Effects of nitrogen limitation in cyanobacterium, *Westiellopsis iyengarii* Jeejibai

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Nitrogen limitation in cyanobacterium, *W. iyengarii* induced by flushing the cultures with a mixture of Ar-C0₂ (99:1 v/v) resulted in drastic reduction in chlorophyll, carotenoid and phycoerythrin pigments within 48 hr and this was accompanied by significant loss of photosynthetic activity. The reduction in pigment levels was in step with the break-down of cellular proteins (storage) including phycobiliproteins. However, heterocyst differentiation in Ar-flushed cultures was stimulated with a simultaneous increase in acetylene reduction activity (ARA). When chloramphenicol (CAM) was added to the Ar-flushed cultures, ARA was completely abolished, indicating a rapid turn-over of nitrogenase proteins. It is suggested that nitrogenase synthesis in newly developed heterocysts was necessary to maintain the level of ARA in cultures.

Cyanobacteria, a unique group of gram-negative bacteria, evolved 2×10⁹ years ago, are atypical among the prokaryotes due to the possession of specific cells called ‘heterocysts’ for nitrogen fixation. Biological nitrogen fixation carried out in heterocyst is catalysed by ‘nitrogenase’ which is extremely sensitive to oxygen. A thick three-layered glycolipid wall around heterocyst and the absence of oxygen-evolving light-harvesting photosystem-II pigments, viz. phycobiliproteins (PBPs) afford a reducing environment necessary for nitrogenase activity. The wall of heterocyst is in fact, differentially permeable to both oxygen and nitrogen, making an ideal micro-aerophilic condition inside.

Nitrogenase has a larger MoFe protein with substrate and binding sites and, a smaller Fe-protein, responsible for reduction of substrate. For the whole process of dinitrogen reduction to take place in heterocysts, cyanobacteria absolutely require Mo and Fe - the basic components of nitrogenase, substrate-dinitrogen, ATP, reducing equivalents and the co-factor, Mg²⁺. If any of the above requirements essential for nitrogenase are not available, growth and nitrogen fixation by heterocystous cyanobacteria would be impaired, leading to nitrogen limitation of the cultures. The response of cyanobacteria is quick in terms of loss of photosynthetic pigments, complete elimination of 33 and 75 kDa polypeptides associated with photosynthetic membranes, lowered photosynthetic rate, diminished protein content of cells, accumulation of glycogen, decrease in the pelletable Rubisco protein, and low level of cytochrome c granular protein. In an earlier study, it was reported that cyanobacteria could sustain nitrogenase activity even if Mo was eliminated from the growing medium. The absolute requirements of Mo was not necessary for these forms at least for sometime due to their capacity to accumulate Mo inside cells. In the present study, another important requirement for nitrogenase-substrate-N₂ is eliminated from the atmosphere by flushing cultures of *Westiellopsis iyengarii* with Ar-CO₂ (99:1 v/v). The response of cyanobacterium is reported here.

An axenic culture of *Westiellopsis iyengarii* Jeejibai isolated and maintained at Centre for Advanced Study in Botany, University of Madras was grown in N-free Allen and Arnon’s medium. The cultures were grown at room temperature of 35±2°C and illuminated continuously with warm white fluorescent lamps (5300 K, Philips 40 W) at an intensity of 75 W/m². The cultures were bubbled with sterile humidified air and log-phase cultures were used for all experimental studies. Nitrogen in the growing atmosphere of flasks was removed by flushing the cultures with a special gas mixture of Ar-CO₂ (99:1 v/v) obtained from Indian Oxygen Ltd., Tondiarpet, Chennai. The flow rate of gas mixture was controlled through a flow-meter maintained at 30 ml/min. This flow rate was optimized earlier for nitrogen exclusion studies. Aliquots of cultures were withdrawn at regular intervals of 6, 12, 24, 48, 72 and 96 hr and analysed for various parameters. Chlorophyll content of the cultures was determined by a modified procedure of Arnon. Carotenoids were
determined using an absorption coefficient \( \alpha =200 \) at 460 nm. After the extraction of chlorophyll, the pellet was suspended in 0.05 M phosphate buffer, pH 8 and sonicated to release phycobilin pigments. Phycocyanin (PC) was determined by a spectrophotometric procedure given earlier. Photosynthetic rate of cultures was measured as oxygen evolution in a Clark type oxygen electrode and calculated using Rawson's nomogram. Whole cell nitrogenase activity was measured by acetylene reduction assay. Heterocysts were counted with light microscope and frequency expressed as percentage of vegetative cells. The total cellular protein was determined by Lowry's method. Chloramphenicol (CAM) was filter-sterilized and added at 100 \( \mu \)g/ml to the cultures, when needed. Statistical analysis had been done and the \( p \) values were ascertained to indicate the significance of results.

Flushing the cultures of \( W. iyengarri \) with a mixture of Ar-CO\(_2\) to eliminate \( N_2 \), enhanced acetylene reduction activity (ARA) within 6 hr. The time-course study (Fig. 1) of ARA revealed that it continued to rise up to 48 hr and decreased thereafter. At 96 hr, maximum reduction in ARA was noted in Ar-flushed cultures. In the air-grown cultures (control), ARA registered a gradual increase throughout the experimental period. When compared, Ar-flushed cultures showed 24% increase in ARA over air-grown at 48 hr. It is known that nitrogenase activity is directly proportional to the number of heterocysts under normal conditions. Since nitrogenase activity measured as ARA was higher during 48 hr of Ar-flushing and diminished later, it was necessary to see if the initial enhancement was due to an increase in heterocysts only due to maximal activity of the preformed heterocysts. Therefore, heterocyst counts were made in the cultures of \( W. iyengarri \) flushed with Ar-CO\(_2\) and air.

There was an alteration in the number of heterocysts during Ar-flushing (Fig. 2). A 40% increase in its frequency was observed at 48 hr. After this period, the heterocyst frequency gradually decreased and was only 63% of initial at 96 hr. Increased production of heterocysts and subsequent elevation in nitrogenase activity in the absence of nitrogen are in full agreement with earlier reports. The recording of low heterocyst frequency beyond 48 hr must be due to bleaching of cultures, associated with lysis of vegetative cells and heterocysts. The layers of heterocyst wall got separated and sometimes the wall was broken. Only those heterocysts, which did not show these degradative changes, were counted and hence this low value.

Even though, nitrogenase activity measured as ARA was high, elimination of substrate \( N_2 \), halted the process of nitrogen fixation itself. This causes a dearth of nitrogen in cell forcing it to undergo certain degradative changes. In Fig. 3, a comparative account on the pigment content, photosynthetic rate and cellular level of protein is presented. At 48 hr, when nitrogenase activity was high, the absence of nitrogen fixation per se caused 80% loss of chlorophyll. Along with it, the level of carotenoid was also lowered upon Ar-flushing and the cultures had only 7.6 \( \mu \)g carotenoid/ml culture. PC, a major PBP in \( W. iyengarri \) was drastically reduced once, \( N_2 \) was eliminated from the culture vessel. As shown in Fig. 3, only 45% of PC could be retained by Ar-flushed cultures at 48 hr. It is known earlier that the first sign of nitrogen starvation in many species like \( Anacystis nidulans \), \( Aphanocapsa 6308 \) (ref. 39), \( Synechococcus 6301 \) and \( Tolypothrix tenuis \) is the degradation of photosynthetic pigments—chlorophyll, carotenoid and PBPs. Breakdown of the light harvesting pigments hampered photosynthetic process as was evident from the sharp decline of
photosynthetic oxygen evolution in Ar-flushed cultures of _W. iyengarii_ (Fig. 3).

Under conditions of nitrogen limitation, specific proteases have been known to be induced in cyanobacteria\(^8\) that degrade PBPs. The low PC content in _W. iyengarii_ during Ar-flushing suggests that specific proteases might have been triggered. The breakdown of PC could be stopped once these proteases are prevented from being synthesized or acting during nitrogen limitation. In order to check this possibility, CAM was added to Ar-flushed cultures from the initial stage itself. Once CAM was added, ARA was completely eliminated in Ar-flushed cultures (Fig. 1). The presence of CAM also maintained the level of pigments in Ar-flushed cultures, comparable to those of control (Fig. 3). However, CAM-addition did not protect nitrogenase from being degraded in _W. iyengarii_ as its activity was lost within 24 hr (Fig. 1), in comparison to Ar-flushed cultures that did not receive CAM. It means that the turn-over of nitrogenase was rapid especially, in the absence of its substrate (N\(_2\)) and CAM arrested the synthesis of new nitrogenase.\(^\)\(^9\) It is inferred that nitrogen limitation induced by Ar-flushing could stimulate the synthesis of nitrogenase and heterocyst differentiation, perhaps, at the expense of other cellular protein reserves, notably PC.\(^10\) This view could well be supported by the overall breakdown of total cellular protein (Fig. 3) in Ar-flushed cultures and its maintenance in CAM-added cultures.

It is to be noted that nitrogen limitation brought about drastic changes in vegetative cells so that most of them could potentially transform into heterocysts.

The drastic changes include, gradual loss of PC and lowered photosynthetic oxygen evolution—notable features observed during heterocyst differentiation.\(^6\)\(^11\) But, here the cultures of _W. iyengarii_ could not withstand nitrogen limitation, as the process of nitrogen fixation itself was impaired in the absence of substrate.

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References

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