0-10 μM) CCCP in the reaction medium. The DCMU effect on hydrogen evolution and uptake activities (panel B) was conducted on 7 day old culture of *Nostoc* sp. grown in BG11<sub>0</sub> medium. The hydrogen evolution (open circles) and uptake (closed circles) activities of *Nostoc* sp. were measured as described in Materials and Methods in presence of various concentrations of (0-0.4 μM) DCMU in the reaction medium. The hydrogen evolution/uptake ratios (closed triangles) were calculated using the mean values of both activities at each DCMU concentration. Experiments were repeated three times and mean values and standard errors are shown.

**Hydrogen and oxygen evolution and uptake activities of *Nostoc* sp. grown in 2,4-D treated BG11<sub>0</sub> culture medium:**

Enhanced rates of the hydrogen evolution activity of bidirectional hydrogenase was recorded in BG11<sub>0</sub> in *Nostoc* cells grown cells with increasing

the concentration of 2,4-D. However, uptake hydrogenase exhibited no significant change in response to 2,4-D (Fig. 6A). Both net photosynthetic activity ( $P_N$ ) and true photosynthetic activity ( $P_t$ ) of *Nostoc* sp. decreased with increasing the 2,4-D concentration in the culture medium (Fig. 6B). In contrast, the rate of *Nostoc* sp. respiration increased with increasing the 2,4-D concentration in the culture medium (Fig. 6B). 2,4-D decreased  $P_t/R$  ratio as its concentration was increased. The PSI activity increased in *Nostoc* grown in 2,4-D treated culture medium (Fig. 6C).



**Figure (6):** Effect of 2,4-D on hydrogen and oxygen evolution and uptake activities and PSI activity of *Nostoc*. Hydrogen evolution and uptake activities (Panel A) were measured in *Nostoc* treated with various concentrations of 2,4-D on growth culture. Hydrogen evolution (open circles) and uptake (closed circles) activities were measured on 7 day old culture of *Nostoc* grown in BG11<sub>0</sub> medium with various concentrations of 2,4-D. The effect of 2,4-D on photosynthetic oxygen evolution and respiratory oxygen uptake (Panel B) were measured in *Nostoc* treated with various concentrations of 2,4-D on growth culture. Net photosynthesis ( $P_N$ ) (open circles) and respiration ( $R$ ) (closed circles) was measured on 7 day old culture of *Nostoc* grown in BG11<sub>0</sub> medium with various concentrations of 2,4-D. The true photosynthesis ( $P_t$ ) (open squares) was calculated by the summation of both net photosynthesis and respiration. The photosynthesis/respiration ratio ( $P_t/R$ ) (closed triangles) were calculated using the mean values of true photosynthesis and respiration at each 2,4-D concentration in the culture medium. The effect of 2,4-D on PSI activity of *Nostoc* (Panel C) was investigated on 7 day old culture of the cyanobacterium grown in BG11<sub>0</sub> medium with various concentrations of 2,4-D. Experiments were repeated three times and mean values and standard errors are shown.

## ***Discussion***

Induction and enrichment of heterocysts might be favorable for hydrogen production not only because of their internal nitrogenase/uptake system but might rather due to their role in enhancing oxygen consumption and creating anaerobiosis. Earlier, **Tiwari *et al.* (1981)** reported the inductive effect of 2,4-D on heterocyst formation but apart from hydrogenases activity. Here we combined both; heterocyst induction with hydrogenases activity. Furthermore, we are reporting the unusual heterocyst formation in the presence of nitrate, a regular inhibitor of heterocyst formation. The formation of two adjacent heterocysts was rarely observed in 2,4-D treated *Nostoc* sp. SAG 2306 but never in untreated cultures. The decrease in chl *a*/dry mass by 2,4-D might be attributed to the increase in heterocysts frequency (heterocysts have lower chl *a* content but higher dry mass than vegetative cells). 2,4-D recovered the inhibitory effect of up to 90  $\mu\text{M}$  nitrate nitrogen on heterocysts formation. These results suggests that 2,4-D can be used as an artificial inducer/signal for heterocysts formation in *Nostoc* sp SAG 2306 even in the presence of combined nitrogen. The stimulatory effect of 2,4-D on heterocyst formation could not be recorded in two extreme cases: zero and  $\geq 120$   $\mu\text{M}$  nitrogen. The first because there was no inhibitory effect to overcome (heterocysts already existed) and the latter because the concentration of 120  $\mu\text{M}$  nitrogen was too high to overcome its inhibitory action. Despite its inductive role on heterocyst formation, the 2,4-D-induced heterocysts seem fragile and detach from the filament, losing nutrient exchange with vegetative cells and hence function. This phenomenon was also observed in BG11<sub>0</sub> grown *Nostoc* cells. Such detachment of heterocysts is mostly attributed to aging when the culture reaches a stationary phase of growth and cell division possibly reducing the culture requirement for nitrogen. In this respect, **Ning and Xu (2004)**, **Ow *et al.* (2008)** and **Cardina *et al.* (2007)** reported detailed studies concerning heterocyst-vegetative cells transitions and interlinks. Maintaining heterocysts attached would be a target of a future study. The increase in growth as a dry mass in 2,4-D-treated cultures of *Nostoc* sp might be attributed to the increase in heterocysts frequency with their relatively higher dry mass than vegetative cells.

2,4-D has a stimulating role in vegetative cell transformation into hetrocysts as revealed by the increase in heterocysts frequency.

Reducing oxygen evolution is a prerequisite for increasing the hydrogen productivity because the cyanobacterial hydrogen evolving enzyme systems are highly sensitive to minute concentrations of oxygen. The decrease in photosynthesis/respiration ratio with increasing the 2,4-D concentration reduces the cellular oxygen partial pressure. Photosynthetic control of hydrogen metabolism revealed a strong correlation between  $P_N$  and hydrogen metabolism (**Abdel-Basset and Bader, 1997; 1998; 1999; 2008; Abdel-Basset *et al.*, 1998**).

The increase in PSI activity in *Nostoc* sp SAG 2306 treated with 2,4-D possibly leads to an increase in reductant supply for hydrogenases. Since phycobilisomes are the light harvesting complex of PS II in cyanobacteria (**Katoh and Gantt, 1979; Himadri and Louis, 1984; Kura-Hotta *et al.*, 1986; Bald *et al.*, 1996**), the decrease in photosynthesis of *Nostoc* sp. treated with 2,4-D might be attributed to the decrease in phycocyanin/chl *a* ratio indicating a decrease in phycobilisomes content and subsequently a decrease in oxygen evolution occurred. Such decrease in photosynthetic oxygen evolution and phycocyanin/chl *a* ratio was associated with an increase in hydrogen evolution activity in *Nostoc* treated with 2,4-D. The investigation of reducing the phycobilisomes content on oxygen and hydrogen evolution in a mutant model cyanobactium with low phycobilisomes content would be of interest in future studies of hydrogen production.

The cyanobacterium under investigation showed a conspicuous activity of both uptake and bidirectional hydrogenases with low  $K_m$  values. The two enzymes showed different characteristics in optimum pH, temperature and response to the herbicide and PSII blocker DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) and the photosynthetic uncoupler CCCP (carbonyl cyanide *m*-chlorophenylhydrazone). The optimum temperature for hydrogen evolution in *Nostoc* was between 25-35 °C. In general the optimum temperature for hydrogen



production for most cyanobacterial species is between 30–40 °C and varies from species to species of cyanobacteria (**Dutta et al., 2005**). For example, *Nostoc* cultured at 22 °C showed higher rates of hydrogen production than at 32 °C (**Ernst et al., 1979**), while *Nostoc muscorum* SPU004 showed optimum hydrogen production at 40 °C (**Datta et al., 2000**). *Anabaena variabilis* SPU 003 on the other hand show optimum hydrogen production at 30 °C (**Moezelaar and Stal, 1994; Serebryakova et al., 2000**).

Although the enrichment of heterocysts would be one of the routes for increasing the hydrogen productivity in heterocysteous cyanobacteria, a serious obstacle remains to overcome in future studies is the detachment of heterocysts from vegetative cells to maintain energy and nutrient exchange between both cells as a gate of hydrogen production.

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إثراء عدد الحويصلات المغايره في عزلة جديدة من النوستوك *Nostoc sp* بواسطة 2,4 - ثنائي كلورو فينوكسى حامض الخليك وأثرها اللاحق على تغيير نسبة التمثيل الضوئي / التنفس وأنشطة انزيمات الهيدروجينيز.

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الحويصلات المغايره هي خلايا متخصصه لتثبيت النيتروجين وتوجد في بعض انواع البكتيريا الخضراء المزرقه الخيطية ولها القدره التركيبية والفيسيولوجية على تخليق بيئه لاهوائية بداخلها. تشير النتائج في هذه الدراسه الي امكان استخدام مبيد الأعشاب الأوكسينى (2,4 - ثنائي كلورو فينوكسى حامض الخليك) لخفض نسبه الاكسجين في مزارع النوستوك (*Nostoc sp* SAG 2306) بهدف زياد نشاط انزيمات الهيدروجينيزويحقق ذلك عن طريق إثراء عدد الحويصلات المغايره. و قد ادت معامله البكتيريا الخضراء المزرقه الخيطية *Nostoc sp* SAG 2306 و المنماه في وسط غذائي خالي من مركبات النيتروجين بتركيزات مختلفه من 2,4 - ثنائي كلورو فينوكسى حامض الخليك الي زياده عدد الحويصلات المغايره الي 19%. علاوة على ذلك فقد تسبب 2,4 - ثنائي كلورو فينوكسى حامض الخليك في استحداث تكوين الحويصلات المغايره في وجود النيتروجين في صورة نترات بالوسط الغذائي بتركيزات تصل إلى 90 ميكرومولر. وقد حدثت زياده في عمليه التنفس ونقص في نسبه التمثيل الضوئي / التنفس في مزارع النوستوك المعامله بماده 2,4 - ثنائي كلورو فينوكسى حامض الخليك وقد صاحب ذلك زياد في أنشطة انزيمات الهيدروجينيز. وقد تسببت ماده 2,4 - ثنائي كلورو فينوكسى حامض الخليك في نقص نسبة صبغ الفيكوسيانين / صبغ الكلوروفيل (أ) وكان ذلك مصحوبا بانخفاض التمثيل الضوئي مما يوحي بان الانخفاض في صبغ الفيكوسيانين والذي يشكل عنصرا رئيسيا من عناصر (phycobilisomes) المجمع الضوئي في النظام الضوئي الثاني لاوليات النواة الخضراء المزرقه نتيجة المعامله بماده 2,4 - ثنائي كلورو فينوكسى حامض الخليك له تأثير سلبي على انتاج الأوكسجين ولها أثر إيجابي على أنشطة انزيمات الهيدروجينيز.وإجمالا فان النقص في نسبة صبغ الفيكوسيانين / صبغ الكلوروفيل(أ) و النقص في انتاج الاكسجين (التمثيل الضوئي) والنقص في نسبه التمثيل الضوئي / التنفس وكذلك الزيادة في معدل التنفس والزياده في عدد الحويصلات المغايره في مزارع النوستوك المعامله بماده 2,4 - ثنائي كلورو فينوكسى حامض الخليك قد ساهم كل ذلك في تكوين الظروف الاهوائية الملائمه لأبيض الهيدروجين.