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Optimization of Conditions for Photoproduction of Ammonia from Nitrate by *Anacystis nidulans*

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The effect of several relevant environmental factors influencing the photoproduction of ammonia from nitrate by *Anacystis nidulans* cells treated with the glutamine synthetase inhibitor L-methionine-DL-sulfoximine has been investigated. The optimal ratio between L-methionine-DL-sulfoximine concentration (micromolar) and cell density (micrograms of chlorophyll per milliliter) was around 1, the process taking place at maximal rate at a temperature of about 40°C, within the pH range of 7 to 10. Ammonia production was stimulated by CO_2 or bicarbonate and was not affected by the accumulation of ammonia in the medium up to concentrations of 30 mM. The rate of ammonia production was found to be determined by the interaction of at least four factors, namely, irradiance and the density, depth, and turbulence of the cell suspension. Ammonia photoproduction from nitrate and water represents an interesting process for the conversion of light energy into chemical energy, which can operate at high efficiency, around 30% of its theoretical maximum.

The photoproduction of ammonia from nitrate and water by photosynthetic organisms is a light-driven process, which represents a net and significant conversion of sunlight energy into stored chemical energy, and takes place according to the following equation:

$$HNO_3 + 4H_2O \xrightarrow[8e]{16\,h\nu} NH_3 + 2O_2 + 3H_2O$$

$$(\Delta E_0' \text{ [pH 7]} = -0.47 \text{ V}; \Delta G_0' \text{ [pH 7]} = 360 \text{ kJ/mol})$$

where $\Delta E_0'$ is the standard redox potential change, and $\Delta G_0'$ is the standard free energy change.

The photosynthetic reduction of nitrate to ammonia occurs in two steps: first, nitrate is reduced to nitrite in a two-electron reaction catalyzed by the molybdoprotein nitrate reductase, and then nitrite is reduced to ammonia in a six-electron reaction catalyzed by the iron protein nitrite reductase (8, 13, 14, 24). The process of nitrate reduction, as it takes place in the blue-green algae (cyanobacteria), with reduced ferredoxin acting as the immediate electron donor for both partial reactions, is one of the simplest examples of photosynthesis (4).

The steady photoproduction of ammonia from nitrate by whole cells of the cyanobacterium *Anacystis nidulans* has been achieved by inhibiting glutamine synthetase, the first enzyme of the main ammonia assimilation pathway in this strain, with the glutamate analog L-methionine-DL-sulfoximine (MSX) (17).

This paper deals with the optimization of ammonia photoproduction from nitrate by MSX-treated *Anacystis* cells with regard to various environmental factors and shows that this simple photosynthetic process can take place at high efficiency, around 30% of its theoretical maximum.

MATERIALS AND METHODS

Growth of cells. *A. nidulans* 1402-1 was grown photoautotrophically, under continuous lighting (Sylvania daylight fluorescent tubes, giving an irradiance value of 25 W/m^2 at the surface of the culture vessels), at 40°C on a synthetic medium containing nitrate as the nitrogen source (10) in a stream of 2% CO_2 in air (vol/vol), which was bubbled through the culture at a flow rate of 1 liter/liter of cell suspension per h.

Ammonia production experiments. Cells from 2-day old cultures containing 20 to 25 μg of chlorophyll *a* (Chl) per ml were used. The cells were harvested by centrifugation, washed with culture medium, and finally suspended in the same medium to a density of 8 to 10 μg of Chl per ml. Enough MSX was then added to the cell suspension to achieve an MSX concentration (micromolar) to cell loading (micrograms of Chl per milliliter) ratio of 1, except where indicated. Unless otherwise indicated, the values of ammonia production rate reported herein remained constant throughout the corresponding experiments.

Trapping of ammonia. The pH of the medium determined the proportion of each form of the acid-base

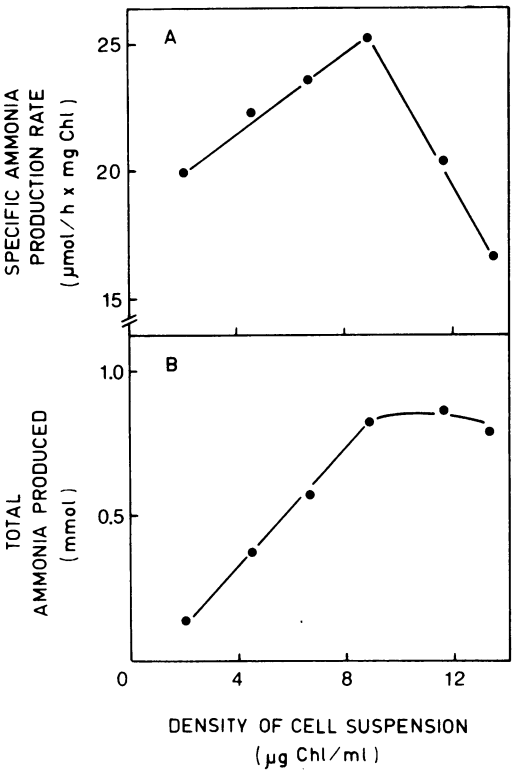

FIG. 1. Effect of the density of the cell suspension on ammonia photoproduction by *A. nidulans*. Cell suspensions (150 ml) at the indicated cell loading were supplemented with MSX. The MSX concentration (micromolar) to cell loading (micrograms of Chl per milliliter) ratio was 1 in all cases. After 24 h under culture conditions, ammonia in the medium was determined. (A) Specific ammonia production rates. (B) Total ammonia production.

couple NH_4^+-NH_3 (pK_a 9.3). Since ammonia is a very hydrosoluble gas, it distributed itself between the liquid phase and the gas phase and could be easily removed at high pH from the medium in which it was formed, to be recovered as ammonium ion in an acid solution. Some experiments were carried out in vessels open to air. The pH of the corresponding cell suspensions was about 7, a condition under which less than 5% of the total ammonia produced was lost. In those experiments in which the pH was higher than 7, the vessels used for ammonia production were sealed hermetically with stoppers pierced across by two glass tubes, one of which reached the bottom of the vessel, allowing the gas mixture to bubble through the cell suspension. The second, shorter glass tube, with its lower end remaining above the suspension, delivered the gas from the vessel atmosphere to two containers linked in series; the first vessel was empty for the condensation of water, and the second one contained 10 ml of 1 N H_2SO_4 for fixation of the ammonia gas as ammonium ion.

Assays of enzyme activities. Nitrate reductase and glutamine synthetase (transferase) activities were determined in toluenized cells as described previously (17).

Analytical methods. Ammonia was determined as described by Solorzano (23). Nitrite was estimated as described by Snell and Snell (22). γ-Glutamyl hydroxamate was estimated after its reaction with $FeCl_3$ in acid medium (21). The packed cell volume was estimated in calibrated hematocrit tubes; cellular protein was estimated by a modification of the Lowry procedure (2) after pretreating the cells with 10% (wt/vol) trichloroacetic acid. Chl was determined spectrophotometrically in methanolic extracts with the extinction coefficient given by MacKinney (15). The Chl and protein contents of MSX-treated *A. nidulans* cells were fairly constant, with values of 5.8 to 6.2 μg of Chl and 110 to 130 μg of protein per μl of packed cell volume (17). Irradiance measurements were made on the surface of the containers in the absence of cells by using an ISY-Kettering model 65A radiometer.

RESULTS

Effect of MSX concentration. Treatment of cells of the cyanobacterium *A. nidulans* with the glutamine synthetase inactivator MSX inhibited ammonia assimilation and resulted in the excretion of ammonia reduced from nitrate by the cells and its accumulation in the outer medium (5, 17). From this experimental study, it was established that the optimal ratio between MSX concentration (micromolar) and density of the cell suspension (micrograms of Chl per milliliter) was about 1. Starting with this relation, the maximal rates of ammonia production were maintained for 30 to 35 h. The MSX effect ceased thereafter, and, as a consequence, not only did ammonia production also cease, but ammonia previously accumulated in the medium was taken up by the cells (18). Lower MSX concentration/cell density ratios also allowed maximal rates of ammonia production, but the production period was then shorter; e.g., for a 0.3 ratio, the process went on for about 20 h. Higher ratios resulted in both decreased rates and shorter periods of ammonia production. Preliminary evidence indicated that high ratios presented toxic effects which led first to a loss of nitrate reductase activity and later to cell lysis (data not shown).

Effect of the density of the cell suspension. Algal cell loading was first optimized under culture conditions. Figure 1 shows specific rates and total ammonia production as a function of the density of the cell suspension. Around 9 μg of Chl per ml was the best loading for both maximal specific rates (Fig. 1A) and maximal total production (Fig. 1B). Density values lower or higher than 9 μg of Chl per ml resulted in a decrease of the specific rate of ammonia production. The diminished rates recorded at lower densities might well be a consequence of overexposure of the cells to light, whereas those observed at higher culture density values might be

caused by self-shading of the cells. Total ammonia production increased practically in proportion with density of the cell suspension up to 9 μg of Chl per ml, where it reached a plateau. Thus, although the efficiency of each single cell decreased (lower specific production rates) for density values higher than the optimal, this loss was compensated by the higher cell loading in such a manner that, under the chosen conditions, total ammonia production was of the same order of magnitude for culture density values between 9 and 14 μg of Chl per ml.

Effect of temperature. The effect of temperature on ammonia production by MSX-treated *Anacystis* cells was studied along a wide thermal range. The results obtained (Table 1) indicate that this algal strain can produce ammonia at maximal rates at temperatures around 40°C; lower and higher temperatures resulted in decreased rates.

Effect of pH. The influence of the pH of the medium on ammonia production by MSX-treated *Anacystis* cells was studied within the pH range between 7 and 10. As shown in Table 2, the production rate increased gradually with the pH. The results also show that the amount of ammonia passing to the gas phase increased with the pH, as expected from the pK_a value of this acid-base pair. Whereas at a final pH of 10.0, up to 25% of the total ammonia produced by the cells was recovered in the acid solution, only about 10% could be trapped at a final pH of 7.3. In all cases, the pH of the medium decreased about 0.5 U during the experiment.

Effect of ammonia concentration. In addition to its specific antagonistic effects on nitrate metabolism, which are overcome in *Anacystis* cells by treatment with MSX (5, 10, 17), high ammonia concentrations exerted other negative effects on cellular metabolism (11, 16) which resulted in a decrease in ammonia production. Such toxic effects became apparent when MSX-treated *Anacystis* cell suspensions containing 9 μg of Chl per ml were incubated in nitrate-containing medium with different concentrations of ammonia (0 to 60 mM NH_4Cl); the increase in the ammonia content of the medium was estimated after 20 h. The results obtained show that the ammonia production rate was unaffected by ammonia in the range from 0 to 30 mM. Nevertheless, higher concentrations resulted in a 50% decrease in ammonia production at 40 mM NH_4Cl and no ammonia production at 60 mM NH_4Cl (data not shown). That the inhibitory effect was due to ammonia was confirmed by experiments in which KCl was substituted for NH_4Cl; no apparent effect of the former salt on the ammonia production rate was observed.

Effect of CO_2 or bicarbonate. It has previously been reported (17) that in MSX-treated *Anacystis* cells with saturating CO_2, about two-thirds of total photosynthetic electron flow is driven to CO_2 fixation and about one-third to nitrate reduction (17). The results in Table 3 show the requirement of CO_2 or bicarbonate for optimal ammonia production. When common air was supplied to the cell suspension, the amount of ammonia produced was fivefold lower than that obtained with air supplemented with saturating CO_2 (2% [vol/vol] CO_2 in air at a flow rate of 1 liter/liter of cell suspension per h). Although bicarbonate (up to 50 mM) did not stimulate ammonia production when it was added to cell suspensions sparged with CO_2-enriched air, its stimulating effect became evident when it was added to cell suspensions sparged with unsupplemented air. It is worth mentioning that cells which had been adapted to low CO_2 tensions by being grown with plain air for at least 50 generations still exhibited a CO_2 requirement for

TABLE 1. Effect of temperature on ammonia photoproduction by MSX-treated *A. nidulans* cells[a]

Temp (°C)	Specific ammonia production rate (μmol/h per mg of Chl)
20	29
25	38
30	45
35	52
40	58
45	37
50	26

[a] A suspension of cells pretreated with 10 μM MSX for 4 h was used. Cells were harvested, washed, and suspended in MSX (10 μM)-containing fresh culture medium. Samples (15 ml each) of the cell suspension (10 μg of Chl per ml) were then placed in conical flasks (100-ml capacity) and preincubated in the dark at the indicated temperature for 15 min in a Warburg bath. The ammonia production assay was started by switching on the light (tungsten lamps, which supplied an irradiance of 100 W/m^2 on the flask bottom). The incubation time under illumination was 20 min.

TABLE 2. Effect of pH on ammonia photoproduction by MSX-treated *A. nidulans* cells[a]

pH		Total ammonia produced (mmol)	Ammonia fixed in acid (% of total)
Initial	Final		
8.0	7.3	0.94	9.8
8.5	8.0	0.98	14.7
9.5	9.0	1.04	18.6
10.5	10.0	1.09	24.7

[a] *A. nidulans* cell suspensions (150 ml) containing 8.8 μg of Chl per ml and buffered with 0.1 M $NaHCO_3$-Na_2CO_3 at the initial pH indicated were treated under culture conditions with 10 μM MSX. Final pH and total ammonia produced (ammonia in medium plus ammonia fixed in acid) were estimated after 26 h.

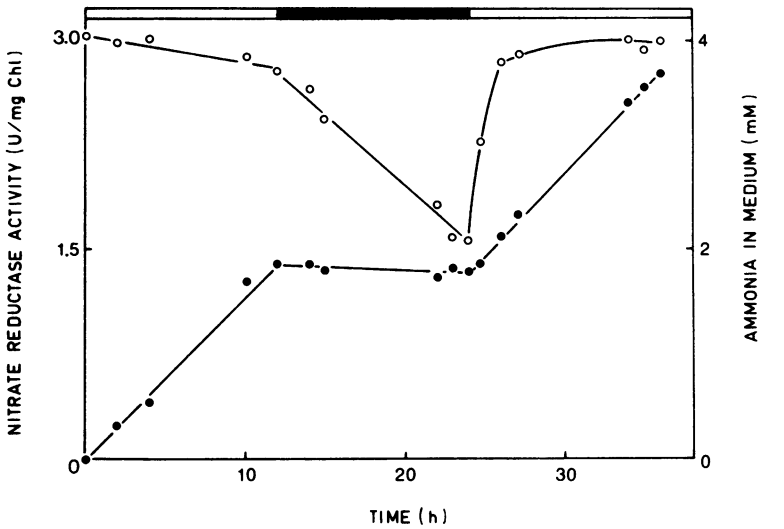

FIG. 2. Effect of light and darkness on ammonia production and nitrate reductase activity in *A. nidulans*. Cell suspensions (150 ml) containing 9 μg of Chl per ml were treated with 10 μM MSX and kept under normal culture conditions either in the light (□) or in the dark (■). Nitrate reductase activity in the cells (○) and ammonium in the medium (●) were estimated in samples withdrawn at the times indicated.

achieving maximal ammonia production. Actually, when these cells were supplemented with CO_2 or bicarbonate, the production rates were only about one-half of those of cells grown with CO_2-enriched air (data not shown).

Effect of light. Ammonia production by MSX-treated *Anacystis* cells is dependent on light (18; E. Flores, M. G. Guerrero, and M. Losada, submitted for publication). To mimic natural conditions, the cells were subjected to alternated cycles of 12 h in the light and 12 h in the dark. Figure 2 shows the results obtained. It can be seen that ammonia was produced linearly and at a high rate (25 to 30 μmol/mg of Chl per h) during the light period, but the production became negligible in darkness. Prolonged dark incubation also resulted in a progressive loss of the nitrate reductase activity level, which decreased to about 50% of its initial value after 12 h in darkness. It is worth noting that cellular Chl and protein levels remained constant throughout the experiment; they were not affected during the incubation periods in darkness. Illumination of the system after a dark period allowed restoration of the initial rate of ammonia production, as well as recuperation of the normal nitrate reductase activity level, in a process which seemed to be dependent on protein synthesis, since it did not take place in the presence of chloramphenicol or rifampin (data not shown).

The effect of the level of irradiance on the rate of ammonia production by MSX-treated *Anacystis* cell suspensions containing 10 μg of Chl per ml is shown in Fig. 3. The production rate increased linearly with irradiance up to about 60 W/m^2. Although direct proportionality was lost thereafter, the rate continued to increase until it reached a plateau in the illumination range between 150 and 300 W/m^2 and decreased slowly afterwards. The maximal rate of ammonia production at saturating light intensity (60 μmol/mg of Chl per h) was about threefold higher than that obtained at 30 W/m^2.

Effect of suspension depth. Figure 4 shows ammonia production by MSX-treated *Anacystis* cells as a function of the depth of the suspension

TABLE 3. Requirement of CO_2 or bicarbonate for effective ammonia production by MSX-treated *A. nidulans* cells[a]

Gas phase	$NaHCO_3$ added (mM)	Ammonia produced (mmol)
Air-CO_2	0	0.77
	50	0.77
Air	0	0.15
	30	0.33
	60	0.44
	120	0.51
	240	0.69

[a] *A. nidulans* cell suspensions (150 ml) containing 8.6 μg of Chl per ml were treated with 10 μM MSX and kept under culture conditions for 26 h, the medium being supplemented with bicarbonate at the indicated concentrations. The gas bubbled through the cell suspensions was either an air-CO_2 mixture (98:2 [vol/vol]) or just air, as indicated.

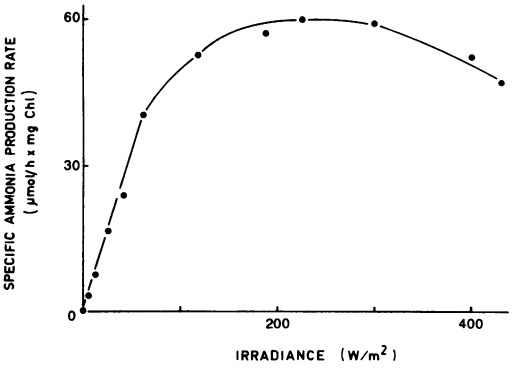

FIG. 3. Photoproduction of ammonia by *A. nidulans* at different irradiance levels. A cell suspension (180 ml) containing 10 μg of Chl per ml was treated with 10 μM MSX for 4 h under culture conditions. After this time, samples were withdrawn, and the cells were harvested, washed, and suspended at the same cell density in fresh culture medium supplemented with 10 μM MSX. The cell suspensions were then placed in Lucite cuvettes (1.5-cm lightpath) and kept for 30 min at 40°C under continuous bubbling with an air-CO_2 (98:2 [vol/vol]) mixture and illumination from a slide projector equipped with a 150-W tungsten lamp, placed at a distance from the cuvette to provide the indicated irradiance values at its surface.

with a cell loading of around 9 μg of Chl per ml and illumination of 70 W/m^2 (white light). Under such conditions, the specific ammonia production rate decreased linearly with the suspension depth, from a value of 30 μmol/mg of Chl per h for 1 cm to 11 μmol/mg of Chl per h for 10 cm (Fig. 4A). Nevertheless, total ammonia production increased hyperbolically with the volume of the suspension as a function of the container depth (Fig. 4B). When the suspension depth increased excessively, e.g., 20 cm, not only did the specific ammonia production rate become very small (2 μmol/mg of Chl per h), but total ammonia production was then of the same order as that registered for a suspension of 3 cm.

Effect of turbulence. The effect of sparging the cell suspension with a CO_2-air mixture was studied in 50-liter containers, 50 cm in depth, with the top surface (0.1 m^2) open to air and exposed to an illumination of 500 W/m^2 (white light). Table 4 shows that the ammonia production rate increased in response to turbulence from a value of 2 μmol/mg of Chl per h without any bubbling to a value of around 40 μmol/mg of Chl per h for a gas flow of 250 to 330 liters/h. Maximal ammonia production under these conditions was obtained with a cell loading of 3 μg of Chl per ml and amounted to about 160 mmol (2.7 g) in 24 h. It is worth noting that the positive effect of turbulence on ammonia production cannot be ascribed only to an increased supply of CO_2, since the latter was saturating in all cases.

DISCUSSION

Optimization of the ammonia photoproduction process by a suspension of MSX-treated *A. nidulans* cells has allowed us to establish that the yield of the process is affected by at least four factors, namely, irradiance and the density, depth, and turbulence of the cell suspension. The turbulence, density, and depth of the cell suspension alone could influence the observed variations in the production of ammonia, independent of the effect of light. Nevertheless, the common element determining the rate of ammonia production might be the amount of light available to each cell of the suspension, which in turn would be affected by each of the above factors. The results in this paper can be interpreted in terms of the latter assumption.

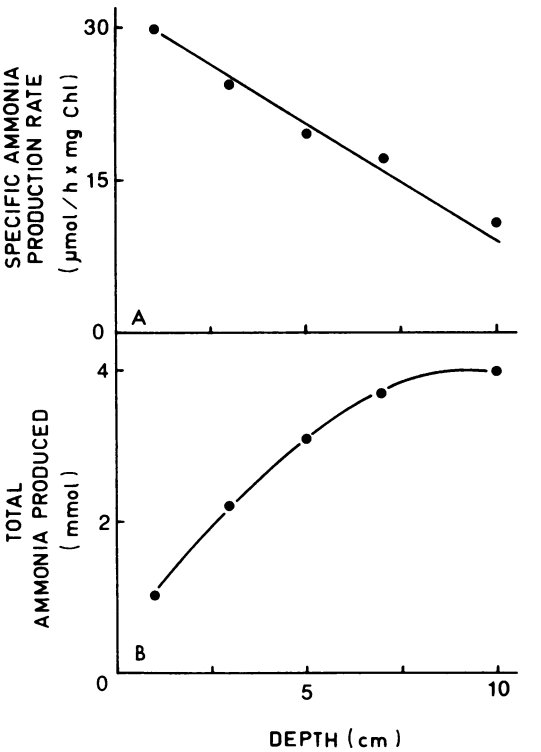

FIG. 4. Effect of cell suspension depth on ammonia photoproduction by *A. nidulans*. Cell suspensions containing 8.9 ± 0.2 μg of Chl per ml were treated with 10 μM MSX and placed in containers of the indicated depth, in which the surfaces (14 by 12.5 cm) were exposed to fluorescent light (70 W/m^2). Other conditions were the same as those for standard cultures. Ammonia produced was determined after 24 h. (A) Specific ammonia production rates. (B) Total ammonia production.

TABLE 4. Effect of turbulence on ammonia production by MSX-treated *A. nidulans* cells[a]

Gas flow (liters/h)	Ammonia production	
	Specific rate (μmol/h per mg of Chl)	Total amount (mmol)
0	1.9	9.2
86	18.0	68.2
180	34.6	138.1
268	41.5	155.8
333	42.1	158.2

[a] *A. nidulans* cell suspensions (50 liters) containing 3 ± 0.2 μg of Chl per ml were treated with 3 μM MSX and incubated for 24 h under the continuous lighting supplied by a bank with eight tungsten lamps (Osram; 150 W) placed 60 cm above the top surface of the container, at which point the irradiance value was 500 W/m^2.

In general, increases in either density or depth of the cell suspension over optimal values resulted in decreased specific rates of ammonia production; these negative effects could be a consequence of deficient illumination. In fact, the low specific production rates obtained for high values of either density or depth of the cell suspension did increase when the irradiance reaching the surface of the suspension was also increased (data not shown). On the other hand, increases in either irradiance or turbulence resulted, up to certain limits, in increases in specific rates of ammonia production; these positive effects might be a consequence of the appropriate exposure to light of the cells. The positive effect of turbulence can be explained in terms of lengthening the time of exposure to light of each individual cell in the suspension. Overexposure of cells to light may, however, result in lower specific rates of ammonia production, as a consequence of negative effects on general cellular metabolism, e.g., photooxidation (1, 6), pigment bleaching (1), and inhibition of nitrate uptake (P. Candau, personal communication). Similar behavior related to light in response to the above factors has also been described with regard to the growth of algae (20).

CO_2, usually supplied by bubbling the gas through the cell suspension, is a requirement for achieving maximal ammonia production rates, but it can also be replaced total or partially by the addition of $NaHCO_3$ to the medium, a circumstance that is particularly advantageous when large volumes are employed. Similar observations in this context have been reported (6) regarding the growth of *Anacystis*.

It is interesting to compare the maximal theoretical efficiency of ammonia photoproduction with the values experimentally obtained. A scheme of the successive energy losses involved in photosynthesis (3, 9), as applied to this particular process, is presented in Table 5. It should be noted first that, in general, only around 30% of the incident solar energy can be used in photosynthesis. Since reduction of 1 mol of nitrate to ammonia by water requires 16 Einsteins of photochemically active light with an energy content of 2,815 kJ (red light of 700 nm), it can be calculated (12, 13; see also the equation in the Introduction) that only 13% of the photochemically active energy remains stored as chemical energy in the reaction products. The maximal theoretical efficiency of the photosynthetic reduction of nitrate to ammonia is thus around 4%.

Considering a cell suspension system covering an area of 1 m^2 exposed to white light (500 W/m^2) for 24 h, the radiant energy which strikes its surface is 43,200 kJ/day. Since only 4% of the incident energy (1,728 kJ/day) can be used for ammonia synthesis from nitrate and the chemical energy stored in ammonia is 360 kJ/mol, it can be calculated that the maximal achievable production of ammonia is 4.8 mol/m^2 per day, i.e., 81.6 g/m^2 per day.

In the experiments described in Table 4, carried out with *Anacystis* cell suspensions in 50-liter containers with an area of 0.1 m^2 exposed to white light (500 W/m^2), a net production of 160 mmol of ammonia per day was obtained. From a simple extrapolation, the production of 1.6 mol (27.2 g)/m^2 per day can be calculated, which represents as much as 30% of the maximal expected yield. A more ambitious dual extrapolation in area and time would render a yield of about 50 ton/ha (5 kg/m^2) per year.

The moderately high optimal temperature and pH, as well as the absence of any added organic carbon source, represent restrictive conditions for the growth of other organisms which might interfere with the process. In fact, under such conditions and in the absence of special sterility measures, ammonia production remained at its maximal rates for days (18).

TABLE 5. Successive energy losses in photosynthetic ammonia production from nitrate

Cause of loss	Remaining energy (%)
Incident solar energy	100
47% loss due to unuseful photons outside the photosynthetically active region	53
30% loss due to incomplete absorption, reflection, and refraction	37
20% loss due to degradation of absorbed photons to excitation energy at 700 nm	30
87% loss due to conversion of excitation energy at 700 nm to chemical energy stored in ammonia by nitrate reduction with water	4

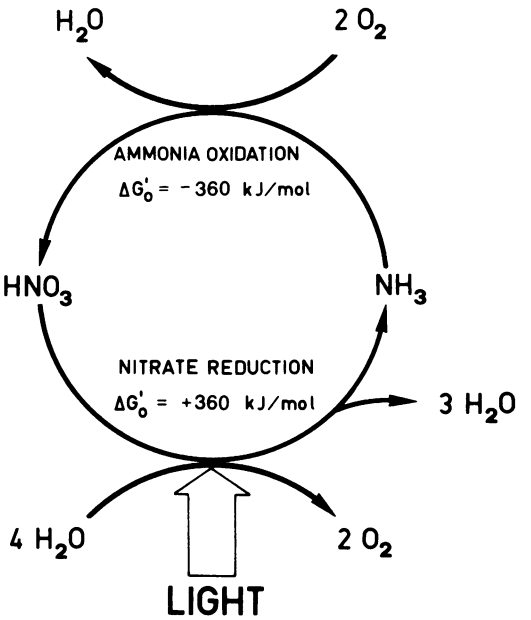

FIG. 5. Proposed cycle to harness sunlight energy through the synthesis of ammonia from nitrate. $\Delta G_0'$ at pH 7 is shown.

The above results indicate that MSX-treated *Anacystis* cells represent an original and valuable system for the bioconversion of solar energy into chemical energy. The cyanobacterial cells can thus be considered as microfactories that, under conditions of high efficiency, can synthesize ammonia from nitrate and water at environmental temperature and pressure. A similar system for the synthesis of ammonia from atmospheric nitrogen has also been developed in our laboratory with MSX-treated N_2-fixing cyanobacteria (7, 19).

As schematically represented in Fig. 5, the production of ammonia from nitrate and water by photosynthetic organisms constitutes an endergonic process driven by sunlight energy. The resulting ammonia can be oxidized back to nitrate in reactions that release the stored energy and regenerate the initial substrate. The convenient integration of both processes would represent a cycle for the capture of light energy and its conversion into another readily usable form of energy (7, 13, 17, 19).

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