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Photoproduction of Ammonia by Cyanobacteria

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Nitrogen-fixing cyanobacteria have the ability to convert solar energy into chemical energy-ammonia, at the expense of sunlight and water alone. Cultures of the cyanobacteria *Gloeocapsa* and *Mastigocladus laminosus* excrete newly fixed ammonia when treated with L-methionine-DL-sulphoximine (MSX). This inhibitor, a glutamate-analogue, induces ammonia excretion by inhibiting the activity of glutamine synthetase (GS), the key enzyme in ammonia assimilation. A single addition of 10 μM MSX induces ammonia excretion for 6 days in *M. laminosus* and 15 days in *Gloeocapsa*. While the GS of *Gloeocapsa* gets inactivated irreversibly by MSX-treatment, that of *M. laminosus* recovers from the 5th day. In addition, *M. laminosus* excretes ammonia when incubated in light or dark and also on treatment with combined nitrogen. *Gloeocapsa* excretes ammonia only in the presence of light. This process of photo-production of ammonia by these cyanobacteria offers great promise for their use as biofertilizers in rice paddies.

Key Words: Cyanobacteria, Photoproduction, Ammonia, L-methionine-DL-sulphoximine, Biofertilizer, Glutamine synthetase-inhibition

Introduction

Nitrogen-fixing cyanobacteria have the unique ability to fix dinitrogen into ammonia at the expense of sunlight and water alone. The fixed ammonium is the primary source of all nitrogenous fertilizers. Except symbiotic nitrogen-fixing cyanobacteria, in most of the free-living, nitrogen-fixing cyanobacteria, ammonia is never excreted in the medium under normal physiological conditions and most of the fixed nitrogen is available only after cell death. Cells of *Nostoc* and *Anabaena azollae* growing symbiotically in the lichen *Peltigera canina* and the water fern *Azolla* respectively, release a high percentage of the nitrogen that they fix to the Symbiont (Stewart et al. 1980). This suggests that cyanobacteria can tolerate to some extent, the release of nitrogen from the cells. Such a release is also sought to be compensated by an increase in nitrogenase activity. The mechanism by which nitrogenase activity is increased is still not clear. However, two possibilities appear feasible. First, the repression of

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nitrogenase by combined nitrogen apparently acting through a product of GS action (Stewart & Rowell 1975) is removed. Secondly, inhibition of the primary ammonia assimilating enzyme GS causes the release of freshly formed ammonia out of the cells and creates a nitrogen starvation condition. This drives the organism towards anoxygenic photosynthesis, which is conducive for nitrogenase action.

Release of ammonia by cells treated with L-methionine-DL-sulphoximine (MSX) has been reported among cyanobacterial species (Stewart & Rowell 1975, Ramos et al. 1982a). MSX, a glutamate-analogue, induces ammonia excretion by inhibiting glutamine synthetase (GS), the key ammonia assimilating enzyme. This paper reports the sustained production of ammonia by two species of nitrogen-fixing cyanobacteria—*Gloeocapsa* sp., and *Mastigocladus laminosus* Cohn, and discusses the feasibility of transferring this technology to the rice field.

Materials and Methods

Organisms and Growth Conditions: *Mastigocladus laminosus* Cohn. (CCAP 1447/1) was grown at 37 ± 2°C under continuous illumination (40W, Cool daylight) of 3000 lux. *Gloeocapsa* sp. (IUCC, LB 795) was grown at 25 ± 1°C and illuminated with cool white fluorescent lamps at an intensity of 500 lux and 12 : 12 hr LD. Both cyanobacteria were grown as semi-continuous cultures in Allen and Arnon's (1955) medium free of combined nitrogen or amended with 20 mM KNO_3, and aerated continuously with a stream of sterile humidified air.

Ammonia production experiments: Cells containing 15–30 μg Chl/ml were harvested by centrifugation, washed with fresh culture medium and resuspended in the same medium to a density of 5–6 μg Chl/ml. MSX was added to the cell suspensions at the final concentrations of 10 and 100 μM and incubated under standard culture conditions in light or in dark (wrapped in aluminium foil). MSX was prepared freshly in distilled water as required and sterilized by filtration.

Enzyme extraction: Cell-free extracts were prepared at 4°C after harvesting the cyanobacteria by centrifugation, washing twice and resuspending in 10 mM Imidazole-HCl, *p*H 7.5. Cells were then disrupted in an ice-bath by sonic oscillation in MSE 150W Ultrasonic Disintegrator and debris removed by centrifugation at 20,000 × g for 30 min.

Enzyme assays: The transferase activity of glutamine synthetase (E.C 6.3.1.2) was performed according to Shapiro and Stadtman (1970) at *p*H 7.5. The Mn^{2+}-concentration was reduced 10-fold to 0.3 mM to prevent $Mn_3(AsO_4)_2$ precipitation.

Analytical methods: Ammonia was estimated by the phenol-hypochlorite method of Solorzano (1969). Protein was determined according to Lowry et al. (1951) after pretreating the extract with 10% (W/V) trichloroacetic acid. Bovine serum albumin served as standard.

Results

Nitrogen-grown, light-incubated cultures of *Gloeocapsa* and *M. laminosus* possess high levels of glutamine synthetase. On treatment with MSX, GS activity decreases. In *Gloeocapsa*, MSX acts as an irreversible inhibitor and GS activity does not recover even on prolonged incubation (figure 1). In *M. laminosus*, on the other hand, following the initial lowering of activity, GS activity starts increasing from the 5th day (figure 2). MSX, even at 10 μM brings about nearly total inhibition of GS in both cyanobacteria (table 1).

Under normal culture conditions, ammonia is not excreted into the medium by both *M. laminosus* and *Gloeocapsa*. Filaments of *M. laminosus* excrete ammonia, 4 hr after the addition of MSX (figure 3); excretion

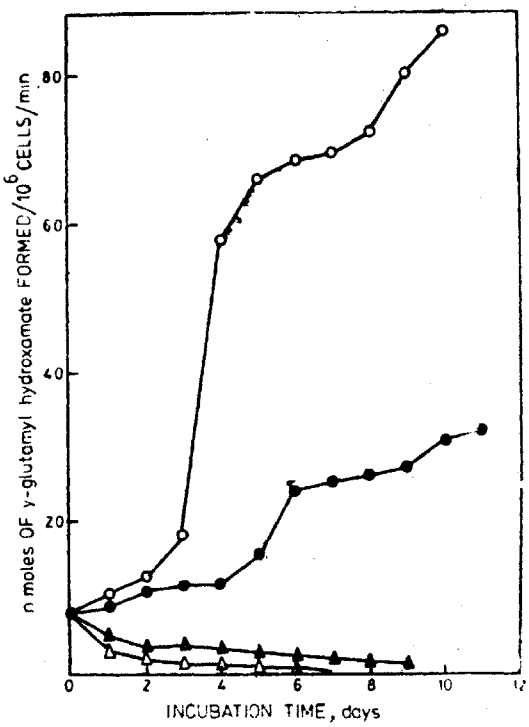

Figure 1 GS activity of N_2-grown *Gloeocapsa* incubated with (*triangle*) or without (*circle*) or 10μM MSx under light (*open symbols*) or dark (*closed symbols*).

Table 1 *Percentage inhibition of GS by* 10 μ*M MSX*

Day	*M. laminosus*		*Gloeocapsa*	
	Light	Dark	Light	Dark
1	32	24	80	72
2	66	40	88	78
3	89	67	94	86
4	94	69	99	88
5	94	78	99	92
6	85	72	100	94
7	—	—	100	96
8	—	—	100	96

Both cyanobacteria were grown in N-free medium
Percentage GS inhibition was computed with reference to MSX-untreated cultures

continues for 8 days when treated with 10 μM MSX. The period of ammonia excretion can be prolonged (to 13 days) by the addition of 100 μM MSX. In the former condition, the average production is $\pm$ 8 nmoles NH_3/μg Chl/day while in the latter the rate is nearly five-fold more. Maximum ammonia production is noticed on the 4th day when GS gets inactivated to its maximum. The rate of ammonia release slows down thereafter coinciding with the recovery of GS activity. MSX-treated cultures of *Gloeocapsa*

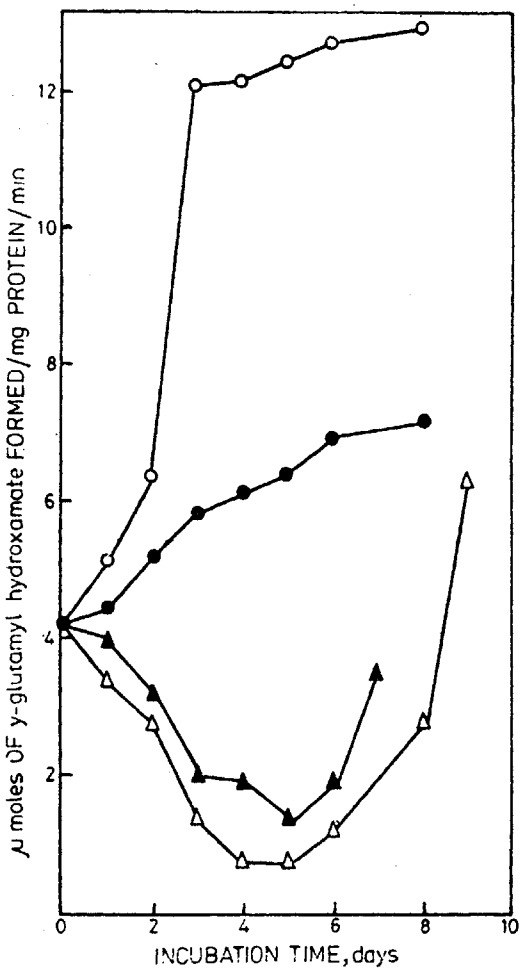

Figure 2 GS activity of N_2-grown *M. laminosus* incubated with (*triangle*) or without (*circle*) 10 μM MSx under light (*open symbols*) or dark (*closed symbols*).

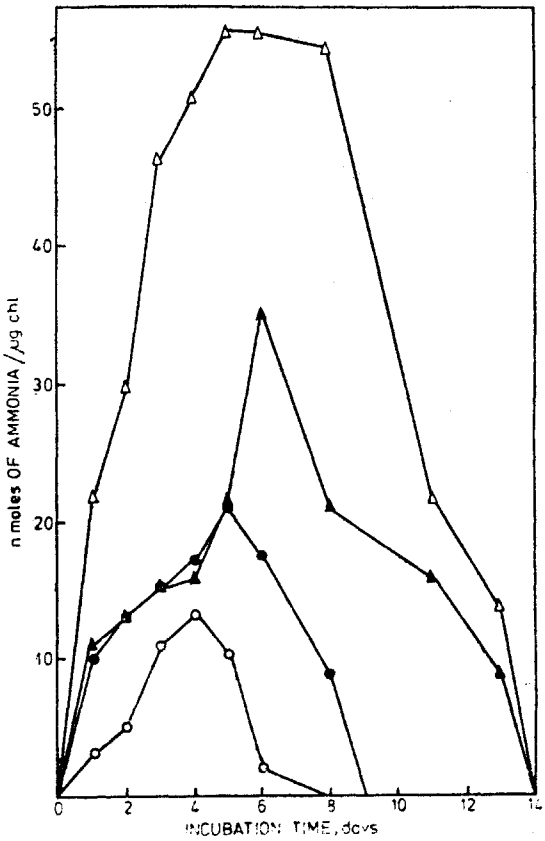

Figure 3 Excretion of ammonia by N_2-grown *M. laminosus* incubated with 10 µM (*circle*) or 100 µM (*triangle*) MSx in light (*open symbols*) or dark (*closed symbols*).

excrete ammonia only from the 3rd day (figure 4). Excretion continues for 15 days with peak production on the 6th day when GS gets totally inactivated, with an average production of 220 nmoles NH_3/µg Chl/day.

GS activity is lowered by 50% and 65% in *M. laminosus* and *Gloeocapsa* respectively (figure 1 and 2) on transfer of light-grown cells to the dark. The inactivation by MSX is also lower than in light-grown cells. The overall pattern of GS inhibition and ammonia production are otherwise similar. In *M. laminosus*, ammonia excretion in dark appears to be independent of MSX concentration, the average being 15 and 17 mnoles NH_3/µg Chl/day at 10 and 100 µM MSX respectively. In *Gloeocapsa*, ammonia production in dark is negligible in comparison to light-incubated cells.

When these nitrogen-fixing cyanobacteria are grown on combined nitrogen (20mM NO_3^-), they show comparable rates of GS activity (figure 5 and 6) and nearly the same amount of ammonia production (in *Gloeocapsa*). In *M. laminosus*, the cultures show 3-fold and 5-fold increase in ammonia production when treated with 10 and 100 µM MSX respectively (figure 5).

Discussion

Inhibition of GS and excretion of ammonia in response to MSX-treatment have been reported in bacteria (Brenchley 1973, Weare & Shanmugam 1976) and in cyanobacteria like *Anabaena cylindrica* (Stewart & Rowell

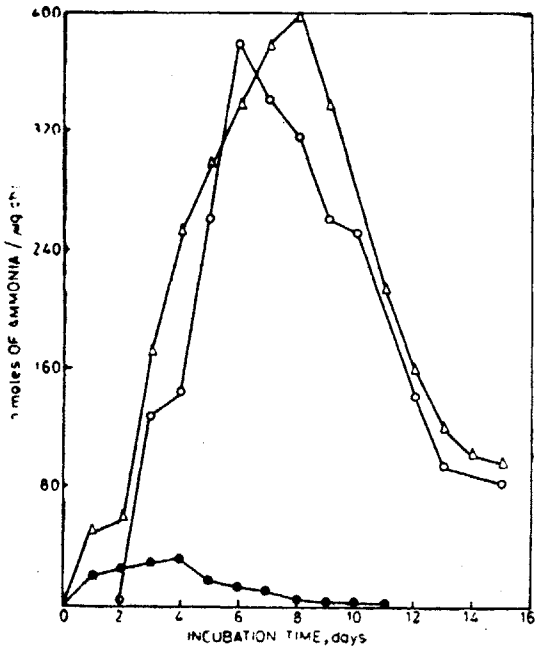

Figure 4 Excretion of ammonia by N_2-grown (*circle*) or NO_3^--grown (*triangle*) *Gloeocapsa* incubated in light (*open symbols*) or dark (*closed symbols*) with 10 µM MSx.

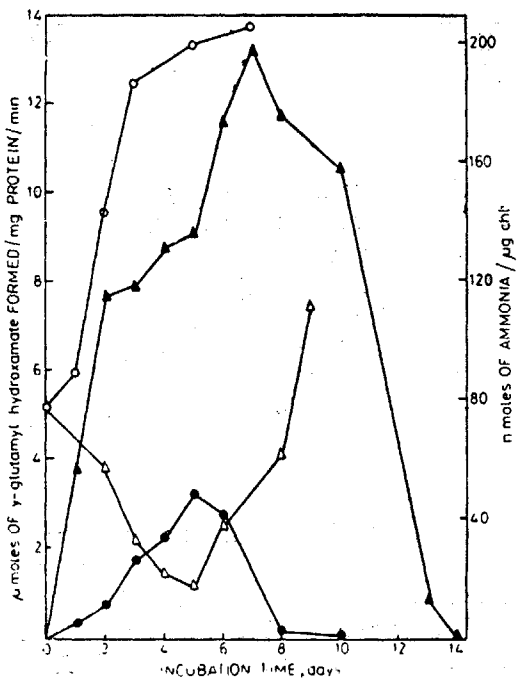

Figure 5 GS activity (*open symbols*) and ammonia excretion (*closed symbols*) by NO_3^--grown *M. laminosus* incubated in light without (o) or woth 10 µM (△●) or 100 µM (▲) MSx.

1975) and *Anacystis nidulans* (Ramos et al. 1982a). Our results show that the nitrogen-fixing cyanobacteria, *M. laminosus* and *Gloeocapsa* are capable of excreting ammonia when treated with MSX. In fact, these cyanobacteria are superior to the other two cyanobacteria mentioned above in being able to produce ammonia continuously for prolonged periods of time (6 days by *M. laminosus* and 15 days by *Gloeocapsa*) in response to a single addition of 10 µM MSX. It is interesting that while MSX acts as a reversible inhibitor in *M. laminosus*, it inactivates GS irreversibly in *Gloeocapsa*. However, GS of the other unicellular cyano Bacterium *A. nidulans* is reversibly inhibited by MSX (Ramos et al. 1982b).

Nitrogen-fixing cyanobacteria are known to fluorish in rice fields where they contribute to the nitrogen economy of the soil. Increase in grain yield by 10–20% has been reported in response to "algalisation" without any added chemical nitrogen fertilizer. The algal technology developed at the IARI has been shown to be capable of providing 20–30 kg of biologically fixed nitrogen per hectare of rice per season (Venkataraman 1972, 1981). This quantity of nitrogen is inadequate to maintain the rice crop and has to be supplemented with other nitrogenous fertilizers. Our results show that MSX treatment leads to the release of substantial

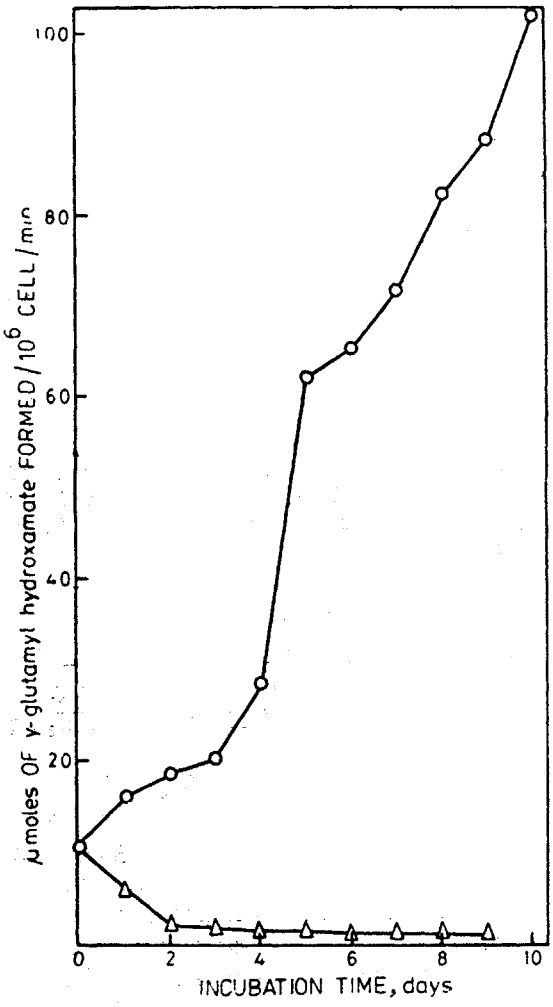

Figure 6 GS activity of NO_3^--grown *Gloeocapsa* incubated in light with (△) or without (o) 10 µM MSx.

quantities of ammonia, a form of nitrogen easily assimilated by higher plants. *M. laminosus* is more promising since its GS recovers once the MSX-effect ceases. This would enable the cyanobacteria to remain viable and by properly spacing the MSX treatments, rich benefits could be achieved.

The full potential of the use of these cyanobacteria as biofertilizers lies in the fact that they should be able to release freshly fixed ammonia as the symbiotic rhizobia and *Anabaena azollae* do. Hitherto it was believed that the cyanobacteria are capable of contributing the fixed nitrogen only on their death. But the technique of inhibiting the primary ammonia assimilating enzyme, temporarily in *M. laminosus* offers an excellent scope for the exploitation of such cyanobacteria as good biofertilizers, generating valuable nitrogen fertilizer *in situ*, using solar energy alone. This organism being a thermotolerant species is ideal for the tropical region.

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