435k Heterocyst Differentiation and H_2 Production in N_2-Fixing Cyanobacteria

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The worldwide concern about the exhaustion of fossil fuels and the harmful effects of their emissions to the environment is guiding efforts to find new energy alternatives. One of the most promising energy resources is hydrogen (H_2). Cyanobacteria are among the most important candidates for photobiological hydrogen production because of their potentially simple and economical cultivation using sunlight as the only energy source, water as the feedstock (source of electrons and reductant) and air as the source of carbon dioxide (CO_2) and nitrogen (N_2). In N_2-fixing cyanobacteria, nitrogenase is the enzyme complex that produces H_2 as a byproduct of nitrogen fixation. However, nitrogenase is sensitive to oxygen. To overcome this obstacle, some filamentous cyanobacteria have evolved to differentiate specialized cells called heterocysts, which possess a protecting cell envelope to limit O_2 penetration and an inactive photosystem II to prevent O_2 evolution. The differentiation allows nitrogen fixation and H_2 production to take place in the microaerobic environment inside the heterocysts.

Heterocysts obtain carbohydrates from the surrounding vegetative cells, and export fixed nitrogen in return. The activation of nitrogen fixation mechanism is determined by the nitrogen condition of the filament. Heterocyst differentiation occurs at the location that ensures the most efficient production and distribution of fixed nitrogen to the surrounding vegetative cells. When the number of vegetative cells is increased by cell division, the space between heterocysts becomes larger. The process leads to the differentiation of another heterocyst near the center of the newly created chain, to conserve the regular spacing and frequency. Since H_2 is produced only in the heterocysts, understanding heterocyst differentiation is crucial to the improvement of photobiological H_2 production. Although, there have been many genetic studies on heterocyst differentiation, there is still a need for quantitative, system-level description and modeling.

In this study, the heterocystous cyanobacteria *Anabaena flos aquae* and *Anabaena sp*. PCC 7120 were cultivated with different nitrogen concentrations in the fresh media and with continuous illumination of white light at different light intensities (2, 5, 10, 20 and 50 $\mu E/m^2$ s). Periodic samples were taken for determining the vegetative cells concentrations (V, g/L) and heterocyst concentrations (H, g/L). The results were used to develop the model that accounted for heterocyst differentiation under solely nitrogen fixation conditions and also under conditions with external nitrogen sources. In the model, the changes of the (dry-weight) concentrations of vegetative cells and heterocysts are described by the following equations:

$$\frac{dV}{dt} = \mu V - (r_{V\to H})V - k_d^V V \qquad (1)$$

$$\frac{dH}{dt} = (r_{V\to H})V - k_d^H H \qquad (2)$$

In these equations only the vegetative cells are capable of growth via fission, with the specific growth rate of μ (h^{-1}). The increase in H is possible only via the transformation of vegetative cells to heterocysts, with the specific V-to-H transformation rate denoted as $r_{V\to H}$ (h^{-1}). Both vegetative cells and heterocysts are assumed to have constant specific decay rates (h^{-1}), i.e., k_d^V and k_d^H, respectively. The specific growth rate (μ) is modeled to have contributions from both the growth with external N sources (NH_3-N, NO_3^--N, and the N released from decaying cells) and the growth with fixed N, i.e.,

$$\mu = \mu_{max}\left(\frac{N}{K_N + N}\right)\left(\frac{I}{K_I + I}\right) + (r_{NF})(Y_{X/N})\left(\frac{H}{V + H}\right) \qquad (3)$$

The external N-dependent growth assumes the common Monod-type dependency on both N concentration and the available light intensity (I, lux), with μ_{max} (h^{-1}) referring to the maximum specific growth rate and K_N (g/L) and K_I (lux) to the Monod constants for N and I, respectively. The specific rate of fixed N-dependent growth is postulated to be proportional to the specific rate of N_2 fixation (r_{NF}, g N/g H-h), with the proportionality governed stoichiometrically by the cell yield ($Y_{X/N}$, g cells/g N) and the heterocyst fraction in the culture.

The N_2 fixation rate by cyanobacteria has been reported to have both light-dependent and light-independent components. Accordingly, the specific rate of N_2 fixation in the heterocysts (r_{NF}, g N/g H-h) is modeled by the following equation:

$$r_{NF} = \left[r_{NF}^{m}\left(\frac{I}{K_I + I}\right) + r_{NF}^{c}\right] e^{-\left(N/K_{NF}^{i}\right)} \qquad (4)$$

where r_{NF}^{m} is the "maximum" rate of the light-dependent portion of N_2 fixation, and r_{NF}^{c} is the "constitutive" rate of the light-independent portion of N_2 fixation. The inhibition by external N-sources is described by a general, non-specific, exponential function, similar to those for some other inhibitory phenomena in biological systems involving just one modeling parameter, i.e., the inhibition constant of external N to N_2 fixation, K_{NF}^{i} (g/L).

One of the most important stages in the development of this model was the description of the transformation of vegetative cells to heterocysts:

$$r_{V \to H} = \left[(k_{V \to H})\mu\left(\frac{\mu}{Y_{X/N}} - \frac{H}{V} r_{NF}\right) + \mu\left(\frac{H}{V}\right)\right] e^{-\left(N/K_{H}^{i}\right)} \qquad (5)$$

It was assumed that the culture-level driving force for accelerated heterocyst differentiation is the difference between (1) the required specific N-generation rate for supporting the growth under the present light intensity, i.e., $\mu/Y_{X/N}$, and (2) the actual specific (per unit V) N-generation rate by the existent heterocysts (via N_2 fixation), i.e., $r_{NF} \cdot H/V$. As described in eq. (3), the external N sources and the fixed N are treated separately in this model. Therefore, only the vegetative growth on external N and the release of N from dead cells are considered in the mass balance equation for external N sources:

$$\frac{dN}{dt} = -\frac{1}{Y_{X/N}}\left\{\left[\mu_{max}\left(\frac{N}{K_N + N}\right)\left(\frac{I}{K_I + I}\right) - k_d^{V}\right] V - k_d^{H} H\right\} \qquad (6)$$

The common Monod-type dependency on light intensity has been assumed for both cell growth and N_2 fixation. The well-known self-shading effect, which reduces the light penetration as the cell concentration ($X = V + H$) increases, is described in the following equation:

$$I = I_0 e^{-K_X(V+H)} \quad (7)$$

I_0 (lux) is the incident light intensity measured at the surface of cultivation vessel, and K_X is the empirical self-shading constant.

The model developed in this work describes well both the experimental results of this study and those reported in the literature. The insights gained on the basic mechanisms of heterocyst differentiation show that the driving force is acting as the trigger for heterocyst differentiation. When the driving force is zero, the culture grows with balanced N generation and consumption tending to maintain the same H/V ratio. With a positive driving force (i.e., more N deficit), the culture raises the H/V ratio by increasing the heterocyst differentiation rate. This increase is proportional to the specific growth rate in order for the H/V ratio to actually increase, without the increasing H being countered by the increasing V due to vegetative growth.

The on-going second stage of the study is focused on the investigation of the effects of red vs. white light intensities on heterocyst differentiation and H_2 production kinetics. (Note that cyanobacteria absorb preferentially red light near 680 nm). For this purpose, red LED panels were constructed to provide red light to the culture systems.

The results indicated a clear tendency of cyanobacteria to have much higher heterocyst contents when growing under red light alone. The effects of this higher heterocyst content on the H_2 production capability (measured by nitrogenase activity) of cyanobacteria are being examined closely. These results will be used to expand the current model to include the description for the H_2 production kinetics in cyanobacteria.

Finally, and as a third stage of the study, the fact that overexpression of the *hetR* gene leads to increased heterocyst frequency in *Anabaena sp*. PCC 7120 was taken under consideration to create a recombinant strain with variable and controllable heterocyst frequency. Towards this end, the copper responsive *petE* promoter and the coding region of *hetR* gene (coding for the HetR protein involved in heterocyst frequency regulation) were isolated from *Anabaena sp*. PCC7120 using gene specific primers. A recombinant construct was created where in the coding region of the *hetR* gene was fused the isolated *petE* promoter. The fusion construct was cloned onto a puc19 based bacterial expression vector. Separate plasmid constructs carrying either the *petE* promoter or the *hetR* coding region were constructed and used as controls for the study. *Anabaena sp*. PCC7120 is currently being transformed and screened for the strain that would provide a handle to study the effect of controllable heterocyst frequency on the condition of the cells and on H_2 production rates. The outcome of this study will supply the necessary information to complete the quantitative model that will provide the optimal conditions for photobiological production of H_2 via N_2-fixing cyanobacteria.