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U.S. AGENCY FOR INTERNATIONAL DEVELOPMENT

THE USE OF CYANOBACTERIA AS BIOFERTILIZERS

by (the Principal Investigator)

Dr. Sammy Boussiba

in

The Microalgal Biotechnology Laboratory
The Jacob Blaustein Institute for Desert Research
Ben-Gurion University of the Negev

Collaborating investigator

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UNITED STATES-ISRAEL PROGRAM
Project Number C5-233
Grant Number: DPE-5544-G-SS-6036-00

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1. GENERAL INFORMATION ON THE PROJECT

1.1. Name of the project

"Ammonium translocation in cyanobacteria and their possible role as biofertilizers". AID-CDR Grant No. DPE-5544-G-SS-6036-00. The project was carried out in, The Algal Biotechnology Laboratory located in the Blaustein Institute for Desert Research Sede Boker campus, Ben Gurion University of the Negev Israel in collaboration with the University of the Philippines at Los Banos, College, Laguna, Philippines.

1.2. Background and objectives

Background:

The lack of available chemical fertilizers, especially nitrogenous ones at economic prices, is one of the basic problems facing agriculture in developing countries. **N-chemicals account for as much as 30% of the total fertilizers needed for agricultural crops and are often regarded as the limiting factors in food production in developing countries.** It is therefore not surprising that biological nitrogen fertilizers that efficiently transfer nitrogenous compounds from the media to the plants are of great interest in many countries, particularly in tropical Asia where rice is one of the major agricultural crops. Unfortunately, the increasing cost of N-fertilizer and the widening gap between supply and demand of **Nitrogen** in the developing countries have placed heavy constraints on the farmers. **Realizing the influence of energy cost on current and probable future prices of N-fertilizer, the need to stimulate research on alternative sources of nitrogen for rice cultivation is thus imperative.**

The concept of using N_2 fixing CBA as nitrogen biofertilizers in rice fields is not yet fully explored, and some major problems are still limiting the wide utilization of this biofertilization technique:

- The lack of an understanding of the environmental conditions prevailing in the rice ecosystem which in some cases affect the blooming of the algae (either indigenous species or inoculated ones)
- The inability to produce good quality inocula at an economical price.
- The low efficiency of the transfer (utilization) of the fixed nitrogen by the CBA to the rice plants.

Objectives:

I) Isolation and characterization of indigenous strains.

We propose to isolate free-living N_2-fixing cyanobacteria from rice paddies in order to study their optimal growth conditions. Promising strains which exhibit high growth rates in the laboratory will be cultivated outdoors to define the biological factors limiting their productivity.

II) Isolation of N_2-fixing cyanobacteria mutants which continuously release ammonia in the presence of combined nitrogen.

The accumulated nitrogen in the algal cells can be made available to the rice mainly by mineralization when the algal cells are microbially decomposed, while only a small part of this nitrogen is available through exudation when the algae are still alive. Thus it is impossible to distinguish between the demand for N-compounds required for the development of the rice plants and the release of these substances by the cyanobacteria. We suggest, therefore, the development of new strains of these algae which will fix atmospheric nitrogen and continuously release a fraction of it throughout their course of normal growth. Using such strains, it should be possible to control the flow of the nitrogen compounds required for the development of the rice plants from seedling to tillering.

III) Development of new concepts for the utilization of N_2-fixing cyanobacteria as nitrogen biofertilizers in rice paddies:

1.The cultivation of selected species of CBA in pilot plants for inoculum factories that will produce the biofertilizer.

2. The biotechnology of harvesting and transferring the CBA in concentrated form to the rice fields.

1.3. Executive summary

The concept of using N_2 fixing CBA as nitrogen biofertilizers in rice fields is not yet fully explored, and some major problems are still limiting the wide utilization of this biofertilization technique:

- The lack of understanding of the environmental conditions prevailing in the rice species or inoculated ones)
- The inability to produce good quality inocula at an economical price
- The low efficiency of the transfer (utilization) of the fixed nitrogen by the CBA to the rice plants.

During this project, intensive work was carried out to address these problems and good progress was achieved:

1. Several promising strains from rice fields have been isolated and characterized, among which are: *Gleotrichia natans*, indeginous to most rice fields in the Philippines, and *A.siamensis*, one of the fastest natural nitrogen-fixing strains.
2. We isolated a mutant strain from mutagenized *A.siamensis*, which continuously releases ammonia.
3. We have succeeded to produce these strain for the first time in outdoor cultures to obtain inocula of good quality.
4. Storage and transport. Several techniques were tested:

   Air-drying in a non-humid place. In this dried form the BGA can be easily transported.

   Another easy methods of transporting of BGA is by storing spores or akinets.

The progress we have made in improving the biotechnology of using cyanobacteria as N-biofertilizers is now implemented in the Philippines. This information is now available to other third world countries through the publications which summarize our accomplishments (see 3.1).

2. SPECIFIC METHODOLOGY

2.1. Screening and isolation procedures

2.1.1. Screening for indigenous species in rice fields

PHASE 1 - Determination of growth limiting factors: light, pH, temp, nitrogen, phosphate, salinity and predators.

Efficient production of inoculum of high quality.

PHASE 2- Application to rice fields:

a. enrichment and establishment of favorable endogenous species

b. efficient transfer of the fixed-nitrogen to the rice plant.

2.1.2. Mutagenesis using EMS for the isolation of ammonium excreting mutants:

100 ml of culture grown on BG110 medium in mid-exponential phase were harvested by centrifugation at 3,500 rpm at room temp.

The filaments were washed once in BG11 medium containing 5 mM ammonium chloride and suspended in 24 ml of the same medium to concentration of 5E8 cfu/ml.

Cells were sonicated for 15 seconds and washed in BG11 medium containing 5 mM ammonium chloride and suspended in 24 ml of the same medium and then divided into two 12-ml cultures (1-control: following every step of the experiment except addition of EMS; 2- for mutagenesis). EMS was added to the cultures at a final concentration of 1%.

Culture was incubated for 90 minutes in room temp. with continuous illumination.

Culture was washed in BG110 medium and suspended in 12 ml of the same medium.

Filaments obtained after mutagenesis were incubated at 48°C for 40 minutes.

Cells were collected by centrifugation and suspended in 100 ml BG11 medium containing 3 mM ammonium Chloride.

Culture was incubated in light in room temp. with gentle shaking for 10 h to allow segregation of mutagenized chromosome. Plating on 500µm MSX and selection of the survivors on pH indicators dyes as indication for ammonia excretion.

2.2. Laboratory techniques and specific assays

1. Laboratory cultures: The algae were cultivated in 500 ml sterilized glass columns inside a transparent plexiglass circulating water bath. Water temperature was controlled at 30°C. A constant photon flux of 175 $\mu E\ m^{-2}\ s^{-1}$ at the surface of the growth vessel was supplied laterally by a battery of 8 cool-white fluorescent lamps. Continuous aeration was provided by bubbling filtered air containing 1.5% CO_2. Under this condition the pH was maintained at 6.8-7.0. The standard growth medium was BG-11o (Stanier et al., 1971).

Unless otherwise stated, culture was sampled during the logarithmic growth phase for use in the different experiments.

2. Outdoor cultures: 2.5 m^2 oval-shaped ponds with two channels forming a single loop were used. The culture, 250 liters in volume (medium was BG-11o) and 10 cm in depth, was stirred by a paddle wheel. CO_2 was supplied to maintain the pH at a range of 6.5 - 7.5.

3. Nitrogenase activity was estimated by the acetylene reduction method (Stewart, 1967). Samples of 4.6 ml of algal culture, washed in fresh BG-11o medium, were placed in a 25 ml Wheaton bottle sealed with a flanged rubber septum. The Wheaton bottles were subjected to rotary shaking and illuminated with a quantum flux of 75 $\mu E\ m^{-2}\ s^{-1}$, during the assay. Cell suspensions were allowed 10 min of acclimation before injection of C_2H_2. Ethylene was analyzed on an HP 5890 gas chromatograph using a stainless steel column packed with Poropack-N (0.2 cm i.d., 265 cm length). Nitrogenase activity was expressed as μ mol C_2H_4 produced per mg chlorophyll per hour.

3. CONCLUSIONS AND PUBLISHED WORK

3.1. published work based upon work carried out during this project

A. Chapters in Books:

1990 Boussiba, S. Nitrogen fixing cyanobacteria. Proceedings of the Fifth International Symposium on Nitrogen Fixation with Non-Legumes. Florence, Italy. Polsinelli, M., Materassi, R. and Vincenzini, M. (eds.) Kluwer Academic Publishers, pp 487-491.

1990 Boussiba, S. Ammonium transport systems in cyanobacteria. In: Inorganic Nitrogen Metabolism. Ullrich, W.R., Rigano, C., Fuggi, A. and Aparicio, P.J. (eds) Springer Verlag, Berlin, pp. 99-105.

B. Refereed Articles in Scientific Journals:

1988 Boussiba, S. Cyanobacteria as nitrogen biofertilizers: A study with the isolate Anabaena *azollae*.. Symbiosis. 6, 129-138.

1989 Boussiba, S. Ammonium uptake in the alkalophilic cyanobacterium *Spirulina platensis*. Plant Cell Physiol. **32**: 303-314.

1990 Querijero-Palacpac, N.M., Martinez, M.R. and Boussiba, S. Mass cultivation of the nitrogen-fixing cyanobacterium *Gleotrichia natans*, indigenous to rice fields. J. Appl. Phycol. 2: 319-325.

1990 Thomas, S.P., Zaritsky, A. and Boussiba, S. Ammonium excretion by a methionine sulfoximine resistant mutant of the rice field cyanobacterium Anabaena *siamensis* . Appl. Environ. Microbiol. **56**: 3499-3504.

1991 Thomas, S.P., Zaritsky, A. and Boussiba, S. Genetic improvement of *Anabaena siamensis* for ammonium hyperproduction and excretion. Bioresource Technology 38: 161-166.

1991 Boussiba, S. Nitrogen fixing cyanobacteria potential uses. Plant and Soil. **137**: 177-180.

C. Refereed articles (others):

1991 Martinez, M.R., Querijero-Palacpac, N.M., Guevarra, H.T. and Boussiba, S. Production of indigenous nitrogen-fixing blue-green algae in paddy field in the Philippines. Workshop on Mass Culture of Microalgae. November 18, Silpakorn University, Thailand.

D. Meetings and invited lectures

a. Invited Lectures:

1987 Boussiba, S. Ammonium transport in cyanobacteria. EMBO Workshop on Oxygenic and anoxygenic electron transport systems in cyanobacteria (Blue-Green Algae), Cape Sounion, Greece.

1988 Boussiba, S. Ammonium translocation in *Anabaena azollae* and its possible use as a nitrogen biofertilizer. National Council for Research and Development. Nitrogen fixation in symbiotic systems, Finland-Israel (Shoresh).

1989 Boussiba, S. Ammonia uptake and assimilation in cyanobacteria. Adv. Course on Inorganic Nitrogen Metabolism. Napoli, Italy.

1989 Boussiba, S. Ammonium transport systems in cyanobacteria. EMBO Workshop on Comparative structure and function of membranes in chloroplasts and cyanobacteria. Corfu, Greece.

1990 Boussiba, S. Nitrogen fixation in blue-green algae: potential uses. In: Biological Nitrogen Fixation Meeting. Israel Society for Microbiology, Jerusalem, Israel.

1990 Boussiba, S. Ammonium excretion by an MSX-resistant mutant of the rice-field cyanobacterium *Anabaena siamensis*. First European Workshop on the Molecular Biology of the Cyanobacteria. Durdan, France.

1990 Boussiba, S. Nitrogen fixing cyanobacteria - potential uses. Fifth Internat. Symp. Nitrogen Fixation with non-legumes. Florence, Italy.

1991 Boussiba, S. and Martinez, M.R. Ammonium translocation in Cyanobacteria and their possible use as nitrogen biofertilizer. Biological Nitrogen Fixation. Networking workshop USAID meeting of principal investigators. Banff, Canada.

b. Meetings:

1987 Boussiba, S. *Anabaena azollae* as a nitrogen biofertilizer. 4th International Meeting of the French Society of Applied Algology. Villeneuve D'ascq, France.

1988 Boussiba, S. and Gibson, J. Ammonium translocation in cyanobacteria. 6th Symp. on Photosynthetic Prokaryotes. Nordwijkerhout, The Netherlands.

1989 Boussiba, S. Biomass production of nitrogen-fixing cyanobacteria. Adv. Course on Inorganic Nitrogen Metabolism. Napoli, Italy.

1990 Thomas, S., Zaritsky, A. and Boussiba, S. Genetic improvement of *Anabaena siamensis* for ammonium hyperproduction and excretion. 5th Internat. Conf. Soc. Appl. Algol. on: Recent Advances in Algal Biotechnology, Tiberias, Israel.

1990 Palacpac, N., Martinez, M. and Boussiba, S. Mass cultivation of an indigenous nitrogen-fixing blue-green alga *Gleotrichia natans*. 5th Internat. Conf. Soc. Appl. Algol. on: Recent Advances in Algal Biotechnology, Tiberias, Israel.

1991 Martinez, M.R., Marcelino,V.M., Palacpac, N.Q. and Boussiba, S. Outdoor production of nitrogen fixing blue green algae. Workshop on Mass Culture of Microalgae. Silpakorn University, Thailand.

1991 Martinez, M.R., Sarmiento, J.F. and Boussiba, S. Effects of phosphorous on algae and rice growth. Workshop on Mass Culture of Microalgae. Ibid.

1991 Martinez, M.R., Encio, D., Paje, P.P., Guevarra, H.T. and Boussiba, S. Interrelationship of planktonic and colony-forming algae in soil based pond. Ibid.

3.2. Concluding remarks of PI's

Dr. Boussiba:

Our collaboration with the Philippines is well established. Dr. Martinez has visited the Algal Biotechnology Laboratory for several weeks and one of her students, Nirrian Palacpac, has spent several months in the lab in Israel. Actually, Ms. Palacpac was a joint research student of Dr. Martinez and myself for an M.Sc. degree. I have visited their laboratory in the Philippines and set up turbidostats there (brought from Israel) for growing algae under controlled conditions. We have also constructed outdoor ponds 2.5 m^{-2}, similar to those we have in Israel. The facilities installed in Dr. Martinez's laboratory for cultivating microalgae were of great importance for the successful implementation of the completed project.

The progress we have made in improving the biotechnology of using cyanobacteria as N-biofertilizers is now implemented in the Philippines. This information is now available to other third world countries through the publications which summarize our accomplishments (enclosed).

Dr. Martinez : (see letter attached)

3.3 Reprints and other relevant materials related to this project:

*Letters

*Final technical report by Dr. Martinez

* Reprints

P.O. Box 169

September 17, 1991

Prof. Jaime Wisniak
Vice Pres. & Dean of Research & Development
Ben Gurion University
P.O. Box 653
Beer Sheva Israel

Dear Prof. Wisniak:

Thank you so much for having given me the opportunity to work with Dr. Sammy Boussiba on this recently concluded U.S. AID-CDR Grant about "Ammonium Translocation in Cyanobacteria and their Possible Use as Biofertilizers."

I have learned a lot from the collaboration and it is already revolutionizing our Cyanobacteria production with the use of your technologies. However, it may take sometime before we can be proficient in the system. Otherwise, we will still be in the backward stage of dual cultivation of our algae with rice that has a minimal contribution in biomass as compared to the Israel technology.

I did not only learn about technical aspects of the project but I also came to appreciate the "drive" and high motivation for work of my collaborator. It took sometime for me to understand and adjust to the situation but I am very appreciative to him for his efforts and patience. I think the project made me more understand him and the Israeli people in general.

Lastly, the monetary remuneration we got helped a number of people over here - from giving jobs to travel and education.

Thank you and my best regards.

Very truly yours,

Milagrosa R. Martinez

MILAGROSA R. MARTINEZ
Associate Professor & Director

cc: Dr. Sammy Boussiba

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UNIVERSITY OF THE PHILIPPINES
Quezon City, Philippines

DATE 12-12-88 1:57
RECEIVED
DEC 13 1988

NOTIFICATION OF APPROVAL OF TEMPORARY APPOINTMENT

DEC 8 1988

The Board of Regents, on recommendation of the President of the University, approved the appointment of Dr. Sammy Boussiba as Visiting Assistant Professor, Institute of Biological Sciences, University of the Philippines Los Baños, ~~at a salary of~~ without compensation ~~per annum~~ effective 5 May 1988 until 4 April 1989 unless sooner terminated, and subject to pertinent University regulations and the following conditions:

U.P. ARTS & SCI
DATE 12-17-88
OFFICE OF THE

(Original appointment.)

(1017th Meeting, Board of Regents, DEC 8 1988)

NOTED:

RAUL P. DE GUZMAN
Chancellor

EMERLINDA R. ROMAN
Secretary of the University

NOTED:

E. L. ASPI
DEAN

B. T. MERCADO
DIRECTOR, IBS

/sc

Symbiosis, 6 (1988) 129–138
Balaban Publishers, Philadelphia/Rehovot

N_2-Fixing Cyanobacteria as Nitrogen Biofertilizer — A Study With the Isolate *Anabaena azollae*

SAMMY BOUSSIBA

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Received April 17, 1988; Accepted July 7, 1988

Abstract

Anabaena azollae possesses several characteristic features advantageous for application as a nitrogen biofertilizer: fast growth rate (μ–0.0675 h^{-1}; dcubling time of 10.2 h); tolerance to a wide range of temperatures (20–4n°C); ability to grow and to fix nitrogen at optimal values (nitrogenase activity–32 μmol C_2H_4 mg^{-1}chl h^{-1}) over a broad range of pH (6 to 9); growth rate and nitrogenase activity not effected by the presence of 1% NaCl in the growth medium. Its production outdoors in 2.5 m^2 ponds was also tested over several months. Maximum yield of 17.9 g $m^{-2}d^{-1}$ was obtained during the month of August in which the morning and noon temperatures fluctuated between 21–24°C and 31–34°C, respectively the ponds being partially (30%) shaded to decrease light intensity.

Keywords: *Anabaena azollae*, nitrogen fixation, biofertilizer, biomass production, rice paddies

1. Introduction

The use of nitrogen-fixing cyanobacteria as nitrogen biofertilizer in rice fields is of great significance in many countries in the far east, where rice is the major staple diet. Indeed since the first report by De (1939) testing the potential application of these algae as a biofertilizer, many studies have been devoted to introducing this biofertilization technique, in various countries (Venkataraman, 1977, 1986; Roger and Kulasooriya, 1980; Martinez, 1984;

Ley and Qianlin, 1985; Grant et al., 1986; Roger and Watanabe, 1986). To date, however, the use of these algae as N-fertilizer still suffers from some major problems: The inability to produce good quality inocula at an economical price (Watanabe, 1984); the lack of understanding the environmental conditions prevailing in the rice ecosystem which in some cases affect the blooming of the algae, either endogenous species or inoculated ones (Roger and Kulasooriya, 1980); the low efficiency of the utilization of the fixed nitrogen by the rice plants (Watanabe et al., 1987).

This study describes the performance of *Anabaena azollae*, isolated from *Azolla filiculoides*, in relation to its application as N-fertilizer. Data concerning the effect of pH, temperature and salinity (environmental conditions which regulate the abundance of cyanobacteria in rice fields) on the growth rate and nitrogenase activity are presented. The possibility of cultivating this strain outdoors under N_2 fixing conditions during a relatively long period of time was also tested.

2. Materials and Methods

Organism

Anabaena azollae isolated from *Azolla filiculoides*, was donated by E. Tel-Or, Faculty of Agriculture of the Hebrew University at Rehovot, Israel.

Growth conditions

1. *Laboratory cultures*: The algae were cultivated in 500 ml sterilized glass columns inside a transparent plexiglass circulating water bath. Water temperature was controlled at 30°C. A constant photon flux of 175 $\mu E\ m^{-2}s^{-1}$ at the surface of the growth vessel was supplied laterally by a battery of 8 cool-white fluorescent lamps. Continuous aeration was provided by bubbling filtered air containing 1.5% CO_2. Under these conditions, the pH was maintained at 6.8–7.0. The standard growth medium was BG-110 (Stanier et al., 1971). Unless otherwise stated, cultures were sampled during the logarithmic growth phase for use in the different experiments.
2. *Outdoor cultures*: 2.5 m^2 oval-shaped ponds with two channels forming a single loop were used. The culture, 250 liters in volume (medium was BG-110) and 10 cm in depth, was stirred by a paddle wheel. CO_2 was supplied to maintain the pH at a range of 6.5–7.5. *Pond maintenance:* Temperature, dissolved oxygen, and pH in the outdoor cultures were monitored daily. Light intensity ranged from 900 to 1250 $\mu E\ m^{-2}s^{-1}$ between March to August, respectively.

To maintain steady state growth, the culture was bled as required. In all outdoor experiments the biomass concentration was kept between 6–8 mg·chl $liter^{-1}$.

Enzyme assays

Nitrogenase activity was estimated by the acetylene reduction method (Stewart, 1967). Samples of 4.6 ml of algal culture, washed in fresh BG-110 medium, were placed in a 25 ml Wheaton bottle sealed with a flanged rubber septum. The Wheaton bottles were subjected to rotary shaking and illuminated with a quantum flux of 75 $\mu E\ m^{-2}s^{-1}$, during the assay. Cell suspensions were allowed 10 min of acclimation before injection of C_2H_2. Ethylene was analyzed on an HP 5890 gas chromatograph using a stainless steel column packed with Poropack-N (0.2 cm i.d., 265 cm length). Nitrogenase activity was expressed as μ mol C_2H_4 produced per mg chlorophyll per hour.

Other methods

Ash free dry weight (AFDW) and chlorophyll-a were determined as previously described (Boussiba et al., 1987). Protein was determined according to Lowry et al. (1951). Frequency of heterocysts was calculated by microscopic countings and is expressed as % of the total number of cells in the culture.

The effect of temperature on growth was studied in a temperature block maintaining a temperature gradient from 20 to 45°C with 1.5 degree increments between adjacent test tubes. The light intensity at the bottom surface of the tubes was 110 $\mu E\ m^{-2}s^{-1}$.

3. Results

Effects of environmental factors

The effect of different growth conditions on the specific growth rate and nitrogenase activity of *A. azollae* were tested in the laboratory. This isolate grew relatively fast and fixed nitrogen over wide ranges of pH's (Fig. 1). The specific growth rate and the maximum nitrogenase activity being 0.065 h^{-1}, and 32 μmol C_2H_4 mg^{-1} chl h^{-1}, respectively, at PH 7.0. The same effects, high growth rate and nitrogenase activity, could be achieved in cultures growing in air, but in which the pH is controlled to 6.8–7.0. *A. azollae* can tolerate a wide range of temperatures from 20 to 40°C, without its growth rate being adversely affected (Fig. 2). This strain exhibits tolerance to NaCl up to 1% without its growth rate or its nitrogenase activity being affected (Fig. 3).

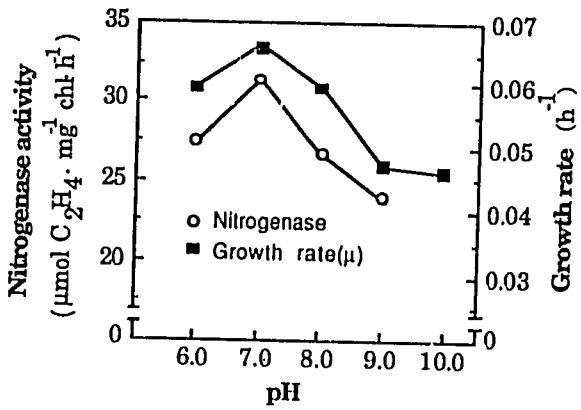

Figure 1. Effect of pH on the growth rate and nitrogenase activity of *Anabaena azollae*.

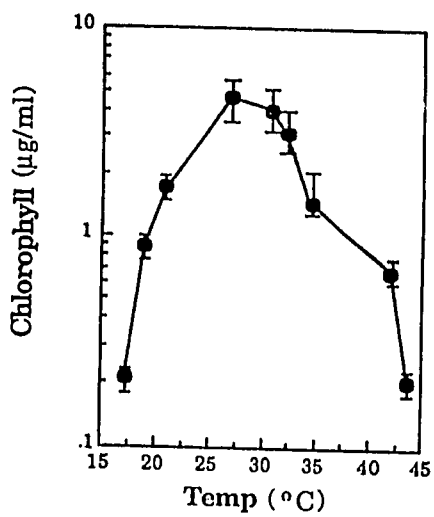

Figure 2. Effect of temperature on the growth of *Anabaena azollae*.

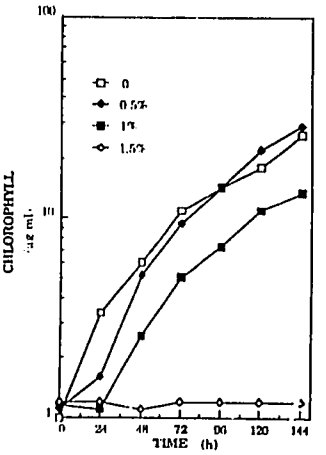

Figure 3. Effect of NaCl on the growth of *Anabaena azollae*. NaCl was added to final concentrations (w/v) as indicated.

Table 1. Outdoor production of *Anabaena azollae* in 2.5 m^2 ponds

Month (1987)	Temperature (°C)	Output rate (g $m^{-2}d^{-1}$)	Nitrogenase* activity	Heterocysts (% of total cells)
March	12–15[1] 23–26[2]	5.6	4.7	4–5
June	18–21 28–31	12.3	5.2	5–6
August	21–24 31–34	17.9	5.8	5–6

[1]morning; [2] noon

*nitrogenase activity – μmol C_2H_4 mg^{-1} chl h^{-1}

Outdoor mass production of Anabaena azollae

Data accumulated at Sede-Boker concerning the mass production of *A. azollae* during several seasons of the year are presented in Table 1. A major effect which controlled the mass production of this nitrogen fixing strain was the temperature fluctuation during the months of production. Maximum yield of 17.9 g $m^{-2}d^{-1}$ was obtained during the month of August during which the morning and noon temperatures were close to optimum. No difference between C/N ratios were observed in the material grown in the lab or outdoors (data not shown).

Some characteristic features of outdoor cultivation of this strain were observed: (1) Relatively small amounts of ammonia were present in the medium during growth (between 0.1 and 0.3 mM). This phenomenon was not observed in the laboratory; (2) significant reduction in frequency of heterocysts (5–7%) and nitrogenase activity (4–7 μmol $C_2H_4 mg^{-1} chl\ h^{-1}$) during growth, compared with laboratory cultures; (3) sensitivity to solar irradiance (1250 $\mu E\ m^{-2} s^{-1}$) which necessitated continuous shading of the pond in the summer (August), reducing light intensity by 30%.

The release of ammonia to the surrounding environment

In one event during the course of growth in outdoor ponds, a sudden drastic drop in temperature occurred (below 10°C). This caused rapid decomposition of the *Anabaena* cells and an increase of ammonia in the growing medium (Fig. 4).

The released ammonia was consumed and promoted the establishment of new species of algae (green) as revealed by microscopic observation and by the total loss of nitrogenase activity (Fig. 4). This situation is comparable to the decomposition of cyanobacteria in rice fields, when the nitrogen compounds are utilized by the rice plants.

4. Discussion

Rice fields continuously undergo environmental changes during maturation of the rice plants (Roger and Kulasooriya, 1980). During the growth cycle of the rice plants, light becomes limiting due to tillering development, and there is an increase of pH from 6 to 7 in the inoculation stages, to 8–9.5 towards the end of growth. Also, due to evaporation, there is a constant increase in salt concentration, while temperature may also fluctuate over a wide range (Venkataraman, 1986). Clearly, these environmental factors may directly affect the growth and development of cyanobacteria in rice fields. In particular, these factors may control nitrogenase activity and therefore affect the performance of these microorganisms as nitrogen biofertilizers. The search for suitable strains which can perform well under the different environmental conditions prevailing in rice fields, should therefore by considered, as the first stage in the development of biotechnology using cyanobacteria as biofertilizer.

The results obtained in this investigation and previously (Zimmerman and Boussiba, 1987), regarding the effect of environmental factors on the growth rate and nitrogenase activity of *A. azollae* give support to the possibility of

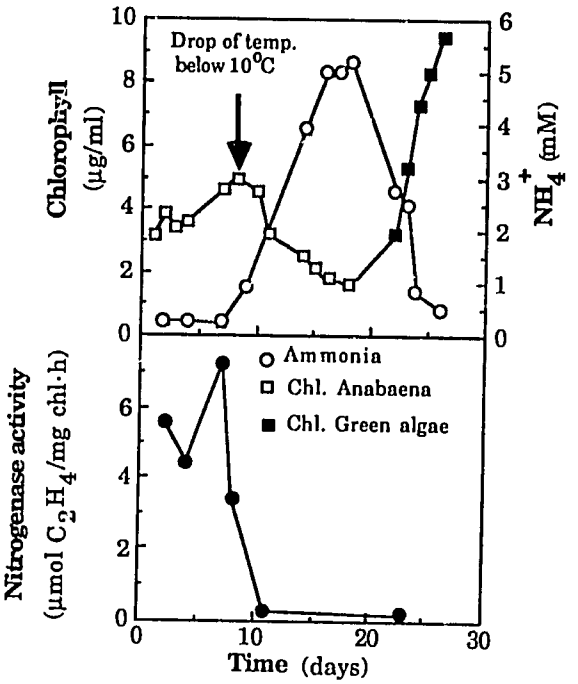

Figure 4. Growth and ammonia release of *Anabaena azollae* cultivated outdoors in 2.5 m^2 pond.

using this isolate as a nitrogen biofertilizer. This strain grows relatively fast (dt of 10.2 hr), but slower than *Anabaena siamensis* another potential biofertilizer strain which grows much faster (dt of about 4.0 hr) (Antarikanonda, 1985). *A. azollae*, however, possesses several other characteristic features which may be considered advantageous; it fixes nitrogen at almost optimal rates over a broad range of pH; tolerates a wide range of temperatures; and can withstand up to 1% NaCl in the growth medium without its growth or its nitrogenase activity being significantly affected. Indeed, these features of *A. azollae* have been described previously (Antarikanonda and Lorenzen, 1983) as the desirable ones, when considering natural isolates of N_2-fixing cyanobacteria to serve as nitrogen biofertilizer.

The next important stage when considering application of N_2-fixing cyanobacteria as nitrogen biofertilizer is mass production of high quality inoculum of the desirable strains (Watanabe, 1984). Data concerning mass production of nitrogen-fixing cyanobacteria are still limited, and the rate of reported production 6-8 g $m^{-2}day^{-1}$ is relatively low (Watanabe, 1959). Recently Fontes et al. (1987) obtained higher rates of production 8 to 13 g (dry weight) $m^{-2}day^{-1}$ using *Anabaena variabilis*. It is imperative to consider these data with great caution since they were obtained in a small scale (0.25 m^2) and over a relatively very short period of time. The highest rate of production in a bigger reactor 2.5 m^2 obtained in this work was 17.9 g (A.F.D.W.) $m^{-2}day^{-1}$. The rate of production was calculated from a culture being at steady state of at least 25 days.

The third stage, which should perhaps be considered the critical one in the selection of the desirable strain, to be used as a nitrogen biofertilizer, is its performance under field conditions. Important factors which should be taken into consideration are: competition with endogenous strains, resistance to pesticides and grazers and, finally, the effectiveness of fixed nitrogen transfer, to the benefit of the rice plants (an example of such a flow of nitrogen during the decomposition of *A. azollae* is documented in Fig. 4). The performance of *A. azollae* in rice fields is now being investigated.

Acknowledgement

This work was supported by AID-CDR Grant No.DPE-5544-G-SS-6036-00.

REFERENCES

Antarikanonda, P. 1985. A new species of the genus *Anabaena: Anabaena siamensis* sp *Novo* (Cyanophycease) from Thailand. *Nova. Hedwigia* **41**: 343-352.

Antarikanonda, P. and Lorenzen, H. 1983. Highly efficient N_2 fixing blue green algae in Thailand: A possible remedy to nitrogen scarcity. *Bangkok MIRCEN News Letters* **3**: 1–6.

Boussiba, S., Vonshak, A., Cohen, Z., Avissar, Y., and Richmond, A. 1987. Lipid and biomass production by the halotolerant microalgae *Nannochhoropsis salina*. *Biomass* **12**: 37–48.

De, P.K. 1939. The role of blue green algae in nitrogen fixation in rice fields. *Proc. Roy. Soc. Lond. B.* **127**: 121–139.

Fontes, A.G., Vargas, M.A., Moreno, J., Guerrero, M.G., and Losada, M. 1987. Factors affecting the production of biomass by a nitrogen fixing blue green alga in outdoor culture. *Biomass* **13**: 33–43.

Grant, I.F., Roger, P.A., and Watanabe, I. 1986. Ecosystem manipulation for increasing biological N_2 fixation by blue-green algae (Cyanobacteria) in lowland rice fields. *J. Biol. Agricult. Horticul.* **3**: 299–315.

Ley, H.S. and Qianlin, W. 1985. Nitrogen fixing blue green algae a source of biofertilizer. In: *Proceedings of the Joint CHINA-U.S. Phycology Symposium*, pp. 479–496.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265–275.

Martinez, M.R. 1984. Algae: biofertilizer for rice. Philippines Council for Agriculture Research and Resources Development (PCARRD). *PCARRD Monitor* **12**: 9.

Roger, P.A. and Kulasooriya, S.A. 1980. *Blue-Green Algae and Rice.* The International Rice Research Institute, Los Banos, Philippines.

Roger, P.A. and Watanabe, I. 1986. Techniques for utilizing biological nitrogen fixation in wetland rice: potentialities, current usage, and limiting factors. *Fertilizer Res.* **9**: 39–77.

Solorzano, L. 1969. Determination in natural waters by the phenolhypochlorite method. *Limnol. Oceanogr.* **14**: 799–801.

Stanier, R.Y., Kunisawa, M.M., and Cohen-Bazire, G. 1971. Purification and properties of unicellular blue-green algae (order Chroococcales). *Bacteriol. Rev.* **35**: 171–205.

Stewart, W.D.P., Fitgerald, G.P., and Burris, R.H. 1967. *In situ* studies on N_2-fixation using the acetylene reduction technique. *Proc. Nat. Acad. Sci. USA* **58**: 2071–2078.

Venkataraman, L.V. 1986. Blue-green algae as biofertilizers. In: *CRC Handbook of Microalgal Mass Culture.* A. Richmond, ed. CRC Press, Inc. Boca Raton, FL, pp. 455–471.

Venkataraman, G.S. 1977. Blue green algae as a biological N-input rice cultivation. In: *Proceedings, National Symposium, Nitrogen Assimilation and Crop Productivity.* Hissar India, pp. 132–141.

Watanabe, A. 1959. On the mass culturing of a nitrogen fixing blue-green algae *Tolypothrix tenuis. J. Gen. Appl. Microbiol.* **5**: 85–91.

Watanabe, I. 1984. Use of symbiotic and free-living blue-green algae in rice culture. *Outlook of Agriculture* **13**: 166-172.

Watanabe, I., de datta, S.K., and Roger, P.A. 1987. Nitrogen cycling in wetland rice soils. In: *International Symposium on Advance in Nitrogen Cycling in Agricultural Ecosystem*, CSIRO, Australia, pp. 1–23.

Zimmerman, W.J. and Boussiba, S. 1987. Ammonia assimilation and excretion in a symbiotic strain of *Anabaena azollae* from *Azolla filiculoides*. *Lam. J. Plant Physiol.* **127**: 443–450.

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Ammonia Uptake in the Alkalophilic Cyanobacterium *Spirulina platensis* [1]

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Ammonia uptake was studied in the alkalophilic cyanobacterium *Spirulina platensis*. In continuous cultures under optimal growth conditions ammonia supported optimal growth (doubling time of 9.3 h), causing a reduction of glutamine synthetase activity to 25% of that found in cultures grown on NO_3^-. Long term (20 min) ammonia uptake assays were performed to study the dependency on metabolism: 1) Ammonia uptake proceeded at the same rates in the light and in the dark, the pH dependency pattern correlating with light-dependent O_2 evolution and dark O_2 consumption. 2) The uptake of ammonia was pH dependent with an optimum at pH 9.3. 3) The uptake was totally dependent upon the activity of glutamine synthetase and was completely inhibited by methoinine sulfoximine.

To study the mechanism by which NH_4^+/NH_3 enters the cells, short term experiments (up to 1 min) were performed at pH 7.0 and pH 10.0: At pH 7.0 the uptake was slow and at a constant rate. At pH 10.0, the uptake did not saturate even at 1 mM ammonia and the kinetics were biphasic, consisting of a fast component lasting less than 5 seconds and of a subsequent slower component. The fast phase was insensitive to methionine sulfoximine, whereas the slower phase was completely inhibited by this compound. We suggest that under optimal (alkaline) pH the entry of ammonia into *Spirulina* cells is likely to be a ΔpH driven diffusion process, continuously supported by its intracellular assimilation.

Key words: Alkalophilic — Ammonia uptake — Cyanobacteria — Methionine sulfoximine — *Spirulina platensis*.

Spirulina platensis is an alkalophilic cyanobacterium, which grows optimally at pH 9.0, but withstands pH values as high as 11.5 (Zarouk 1966). At present it is being extensively cultivated for the production of protein and rare chemicals (Richmond 1986). However, limited information is available concerning its nitrogen metabolism (Boussiba and Richmond 1980) and the uptake of nitrogenous substances at high pH values. The latter is of special interest when ammonia is used as the sole nitrogen source, since this molecule, which is mostly unprotonated at alkaline pH (pK 9.25), was reported to uncouple cyanobacterial photosynthesis (Abeliovich and Azov 1979). We therefore set out to study whether *S. platensis* can grow satisfactorily on ammonia as a sole nitrogen source at an alkaline pH and if so, to characterize the mechanism of its influx.

Ammonium uptake and its retention have been examined in neutrophilic cyanobacteria (Boussiba et al. 1984, Kashyap and Johar 1984, Turpin et al. 1984, Zimmerman and Boussiba 1987, Ritchie and Gibson 1987, Ohmori and Kanda 1987). It has been shown that *Anacystis nidulans* can take up NH_4^+ in the light against a concentration gradient. NH_4^+ uptake was completely inhibited by dark anaerobic conditions and by protonophores. It was also strongly inhibited by -SH reagents and by MSX, a specific and irreversible inhibitor of GS (Rowell et al. 1979). It was concluded (Boussiba et al. 1984) that a) interference with energy supply or with ammonia metabolism limits ammonia entry into the cells; and b) the glutamine synthetase/glutamate synthase enzyme system is the primary ammonia assimilation pathway in *Anacystis nidulans*. The latter conclusion was also arrived at by Ohmori and Ohmori (1988) for *Spirulina platensis*, although they also suggest

Abbreviations: MSX, methionine sulfoximine; GS, glutamine synthetase.

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that in this organism alanine dehydrogenase plays a minor part in the assimilation process. The role of glutamate dehydrogenasse was found by Ohmori and Ohmori (1988) to be negligable, as was generally observed for other cyanobacteria (Guerrero and Lara 1988).

In the present study we found that ammonia (2.5 mM) can support growth at a fast rate (9.3 h doubling time). Ammonia uptake at pH 7.0 and pH 10.0 showed different kinetics. The mechanisms by which ammonia enters the cell of this cyanobacterium are discussed. Please note that term ammonia is used here to cover both the protonated and unprotonated forms of this compounds, while NH_3 or NH_4^+ are used where one or the other forms is meant specifically.

Materials and Methods

Organism and growth conditions—*Spirulina platensis* (LB1475/a), obtained from the Cambridge Culture Collection, was grown in Zarouk's medium (Zarouk 1966). The cyanobacteria were cultivated either in continuous culure as described previosuly (Boussiba and Richmond 1980), or in 500 ml batch cultures, in glass columns. Growth temperature was 35°C; pH was maintained in the range of 8.9-9.1, and the cultures were stirred by means of an air stream (4 liters·min^{-1}) containing 1.5% CO_2. Illumination was provided by cool white fluorescent lamps at a constant light intensity of 180 $\mu E \cdot m^{-2} \cdot s^{-1}$. Cultures grown continuously were allowed to grow for at least 10 doublings, after which they were regarded to be at steady state. Cells from steady state cultures were used for most of the experiments.

Ammonia uptake assays—The experiments were performed as described previosuly (Boussiba et al. 1984) with some modifications. Cell suspensions (15 ml) containing up to 1 mg protein·ml^{-1} were placed in a 50-ml Erlenmeyer flask and agitated gently in a water shaker-bath at 35°C, at a light intensity of 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ provided by overhead cool-white fluorescent lamps. NH_4Cl was added to final concentrations ranging from 10 to 1,000 μM. Samples were drawn out at intervals, filtered through a 25-mm GF/C filter and the ammonia in the filtrate was determined by the phenol alkaline hypochlorite method (Solorzano 1969), as previously described (Boussiba et al. 1984). Uptake assays were prolonged either for 20 min when the uptake was correlated to metabolic activity of the cells, or for short periods (up to 1 min), when the mechanism of uptake was studied. For the determination of pH optimum the following buffers were used: 20 mM phosphate buffer for pH 6.0-8.0 or 50 mM bicarbonate buffer for pH 9.0-10.8. A control experiment showed that at the pH range used there was no disappearance of ammonia from a medium free of cells under the experimental conditions.

Measurements of oxygen evolution and consumption—Cells were washed and resuspended in a fresh Zarouk medium, which was adjusted to different pH values, as outlined above. Suspensions at a concentration of 2.0 μg chlorophyll·ml^{-1} (150 μg protein·ml^{-1}) were used. The rates of oxygen evolution in the light (photosynthesis) and of oxygen consumption in the dark (respiration) were measured at 30°C with a Clark-type oxygen electrode (Yellow Springs, Ohio Instruments Co.) connected to a recorder (Vogor 310). The light intensity at the surface of the electrode cell was 700 $\mu E \cdot m^{-2} \cdot s^{-1}$.

Other methods—Glutamine synthetase assays were performed in concentrated suspensions (1 mg protein·ml^{-1}). Cells were then permeabilized with 2% toluene for one minute and kept on ice for 15 min. Activity was measured as transferase (Sampio et al. 1979). Pigments extraction and quantification were performed as described by Boussiba and Richmond (1979). Total protein was determined by Lowry's procedure (1951). For the determination of ammonia and nitrate in the growth medium, Nessler's methhod, as modified by Abeliovich and Azov (1979), and the Szechrome NAS method (Shilo and Rimon 1982) were respectively employed.

Results and Discussion

Ammonia as a nitrogen source

S. platensis grown in batch culture utilized either nitrate or ammonia (Fig. 1). Except for a 1 day lag in the presence of ammonia, growth rates with the two nitrogen sources were similar. This cyanobacterium can thus assimilate ammonia at pH 9.0 as its sole nitrogen source and does so with the same efficiency as for nitrate. Moreover, when both these nitrogen substrates were present in the growth medium, ammonia was used preferentially (Fig. 1, insert), as has also been observed in neutrophilic cyanobacteria and green algae (Ohmori et al. 1977, Cresswell and Syrett 1979).

Further evidence for the ability of this cyanobacterium to use ammonia as its nitrogen source without its growth being adversely affected was obtained from continuous cultures. Under steady state conditioins, cultures fed with NO_3^- and NH_4^+ exhibited the same doubling time, i.e. 9.3 h (Table 1). GS activity in cells grown on ammonia was only 25% of that found in cells grown on NO_3^- (Table 1), a response generally observed in neutrophilic cyanobacteria growing in the presence of NH_4^+ (Rowell et al. 1979, Tuli and Thomas 1980, Zimmerman and Boussiba 1987).

Ammonia uptake and metabolic activities

When relatively dilute *S. platensis* suspensions (0.3 mg protein·ml^{-1}) were used, ammonia uptake appeared to be linear for over 20 min. It proceeded at similar rates in the light and in the dark (Fig. 2A, B, respectively), exhibiting an identical pH dependency: no activity at

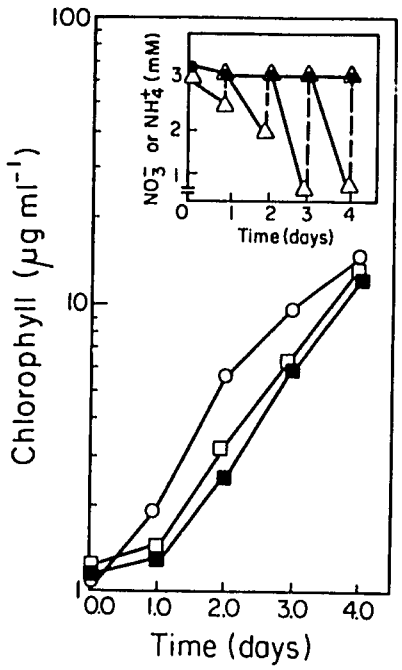

Fig. 1 Effect of the nitrogen source on growth of *S. platensis* in batch cultures. -○-, 3 mM NO_3^-, adjusted daily; -□-, 3 mM NH_4^+, adjusted daily; -■-, 3 mM NO_3^- + 3mM NH_4^+, only NH_4^+ being adjusted daily.
Insert: fluctuations of NO_3^- (●) and NH_4^+ (△) in the medium during growth in the presence of both NO_3^- and NH_4^+. Broken lines, daily adjustment of NH_4^+ to initial concentration.

pH 6.0, an optimum at pH 9.0 and approximately 80% of the optimum at pH 10.8 (Fig. 2). These findings, however, differ from those observed in several neutrophilic cyanobacteria, e.g. *Anacystis nidulans* (Boussiba et al. 1984) and *Anabaena azollae* (Zimmerman and Boussiba 1987) in which NH_4^+ uptake was totally pH independent, exhibiting the same rate between pH 6 and 9. This may indicate that in *Spirulina* ammonia penetrates via a mechanism different from that operating in the latter cyanobacteria.

The same pattern observed for ammonia uptake was found for other metabolic activities, such as light dependent O_2 evolution and dark O_2 uptake (Fig. 2A, B, respectively). These data indicate that ammonia uptake may be related to the overall activities of the cell.

The step in which ammonia uptake is likely to interact with cellular metabolism is its actual assimilation, i.e. the activity of glutamine synthetase. To investigate this possibility the effects of glutamine and MSX (a specific inhibitor of the glutamine synthetase, Rowell et al. 1979), were tested at two pH values. At pH 7.0, a low concentration of MSX (5 μM) completely inhibited NH_4^+ entry almost instantaneously (Fig. 3a), the inhibition being accompanied by a total loss of GS activity (Fig. 3b). At pH 10.0 NH_3 uptake and GS activity were also completely inhibited by MSX (Fig. 3a, b), but much higher concentrations (100 μM) were required, and only after 5 min from the time

Table 1 Steady state growth rate and glutamine synthetase activity of *Spirulina platensis* cultivated under different nitrogen sources

Nitrogen source	(mM)	d.t.[a] (h)	GS activity[b]
NO_3^-	3.0	9.6	0.95
NH_4^+	2.5	9.3	0.20

[a] doubling time.
[b] μmol glutamylhydroxamate·min^{-1}·(mg protein)$^{-1}$.

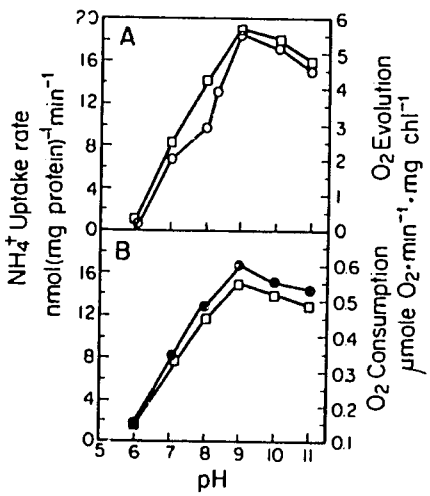

Fig. 2 Effect of pH on ammonia uptake and on O_2 evolution in the light (A) and dark O_2 consumption (B) in *S. platensis*. *Spirulina* cells grown under steady state conditions, were washed in fresh medium and adjusted to different pH values as indicated in Materials and Methods. For the assays of ammonium uptake (20 min), cells were concentrated to 0.3 mg protein·ml^{-1}, and NH_4^+ was added to a final concentration of 50 μM. For oxygen measurements, cells were diluted to 2 μg chl·ml^{-1} (150 μg protein·ml^{-1}). Numbers are average of five different experiments; in all cases standard deviation did not exceed 10%. -□-, NH_4^+ uptake; -○-, O_2 evolution; -●-, O_2 consumption.

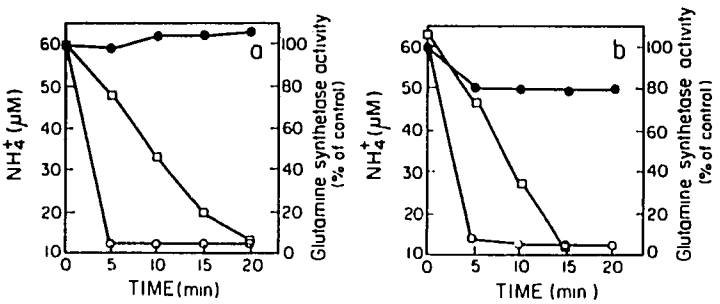

Fig. 3 Effect of MSX on ammonia uptake and glutamine synthetase activity in *S. platensis* at pH 7.0 (a) and pH 10.0 (b). *Spirulina* cells were washed and treated as described in Fig. 2, and concentrated to 0.3 mg protein·ml^{-1}. Ammonium and MSX were added at the beginning of the assay at the following final concentrations: NH_4^+, 60.0 μM; MSX, 5 μM at pH 7.0 and 100 μM at pH 10.0. GS in the control treatments were 1.1 and 0.95 μmol glutamylhydroxamate·(mg protein)$^{-1}$·min^{-1} at pH 10 and pH 7 respectively. Numbers are average of five differents experiments, standard deviation did not exceed 12%. -○-, GS activity; -○-, NH_4^+; -●-, NH_4^+ + MSX.

of application. When added together with MSX at pH 10.0, glutamine alleviated the inhibitory effect of MSX on both ammonia uptake and GS activity, probably by protecting the latter (Table 2). The same results were obtained at pH 7.0 (data not shown). These data further support the assumption (Boussiba and Gibson 1985) that primarily MSX inhibits GS, rather than a direct inhibition of the ammonium carrier (Turpin et al. 1984), and that net ammonia uptake is observed only when conditions permit continuous amidation, i.e. GS activity. These findings differ also from previously reported data in *Klebsiella pneumoniae* (Kleiner and Castorph 1982) suggesting that the ammonium transport system contains a regulatory site for glutamine and its analog MSX.

Ammonia uptake mechanism and the effect of pH

Ammonia uptake was studied within short intervals (1 min) to study its influx mechanism, at pH 7.0 (practically all [> 99%] of the ammonia is protonated) and at pH 10.0 (over 90% unprotonated). The patterns of uptake at these pH values differed greatly: at pH 7.0, typical Michaelis-Menten kinetics were obtained with a K_m of 34 μM and a V_{max} of 22 nmol·(mg protein)$^{-1}$·min^{-1} (Fig. 4). At pH 10.0, the uptake rate increased with ammonia concentration, and saturation was not reached even at 1 mM ammonia (Fig. 4). These data may suggest different mechanisms of ammonia entry into this cyanobacterium but may also reflect different kinetics of glutamine synthetase at the two pH values.

As noted above, linear ammonia uptake were observed at all the pH values tested when dilute cell suspensions were used. When the experiments were conducted using higher

Table 2 Effect of MSX and glutamine on ammonium uptake and glutamine synthetase activity in *Spirulina platensis*

Additions	Uptake rate[a]	GS activity[b] (% of control)
no	22.5	100
50 μM MSX	0	5
500 μM gln	19.7	95
50 μM MSX + 500 μM gln	20.3	92

[a] nmol·min^{-1}·(mg protein)$^{-1}$.

[b] 1.15 μmol glutamylhydroxamate·min^{-1}·(mg protein)$^{-1}$.

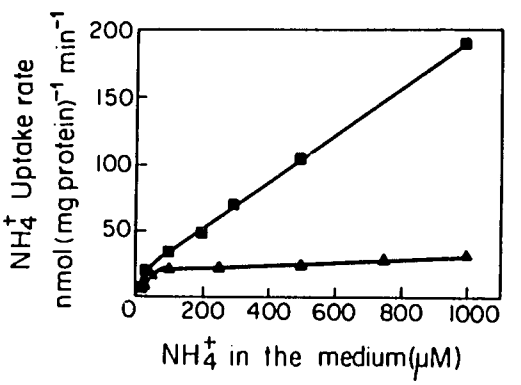

Fig. 4 Effect of pH on ammonia uptake by *S. platensis* as a function of NH_4^+ concentration in the medium. Numbers are average of five different experiments, standard deviation did not exceed 15%. -▲-, pH 7.0; -△-, pH 10.0.

cell densities (1 mg protein·ml^{-1}), an additional phenomenon was observed. At pH 10.0 the uptake consisted of two components: a rapid initial disappearance of ammonia from the medium immediately upon its application, which lasted less than 5 seconds (the shortest period measured), and a subsequent slow phase (Fig. 5). The fast phase was totally insensitive to MSX and took place even when GS was completely inhibited. (Fig. 5). The slow component at pH 10 was totally inhibited by MSX through its effect on GS activity (Fig. 5). At pH 7.0 the uptake was slow and at almost constant rate and completely inhibited by MSX (Fig. 5).

The different uptake patterns observed at these pH values (Fig. 4, 5) suggest the possible involvement of more than one mechanism for the entry of ammonia into *S. platensis*. At pH 7.0 the ammonium ion (NH_4^+) probably crosses the plasma membrane against a concentration gradient (Boussiba et al. 1984) via an active process characterized by saturation kinetics (Fig. 4) as has been suggested also for neutrophilic cyanobacteria (Boussiba et al. 1984, Kashyap and Johar 1984, Turpin et al. 1984, Zimmerman and Boussiba 1987, Ritchie and Gibson 1987, Ohmori and Kanda 1987). At pH 10 a diffusion mechanism which consists of two phases may be responsible for the entry of ammonia. The fast component is driven by ΔpH acidic inside. Preliminary results (S. Boussiba and S. Belkin, unpublished data) indicate that at pH 10.0 the cytoplasmic pH is approximately 8.5. The ΔpH is therefore large enough to support the inward movement of the uncharged ammonia, and lead to its intracellular accumulation (Schuldiner et al. 1972, Gaensslen and McCarty 1971). Indeed when cells were toluenized and the ΔpH collapsed, the fast influx of ammonia was totaly abolished, indicating that uptake of ammonia from the medium was not caused by unspecific binding. Following this initial fast entry, the continuous activity of glutamine synthetase supports further entry by reducing internal ammonia concentration, thus supplying the driving force for the second slower phase. The fast phase was thus apparent only in the presence of high cell densities, since only then were significant amount of ammonia removed from the medium.

The data presented suggest that ammonia can serve as nitrogen source for optimal growth of *Spirulina* at high pH, and that GS is the ultimate regulator for ammonia uptake, most likely through the catalysis of nitrogen assimilation. Further support for the latter assumption is provided by prolonged inhibition of GS by MSX, which causes leakage of ammonia from cells of *S. platensis* (unpublished results) as has been reported in other cyanobacteria (Boussiba et al. 1984, Zimmerman and Boussiba 1987). We suggest that under optimal (alkaline) pH the entry of ammonia into alkalophilic cyanobacteria such as *Spirulina* is primarily a diffusion process driven by the pH gradient on the one hand, and by the GS dependent intracellular ammonia assimilation on the other hand.

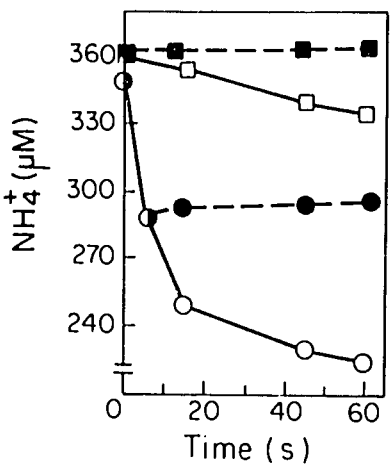

Fig. 5 Effect of MSX on short-term ammonia uptake by *S. platensis* at pH 7.0 and pH 10.0. *Spirulina* cells taken from cultures growing at steady state were washed in fresh medium, resuspended in the appropriate buffers (see Materials and Methods) and concentrated up to 1 mg protein·ml^{-1}. Ammonia concentration at zero time was 350 μM. MSX was added 5 min before ammonium uptake assays started, by which time GS activity was completely inhibited. MSX concentration was 5 μM at pH 7.0 and 100 μM at pH 10.0. Numbers are average of five different experiments, standard deviation did not exceed 15%.
Open symbols without MSX, closed symbols with MSX. -●-, pH 7.0; -○-, pH 10.0.

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References

Abeliovich, A. and Azov, Y. (1979) Toxicity of ammonia to algae in sewage oxidiation ponds. *Appl. Environ. Microbiol.* 31: 801–806.

Boussiba, S. and Gibson, J. (1985) The role of glutamine synthetase activity in ammonium and methylammonium transport in *Anacystis nidulans R-2*. *FEBS Lett.* 180: 13–16.

Boussiba, S., Resch, C. R. and Gibson, J. (1984) Ammonia uptake and retention in some cyanobacteria. *Arch. Microbiol.* 138: 287–292.

Boussiba, S. and Richmond, A. E. (1979) Isolation and characterization of phycocyanins from the blue-green alga *Spirulina platensis*. *Arch Microbiol.* 119: 155–159.

Boussiba, S. and Richmond, A. E. (1980) C-Phycocyanin as a storage protein in the blue-green algae *Spirulina platensis*. *Arch. Microbiol.* 125: 143–147.

Cresswell, R. C. and Syrett, R. J. (1979) Ammonium inhibition of nitrate uptake by the diatom *Phaeodactylum tricornutum*. *Plant Sci. Lett.* 14: 321–325.

Gaenssien, R. E. and McCarty, R. E. (1971) Amine uptake in chloroplasts. *Arch. Biochem. Biophys.* 147: 55–65.

Guerrero, M. G. and Lara, C. (1988) Assimilation of inorganic nitrogen. *In* The Cyanobacteria. Edited by Fay, P. and Van Baalen, C. V. pp. 163–176. Elsevier, Amsterdam.

Kashyap, A. K. and Johar, G. (1984) Ammonium transport in the nitrogen-fixing cyanobacterium *Nostoc muscorum*: interaction with copper and sulfhydryl agents. *J. Gen. Appl. Microbiol.* 30: 267–271.

Kleiner, D. (1985) Bacterial ammonium transport. *FEMS Microbiol. Rev.* 32: 87–100.

Kleiner, D. and Castorph, H. (1982) Inhibition of ammonium (methylammonium) transprot in *Klebsiella pneumoniae* by glutamine and glutamine analogs. *FEBS Lett.* 146: 201–203.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265–275.

Ohmori, M. and Kanda, J. (1987) H^+ and K^+ efflux associated with NH_4^+ uptake in *Anabaena cylindrica* cells in the dark. *J. Gen. Appl. Microbiol.* 33: 311–319.

Ohmori, K. and Ohmori, M. (1988) Ammonium assimilation in the blue green alga *Spirulina platensis*. *Jpn. J. Phycol.* 36: 12–16.

Ohmori, M., Ohmori, K. and Strotmann, H. (1977) Inhibition of nitrate uptake by ammonia in a blue-green alga *Anabaena cylindrica*. *Arch. Microbiol.* 114: 255–262.

Richmond, A. (1986) Microalgalculture. *CRC Critical Review in Biotech.* 4: 369–438.

Ritchie, R. E. and Gibson, J. (1987) Permeability of ammonia, methylamine and ethylamine in the cyanobacterium *synechococcus R-2 (Anacystis nidulans)* PCC 7942. *J. Membrane Biol.* 95: 131–142.

Rowell, P., Sampio, M. J. A. M., Lada, J. K. and Stewart, W. D. P. (1979) Alteration of glutamine synthetase activity in vivo in response to light and NH_4^+. *Arch. Microbiol.* 119: 195–190.

Sampio, M. J. A. M , Rowel, P. and Stewart, W. D. P. (1979) Purification and some properties of glutamine synthetase from the nitorgen fixing cyanobacterium *Anabaena cylindrica*. *J. Gen. Microbiol.* 111: 181–191.

Schuldiner, S., Rotenberg, H. and Avron, M. (1972) Determination of ΔpH in chloroplasts. *Eur. J. Biochem.* 25: 64–70.

Shilo, M. and Rimon, A. (1982) Factors which affect the intensification of fish breeding in Israel. 2. Ammonia transformation in intensive fish ponds. *Bamidgeh Bull Fish Culture* 34: 101–114.

Solorzano, L. (1969) Determination of ammonia in natural waters by the phenolhypochlorite method. *Limnol. Oceanogr.* 14: 799–801.

Tuli, R. and Thomas, J. (1980) Regulation of glutamine synthetase in the blue green alga *Anabaena* L 31. *Biochim. Biophys. Acta* 613: 526–533.

Turpin, D. H., Scott, A. E. and Canvin, D. T. (1984) In vivo nitrogenase regulation by ammonium and methylamine and the effect of MSX on ammonium transport in *Anabaena flosaquae*. *Plant Physiol.* 74: 701–704.

Zarouk, C. (1966) Contribution a l'etude d'une cyanophycee. Influence de divers facteurs physiques et chimiques sur la croissance et al photosynthese de *Spirulina maxima*. Ph. D. Thesis. Univ. of Paris.

Zimmerman, W. J. and Boussiba, S. (1987) Ammonia assimilation and excretion in an asymbiotic strain of *Anabaena azollae* from *Azolla filiculoides Lam*. *J. Plant Physiol.* 127: 443–450.

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Ammonium Excretion by an L-Methionine-DL-Sulfoximine-Resistant Mutant of the Rice Field Cyanobacterium *Anabaena siamensis*

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An ammonium-excreting mutant (SS1) of the rice field nitrogen-fixing cyanobacterium *Anabaena siamensis* was isolated after ethyl methanesulfonate mutagenesis by selection on 500 μM L-methionine-DL-sulfoximine. SS1 grew in the presence and absence of L-methionine-DL-sulfoximine at a rate comparable to that of the wild-type strain, with a doubling time of 5.6 h. The rate of ammonium release by SS1 depended on cell density; it peaked at the 12th hour of growth with 8.7 μmol mg of chlorophyll^{-1} h^{-1} (at a chlorophyll concentration of 5 μg ml^{-1}) and slowed down to almost nil at the fourth day of growth. A similar pattern of release by immobilized SS1 was observed between 12 to 20 h after loading alginate beads in packed-bed reactors at the rate of 11.6 μmol mg of chlorophyll^{-1} h^{-1}. The rate was later reduced significantly due to the fast growth of SS1 on the substrate. Prolonged release of ammonium at the peak level was achieved only by maintaining SS1 under continuous cultivation at low chlorophyll levels (5 to 7 μg ml^{-1}). Under these conditions, nitrogen fixation in the mutant was 30% higher than that in its parent and glutamine synthetase activity was less by 50%. Immunoblot analysis revealed that SS1 and its parent have similar quantities of glutamine synthetase protein under ammonium excretion conditions. In addition, a protein with a molecular weight of about 30,000 seems to have been lost, as seen by electrophoretic separation of total proteins from SS1.

Nitrogen-fixing cyanobacteria are being used as nitrogen biofertilizers in rice fields in countries where rice is the major staple diet (30, 31). Although some strains which thrive in rice fields release small quantities of the major fertilizing product, ammonia, during active growth, most of the fixed products are made available mainly through autolysis and microbial decomposition (18). Under these circumstances, it is difficult to control the flow of nitrogen compounds needed for the development of rice plants. A possible solution to this problem is to develop strains of cyanobacteria which release ammonium continuously.

Mutants of *Anabaena variabilis* and *Nostoc muscorum* resistant to the ammonium analog ethylenediamine and to the L-glutamate analog L-methionine-DL-sulfoximine (MSX) have been reported to release ammonium (11, 12, 22, 24, 26). They exhibit, however, a slower growth rate as compared with that of their parents. Another major problem of using cyanobacteria as biofertilizers is the competition between indigenous and introduced strains, the former generally dominating. It was assumed that ammonium-excreting mutants that had been isolated from strains indigenous to rice fields would overcome the constraints of the rice field environment better than strains derived from other habitats. The reinoculation and establishment of these mutants in rice fields would thus be comparatively more successful.

In the present work, we used a rice field isolate of *A. siamensis* which has a fast growth rate (0.123 h^{-1}) and high nitrogen-fixing capacity (6). This strain also adapts well to temperature fluctuations (25 to 42°C) prevailing in rice fields and to the salinity ranges (1 to 2%) existing in most of the tropical wetland soils (5). We hereby describe the characterization of an ammonium-excreting mutant of *A. siamensis*.

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MATERIALS AND METHODS

Organism. The nitrogen-fixing cyanobacterium *A. siamensis* used in this study, originally isolated in Thailand (6), was obtained from the Sammlung von Algenkulturen, Pflanzenphysiologisches Institute, Universität Göttingen, Göttingen, Federal Republic of Germany, under the signature *Anabaena* sp. strain B 11.82.

Growth conditions. *A. siamensis* was cultivated in AS medium (1) in 500-ml sterilized glass columns placed in a transparent Plexiglas circulating water bath. Water temperature was maintained at 42°C. A constant photon flux of 175 microeinsteins m^{-2} s^{-1} at the surface of the growth vessel was supplied laterally by a battery of eight cool-white fluorescent lamps. Continuous aeration was provided by bubbling filtered air containing 1.5% CO_2. The pH was thus maintained at 7.0 to 7.2. Unless otherwise stated, cultures were sampled during the logarithmic growth phase for use in the different experiments.

Continuous culture experiments were carried out as described previously (7). The cultures were maintained at 5 μg of chlorophyll ml^{-1}.

Immobilization was carried out by using the alginate entrapment method (11) with a few modifications. Cyanobacterial cell suspensions containing 10 μg of chlorophyll ml^{-1} were mixed with an equal volume of 3% sodium alginate solution and added dropwise into 0.1 M $CaCl_2$ solution through a capillary tube to form beads of 2 to 5 mm in diameter. The alginate-entrapped cells were collected after 2 h and kept at 4°C for 12 h. The beads were loaded into packed-bed reactors to collect the ammonium released into the culture medium with a dilution rate of 0.2 h^{-1}.

Growth was followed by means of chlorophyll and protein determinations; the initial inoculum contained 1 μg of chlorophyll ml^{-1}, which corresponded to a protein concentration of 26.3 μg ml^{-1}. Chlorophyll *a* was determined colorimetrically in methanol extracts (17), and protein was determined

by the method of Lowry et al. (16) after digestion with 0.5 N NaOH.

Mutagenesis. Mutagenesis with ethyl methanesulfonate was carried out as reported by Spiller et al. (26). Mutants resistant to MSX were selected on agar plates containing 500 μM MSX. Ammonium-excreting mutants were selected on plates containing the pH indicator phenol red. One of the mutants, designated as SS1, caused a strong change of color on the plates and was used for further studies.

Phycocyanin determinations. Five milliliters of cell suspension was centrifuged at 10,000 × *g* for 10 min at 4°C. The pellet was suspended in the same volume of 20 mM sodium acetate buffer, pH 5.5, containing 3 mM sodium azide and 10 mM disodium EDTA. Cells were broken by sonication at 4°C and centrifuged at 10,000 × *g* for 10 min at 4°C to remove membrane fragments containing chlorophyll. Phycocyanin concentration was calculated from measurements of optical densities at 620 and 650 nm (28).

Heterocyst frequencies. Heterocyst frequencies were determined microscopically. Ten independent counts of about 100 vegetative cells each were made for each sample.

Ammonium uptake. Ammonium uptake was followed for 20 min starting from an initial concentration of 100 μM, as described previously (32). Cell concentration for the treatments corresponded to 120 μg of protein ml^{-1}.

Ammonium determinations. Culture filtrates were collected during growth and analyzed for the amounts of ammonium released into the medium by Solorzano's phenol-hypochlorite method (25).

Enzyme assays. Nitrogenase activities were estimated in intact filaments by the acetylene reduction assay (27). Samples of 4.6 ml of culture were washed, suspended in fresh AS medium, and placed in 25-ml Wheaton bottles sealed with a flanged rubber septum. The Wheaton bottles were placed on a rotary shaker (100 rpm) and illuminated with a quantum flux of 75 microeinsteins m^{-2} s^{-1} during the assay. The filaments were allowed to stand for 10 min before injection of acetylene. Ethylene was analyzed with an HP 5890 gas chromatograph, using a stainless-steel column packed with Porapack-N (0.2-cm inside diameter, 265-cm length). Nitrogenase activities were expressed as micromoles of C_2H_4 produced per milligram of chlorophyll per hour.

Nitrogenase activities in immobilized cyanobacteria were determined as described above by incubating the alginate beads (with 5 μg of chlorophyll ml^{-1}) in 130-ml Wheaton bottles.

Glutamine synthetase (GS) activities were assayed in concentrated suspensions (1 mg of protein ml^{-1}) of cells which were permeabilized with 2% toluene for 1 min and then kept on ice for 15 min. Activity was measured as transferase and expressed in micromoles of γ-glutamyl hydroxamate formed per milligram of protein per minute (23). For in vitro GS activity determinations, 500 μM MSX was added to the enzyme assay mixture.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Exponentially growing cells were harvested and suspended in 20 ml of ice-cold Tris hydrochloride buffer (pH 8.0)–2 mM disodium EDTA–3 mM dithiothreitol–5 mM $MgCl_2$–1 mM phenylmethylsulfonyl fluoride. The cells were lysed by passage through a French pressure cell (113 kg cm^{-2}) and then centrifuged (10,000 × *g*) for 30 min at 4°C to remove debris and unbroken cells. Soluble polypeptides were precipitated by addition of trichloroacetic acid (to 5%), and the pellet recovered after centrifugation was washed with ice-cold acetone. The proteins were suspended in Laemmli's breaking buffer, boiled for 5 min, separated by electrophoresis through 10% (wt/vol) polyacrylamide gels containing 0.1% (wt/vol) sodium dodecyl sulfate (13), and then stained with Coomassie blue G. The molecular weights of standard polypeptides (Sigma Chemical Co.) used were as follows: 200,000 (myosin); 116,250 (β-galoctosidase); 97,400 (phosphorylase *b*); 66,200 (bovine albumin); 45,000 (egg albumin); 31,000 (carbonic anhydrase).

Immunoblot analysis. Following electrophoresis, proteins were transferred electrophoretically to a nitrocellulose membrane filter (0.45 μm; Sigma Chemical Co.) at 110 V for 1 h in an ABN PolyBlot transfer system (American Bioneties) (29). The GS levels in dot blot and Western blot (immunoblot) analyses were detected with the antiserum against purified *Anabaena* sp. strain 7120 GS by the method of Orr and Haselkorn (21) and the reagents of Stratagene Cloning Systems (picoBlue ImmunoDetection Kit) per the kit directions.

Chemicals. MSX was purchased from Sigma, and other chemicals were from E. Merck AG. Sodium alginate was the product of Aldrich Chemical Co. Chemicals used for polyacrylamide gel electrophoresis were acquired from Bio-Rad Laboratories.

RESULTS

Growth characteristics of SS1. SS1 mutant contained chlorophyll (Fig. 1A) and protein (Fig. 1B) at the same level as the parent throughout the growth cycle. At the optimal temperature (42°C), both mutant and wild-type strains grew with a doubling time of 5.6 h, whether the nitrogen source was molecular nitrogen (Fig. 1) or nitrate (data not shown), attaining the stationary phase on day 3. The doubling time was reduced when ammonium or glutamine were used as the sole nitrogen source (about 4 and 4.5 h, respectively). Both strains grew at the same rate when immobilized (Table 1). SS1 exhibited phycocyanin levels (14.0 μg ml^{-1}) significantly higher than in the parent strain (9.6 μg ml^{-1}). Addition of 500 μM MSX to wild-type liquid cultures caused chlorosis (Fig. 1A), followed by filament lysis (Fig. 1B).

Rate of ammonium release. No detectable ammonium was released by the parent strain during active growth (data not shown). The rate of ammonium production by SS1 during the growth cycle in a batch culture (Fig. 2) was the highest at the 12th hour (8.7 μmol mg of $chlorophyll^{-1}$ h^{-1}) and declined during the entry to the stationary phase at day 2; subsequently, the rate was almost nil. In continuous cultures, on the other hand, the rate of ammonium production was constant, 8.7 μmol mg of $chlorophyll^{-1}$ h^{-1}, similar to the rate at the 12th hour of growth in batch cultures (Table 1). A higher rate of ammonium production, 11.6 μmol mg of $chlorophyll^{-1}$ h^{-1}, was obtained in immobilized cells (Table 1).

Ammonium uptake. Both the parent and mutant strains showed the same pattern of ammonium uptake in the absence of MSX (Fig. 3), 32.6 and 30.1 nmol mg of $protein^{-1}$ min^{-1}, respectively. Addition of 200 μM MSX completely inhibited ammonium uptake only in the parent, but did not change the rate of uptake by SS1, 30.6 nmol μg of $protein^{-1}$ min^{-1}, with or without the inhibitor.

Nitrogenase activity. Both strains exhibited identical frequencies of heterocysts (about 19%) and the same pattern of acetylene reducing activity during the growth cycle (Fig. 4). When the cultures became dense, their nitrogenase activities decreased, probably due to shortage of light available to the cells rather than to the phase of growth; the specific activity of the enzyme immediately after diluting the stationary-

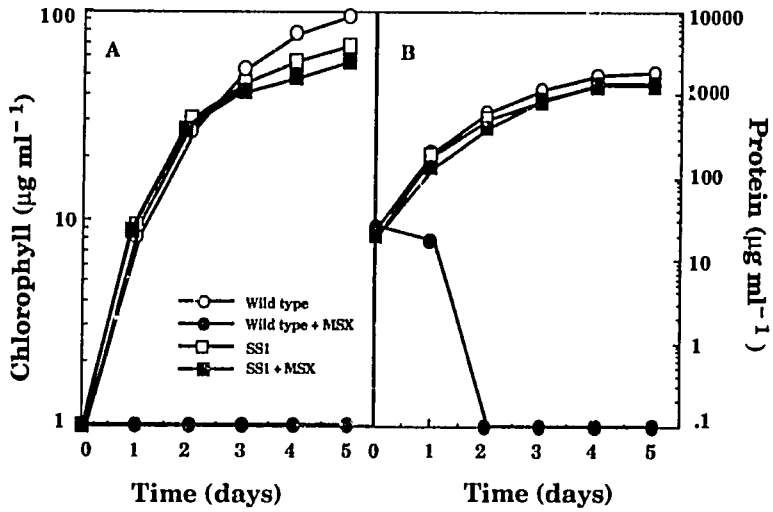

FIG. 1. Growth of the parent *A. siamensis* and its SS1 mutant in the presence and absence of 500 μM MSX, in terms of chlorophyll (A) and protein (B) concentrations.

phase culture was similar to that in log phase (results not shown). Both the parent and mutant strains exhibited acetylene reducing activities in the presence of ammonium, the rate being higher in SS1 (8.9 μmol of C_2H_4 mg of chlorophyll^{-1} h^{-1} versus 1.8 μmol of C_2H_4 mg of chlorophyll^{-1} h^{-1}). Nitrogenase activity was 30% higher in SS1 than in the parent strain at the 12th hour of growth, reaching a value of 35.5 μmol of C_2H_4 mg of chlorophyll^{-1} h^{-1}; in a continuous culture, it was similarly higher (Table 1). The activity in SS1 and not in the parent was further enhanced (16%) by immobilization. Increased nitrogenase activities were observed in other cyanobacterial species following immobilization (8, 19).

GS activity. In the presence of 500 μM MSX, the GS activity of the parent strain was completely inhibited. The mutant strain showed <50% of GS activity in both the presence and the absence of MSX, compared with that of the wild-type strain (without MSX), during the whole growth cycle (Fig. 5) and under immobilizing conditions (Table 1). Similar reduction in SS1 GS activity was also observed during in vitro assay (1.6 μmol of γ-glutamyl hydroxamate mg of protein^{-1} min^{-1} versus 3.1 μmol of γ-glutamyl hydroxamate mg of protein^{-1} min^{-1} in the parent).

Protein analysis. When the total proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gels, the absence of a 30,000-dalton polypeptide was noticed in the profile of SS1 (Fig. 6). Preliminary analysis revealed that it was a soluble cytoplasmic polypeptide (results not shown).

Immunoassay of GS protein. Immunoblot analysis of crude lysates at various dilutions showed similar extents of antigen reaction in the parent and SS1 (Fig. 7A). GS protein levels and its mobility seem to be identical in the parent and SS1 in a Western blot analysis (Fig. 7B).

TABLE 1. Growth rates, enzyme activities, and ammonium excretion rates for the parent and SS1 grown under different growth conditions

Growth conditions[a]	Nitrogenase activity (μmol of C_2H_4 mg of chlorophyll^{-1} h^{-1})	GS activity (μmol of γ-glutamyl hydroxamate mg of protein^{-1} min^{-1})	Rate of NH_4^+ excretion (μmol mg of chlorophyll^{-1} h^{-1})
Batch culture			
Parent	21.0	3.6	0.0
SS1	35.5[b]	1.3	8.7[b]
Continuous culture			
Parent	24.5	3.0	0.0
SS1	35.3	1.4	8.7
Immobilized culture			
Parent	20.5	3.1	0.0
SS1	41.3[b]	1.2	11.6[b]

[a] Doubling time was 5.6 h under all conditions for both parent and SS1.
[b] Measured after 12 h of inoculation.

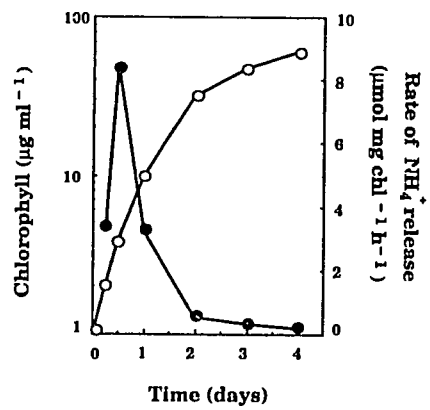

FIG. 2. Chlorophyll content (O) and rate of ammonium release (●) during the growth cycle of the SS1 mutant.

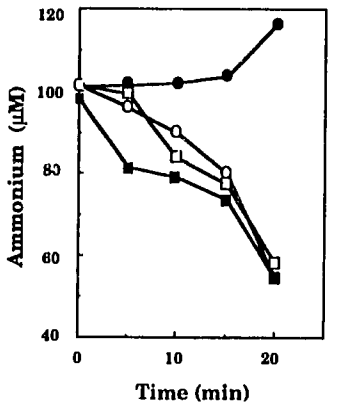

FIG. 3. Ammonium uptake by the parent (O) and SS1 (□). Closed symbols are treatments with MSX.

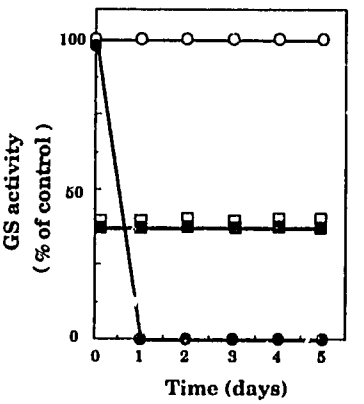

FIG. 5. GS activity in the parent and SS1 strains grown with and without MSX. Closed symbols indicate the addition of MSX. Circles and squares represent the parent and the mutant, respectively.

DISCUSSION

The contribution of nitrogen-fixing cyanobacteria to the productivity of rice fields has long been recognized (30), but the inoculation of nonindigenous cyanobacterial strains is not as successful as expected due to failure to overcome the interspecific competition and environmental constraints. The need thus arises to identify good strains among the native populations of nitrogen-fixing cyanobacteria having high potentials of increasing the yield of rice plants. In this respect, *A. siamensis* proved promising (5) and is already marketed as an algal biofertilizer for rice fields. Its efficiency in increasing the growth and yield of rice plants is apparently due to its high nitrogen-fixing capacity (2, 3). It was reported to release a variety of amino acids during active growth (4), but not ammonium as observed for other nitrogen fixers found in rice fields (30). It does release ammonium into the medium in the presence of MSX, an inhibitor of GS activity (results not shown). Selection of mutants resistant to MSX was found to lead to reduction in GS activity (11). The unassimilated ammonium is consequently released into the medium without induction by MSX (24, 26). It is assumed that a strain releasing ammonium continuously would be a better biofertilizer (14). The MSX-resistant mutant of *A. siamensis* isolated in this study, SS1, seems to conform to this expectation.

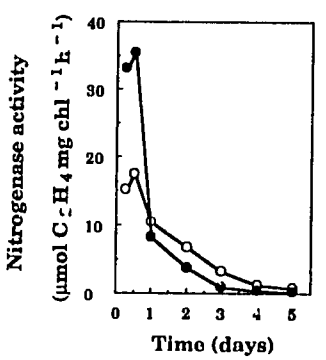

FIG. 4. Nitrogenase activity of the parent (O) and SS1 (●) during growth in batch cultures expressed by acetylene reduction.

SS1 released ammonium due to the high activity of nitrogenase, both being controlled by the cell density of the culture (Fig. 2 and 4). The direct effector was apparently light availability, which became progressively limited as cell density increased. The rate of ammonium release was consequently maximum only during the early log phase of growth in batch cultures. A similar pattern was seen under immobilized conditions.

Based on the above observations, SS1 growing in continuous cultures at low cell densities (chlorophyll value of 5 to 7 μg ml^{-1}) seems an ideal system for sustained ammonium release. The rate of ammonium release by SS1 was lower than the rates obtained for other mutants of *A. variabilis*, i.e., 35 to 50 μmol mg of chlorophyll^{-1} h^{-1} in batch and immobilized cultures (11, 26). No data have been provided, however, on the excretion of ammonium by these mutants under steady-state growth conditions. Hence, comparison of rates of ammonium release by the various mutants is inade-

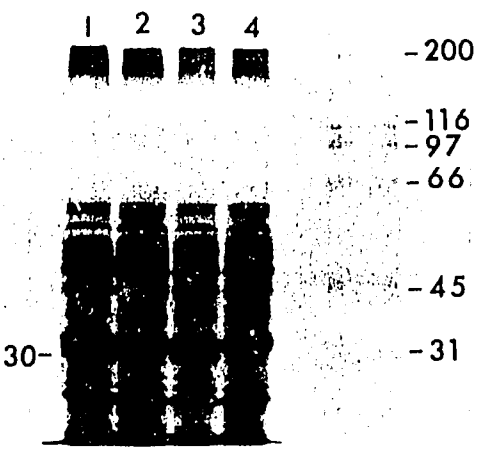

FIG. 6. Separation of total proteins from parent (lanes 1 and 3) and SS1 grown in the absence (lane 2) and presence (lane 4) of 500 μM MSX.

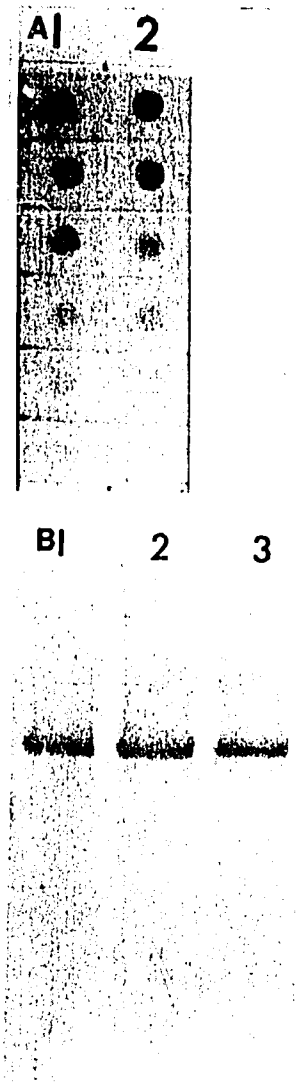

FIG. 7. Immunoblot analysis (A) of total proteins in the parent strain (1) and the mutant SS1 (2). Crude lysates were dotted from top to bottom with the following amounts of protein: 100, 50, 25, 12.5, 6.25, and 3.125 ng. Western blot analysis (B) of GS protein: lane 1, SS1 grown in the absence of MSX; lane 2, SS1 grown in the presence of MSX; lane 3, parent.

quate. The phycocyanin content of SS1 was also higher than of the parent, and it is possible that a certain proportion of the fixed nitrogen is expended for the synthesis of this storage product (7).

Nitrogenase activity in SS1 was 30 and 50% higher than that of the parent during steady-state growth (Fig. 4) and under immobilizing conditions (Table 1), respectively. In the presence of ammonium, SS1 nitrogenase activity was about fivefold higher than that in the parent due to a weaker repression by the end product (22). GS seemed to be defective in SS1 as well, exhibiting <50% of the parent enzyme activity (Fig. 5) without a significant reduction in protein content (Fig. 7A). It therefore appears that the decreased susceptibility of nitrogenase activity to repression by ammonium and the defective activity of GS are related to ammonium excretion in SS1. The ammonium-excreting mutants of *A. variabilis* (9), i.e., SA1, ED81, and ED92, were found to have derepressed nitrogenase and lower GS activities. Analyses of GS and its mRNA in the two ethylenediamine-resistant mutants (9) suggested that one of them (ED92) was a regulatory mutant containing less GS mRNA and consequently less GS protein as found for *A. azollae* growing in symbiosis (20). The other (ED81) is a structural mutant with a catalytically deficient GS, resulting in reduced activities, which synthesizes protein in equal amounts to its parent (12), as found for *Nostoc* sp. strain 7801 growing in symbiosis (19, 15). Immunoblot analysis of crude proteins and Western blot analyses of GS from SS1 revealed that its quantity (Fig. 7A) and mobility (Fig. 7B) are the same as in the parent. A modification similar to that of ED81 may also have occurred in SS1, forming a catalytically deficient GS, but conclusive evidence is yet to be obtained.

A 30,000-dalton soluble cytoplasmic protein was absent from SS1 grown in the presence and absence of MSX (Fig. 6). Its relevance to nitrogen metabolism should be elucidated.

Fertilization of rice plants under laboratory conditions by application of another MSX-resistant mutant of *A. variabilis*, SA1, was successful (14). The shorter doubling time of SS1 and the lack of a lag period at the beginning of the growth cycle as compared with SA1 (26) are obvious assets for SS1 mass cultivation. These characteristics enable efficient production of inoculum material. The usefulness of SS1 as a biofertilizer to rice plants in the original isolate location (Thailand) should be studied further.

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LITERATURE CITED

1. Antarikanonda, P. 1982. Effect of salinity on growth, nitrogen fixation and sodium uptake of rapidly growing N_2-fixing blue-green alga *Anabaena* sp. TA1. Microbios 34:177–184.
2. Antarikanonda, P. 1982. Influence of pretreating the seeds with extract of *Anabaena siamensis* on germination and growth of some rice varieties from Thailand. FAO Int. Comm. 31:37–39.
3. Antarikanonda, P. 1982. Influence of nitrogen fixing blue green alga *Anabaena siamensis* on yield and protein quality of highly yielding rice cultivar (Khao Dowk Mali 4-2-105). German Research Society Report no. 10680, p. 30.
4. Antarikanonda, P. 1984. Production of extracellular free amino acids by cyanobacterium *Anabaena siamensis*. Curr. Microbiol. 11:191–196.
5. Antarikanonda, P. 1985. A new species of the genus *Anabaena*: *Anabaena siamensis* sp. nov. (Cyanophyceae) from Thailand. Nova Hedwigia 41:343–352.
6. Antarikanonda, P., and H. Lorenzen. 1982. N_2 fixing blue green algae (Cyanobacteria) of high efficiency from paddy soils of Bangkok, Thailand: characterization of species and nitrogen fixing capacity in the laboratory. Acta Hydrobiol. Suppl. 63:53–70.
7. Boussiba, S., and A. E. Richmond. 1980. C-phycocyanin as a storage protein in the blue-green alga *Spirulina platensis*. Arch. Microbiol. 125:143–147.

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8. **Brouers, M., and D. O. Hall.** 1986. Ammonia and hydrogen production by immobilized cyanobacteria. J. Biotechnol. 3:307–321.
9. **Ilen, N. T., N. W. Kerby, G. C. Machray, P. Rowell, and W. D. P. Stewart.** 1988. Expression of glutamine synthetase in mutant strains of the cyanobacterium *Anabaena variabilis* which liberate ammonia. FEMS Microbiol. Lett. 56:337–342.
10. **Joseph, C. M., and J. C. Meeks.** 1987. Regulation of expression of glutamine synthetase in a symbiotic *Nostoc* strain associated with *Anthoceros punctatus*. J. Bacteriol. 169:2471–2475.
11. **Kerby, N. W., S. C. Musgrave, P. Rowell, S. V. Shestakov, and W. D. P. Stewart.** 1986. Photoproduction of ammonium by immobilized mutant stains of *Anabaena variabilis*. Appl. Microbiol. Biotechnol. 24:42–46.
12. **Kerby, N. W., G. W. Niven, P. Rowell, and W. D. P. Stewart.** 1987. Ammonia and amino acid production by cyanobacteria, p. 277–286. *In* T. Stadler, J. Mollion, M.-C. Verdus, Y. Karamanos, H. Morvan, and D. Christiaen (ed.), Algal biotechnology. Elsevier Applied Science, London.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685.
14. **Lattore, C., J. H. Lee, H. Spiller, and K. T. Shanmugam.** 1986. Ammonium ion-excreting cyanobacterial mutant as a source of nitrogen for growth of rice: a feasibility study. Biotechnol. Lett. 8:507–512.
15. **Lee, K. Y., C. M. Joseph, and J. C. Meeks.** 1988. Glutamine synthetase specific activity and protein concentration in symbiotic *Anabaena* associated with *Azolla caroliniana*. Antonie van Leeuwenhoek J. Microbiol. Serol. 54:345–355.
16. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
17. **Mackinney, G.** 1941. Absorption of light by chlorophyll solutions. J. Biol. Chem. 140:315–322.
18. **Martinez, M. R.** 1984. Algae: biofertilizer for rice. Philipp. Counc. Agric. Res. Resources Dev. (PCARRD) Monit. 12:9–12.
19. **Musgrave, S. C., N. W. Kerby, G. A. Codd, and W. D. P. Stewart.** 1982. Sustained ammonia production by immobilized filaments of the nitrogen fixing cyanobacterium *Anabaena* 27893. Biotechnol. Lett. 4:647–652.
20. **Nierzwicki-Bauer, S. A., and R. Haselkorn.** 1986. Differences in mRNA levels in *Anabaena* living freely or in symbiotic association with *Azolla*. EMBO J. 5:29–35.
21. **Orr, J., and R. Haselkorn.** 1982. Regulation of glutamine synthetase activity and synthesis in free living and symbiotic *Anabaena* sp. J. Bacteriol. 152:626–635.
22. **Polukhina, L. E., G. N. Sukhurieva, and S. V. Shestakov.** 1982. Ethylenediamine-resistant *Anabaena variabilis* mutants with derepressed nitrogen fixing system. Microbiology 51:90–95.
23. **Samplo, M. J. A. M., P. Rowell, and W. D. P. Stewart.** 1979. Purification and some properties of glutamine synthetase from the nitrogen fixing cyanobacteria *Anabaena cylindrica* and a *Nostoc* sp. J. Gen. Microbiol. 111:181–191.
24. **Singh, H. N., R. K. Singh, and R. Sharma.** 1983. An L-methionine-D,L-sulfoximine-resistant mutant of the cyanobacterium *Nostoc muscorum* showing inhibitor resistant γ-glutamyl-transferase, defective glutamine synthetase and producing extracellular ammonia during N_2 fixation. FEBS Lett. 153:10–14.
25. **Solorzano, L.** 1969. Determination of ammonia in natural waters by the phenol-hypochlorite method. Limnol. Oceanogr. 14:799–801.
26. **Spiller, H., C. Latorre, M. E. Hassan, and K. T. Shanmugam.** 1986. Isolation and characterization of nitrogenase derepressed mutant strains of cyanobacterium *Anabaena variabilis*. J. Bacteriol. 165:412–419.
27. **Stewart, W. D. P., G. P. Fitgerald, and R. H. Burris.** 1967. *In situ* studies on N_2 fixation using the acetylene reduction technique. Proc. Natl. Acad. Sci. USA 58:2071–2078.
28. **Tandeau de Marsac, N., and J. Houmard.** 1988. Complementary chromatic adaptation: physiological conditions and action spectra. Methods Enzymol. 167:318–328.
29. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76:4350–4354.
30. **Venkataraman, G. S.** 1975. The role of blue green algae in tropical rice cultivation, p. 207–218. *In* W. D. P. Stewart (ed.), Nitrogen fixation by free-living microorganisms. Cambridge University Press, London.
31. **Venkataraman, G. S.** 1981. Blue-green algae: a possible remedy to nitrogen scarcity. Curr. Sci. 50:253–256.
32. **Zimmerman, W. J., and S. Boussiba.** 1987. Ammonia assimilation and excretion in an asymbiotic strain of *Anabaena azollae* from *Azolla filiculoides* Lam. J. Plant Physiol. 127:443–450.

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Mass cultivation of the nitrogen-fixing cyanobacterium *Gloeotrichia natans*, indigenous to rice-fields

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Abstract

Gloeotrichia natans, a nitrogen fixing cyanobacterium common in rice fields in the Philippines, was used for studies to establish key features of its physiology and potential production in outdoor cultures. Under optimal growth conditions (38 °C, pH 8.0, no carbon enrichment) the specific growth rate of rice-field isolate was 0.076 h^{-1}. The pH of the medium (between 6.5 and 9.0) did not influence the growth rate, but it did affect phycobiliprotein content, as reflected by a change in colour. At pH 7.0 the culture was green-brown, with phycobiliproteins constituting up to 10% of the total protein, while at pH 9.0 the culture was brownish-black and the pigment content was as high as 28% of the total protein. In outdoor cultures the specific growth rate was related directly to cell density in the range of 0.7–1.5 g dry weight l^{-1} at a rate of stirring of 30 rpm, and inversely related to cell density at half this rate. At a stirring of 30 rpm, daily production of outdoor cultures harvested to maintain cell densities of 0.7, 1.15 and 1.5 g l^{-1} were 14.7, 17.1 and 18.1 g m^{-2} d^{-1}, respectively. This rate of production was maintained for more than 45 days. Phycobiliprotein content in the culture kept at a density of 1.5 g l^{-1} reached 14% of the total biomass.

Introduction

Large-scale production of nitrogen-fixing cyanobacteria (blue-green algae) was aimed originally at expanding the traditional application of diazotrophic heterocystous cyanobacteria as a nitrogen source in rice paddies (Roger & Kulasooriya, 1980). Production was confined to non-lined open ponds, in which the resulting algal product contained a high percentage of soil, and productivity was highly unpredictable in terms of the amount and kind of algal biomass that could be produced (Venkataraman, 1969; Roger & Kulasooriya, 1980).

Interest in the mass culture goes beyond their use as a nitrogen biofertilizer. The cyanobacteria

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also constitute a source of valuable products such as phycobiliproteins, polysaccharides and protein for feed and food (Cohen, 1986). One of the major problems limiting this biotechnology, however, is the fragmentary nature of the information available on the mass production of these microorganisms (Fontes *et al.*, 1987; Boussiba, 1988). Some time ago, we began investigating the potential production of nitrogen-fixing cyanobacteria in outdoor cultures (Boussiba, 1988). The aim of the present study was to achieve efficient production of biomass of *Gloeotrichia natans* for application as N-fertilizer and as a source of biochemicals. This cyanobacterium was chosen since it is common in rice fields in the Philippines.

Materials and methods

Organism and growth medium

The strain of *Gloeotrichia natans* Rabh. ex. Flah. is an isolate from a wetland rice field at Los Bānos, Laguna, Philippines. It was cultivated in BG-11 medium and its variant BG-11_0 (BG-11 with omission of $NaNO_3$) (Rippka *et al.*, 1979). The name is based on the original field morphology, since major morphological changes (loss of large colony form, tapered filaments, akinetes, terminal hairs) occurred upon transfer to the laboratory.

Growth conditions

Laboratory cultures. The cyanobacterium was cultivated in either of two systems: (a) 250 ml flasks, placed on a gyratory shaker at a constant temperature of 30 °C under constant illumination of 75 μmol photon $m^{-2} s^{-1}$; (b) 500 ml glass columns placed in a transparent plexiglass water bath under the following conditions: temperature 30 °C; constant photon flux of 175 μmol photon $m^{-2} s^{-1}$ at the surface of the growth vessel; continuous aeration by bubbling filtered air with or without 1.5% CO_2, yielding pH values of 6.8–9.0, respectively. In some experiments $NaHCO_3$ was added to the culture medium instead of CO_2. The effect of temperature on growth was studied in cultures growing in tubes by means of a temperature block, which maintains a temperature gradient of 16 to 47 °C with 1.0–1.5° increments. The light intensity at the bottom surface of the tubes was 100 μmol photon $m^{-2} s^{-1}$.

Outdoor cultures. Cultures of *G. natans* grown in 12-litre bottles under controlled laboratory conditions (30 °C, 260 μmol photon $m^{-2} s^{-1}$, air bubbling) were used as inocula for 2.5 m^2 outdoor raceway ponds located at Sede Boqer in the Negev desert. The outdoor cultures, 200 litres in volume and 8 cm in depth, were stirred by paddle wheels. During the first two days after inoculation, shade nets (which reduced light intensity by 50%) were used to protect the cultures against the inhibitory effect of high light. The pH was maintained in the range of 8.5–9.5 by introducing CO_2 into the cultures via porous rubber tubes.

Biomass was kept constant by daily removal of a portion of the culture a procedure known as 'bleeding'), thus maintaining the culture at steady state. Measurements of pH, light intensity, temperature and O_2 were made several times during the day.

Analytical methods

Specific growth rate was determined using chlorophyll, protein and ash-free dry weight as growth parameters. Chlorophyll and dry weight were determined as described previously (Boussiba, 1988). Protein content was measured according to Lowry *et al.* (1951) in samples that had been used for chlorophyll analysis and then treated with NaOH. Lipid content was determined as described recently (Boussiba *et al.*, 1988). Carbohydrates were estimated by the anthrone method (Hassid & Abraham, 1957).

The concentration of phycobiliproteins was determined in 25 ml culture aliquots which were centrifuges, washed in 5 ml 0.1 M Na phosphate buffer at pH 7.0, resuspended in the same buffers

and sonicated at a power setting of 150 W. Quantitative evaluations were based on the extinction coefficients according to the method of Bennett and Bogorad (1973). Partial purification of the phycobiliproteins was performed by mixing crude extracts with ammonium sulp' ate (36%) for 30 min at 4 °C, followed by dialysis for 12 h against Na phosphate buffer (10 mM) at 4 °C. The dialysate was then placed on a DEAE cellulose column and eluted with increasing concentrations of phosphate buffer ranging from 10 to 150 mM. The light absorption profile of the different samples was determined with a Milton Roy Spectronic 1201 scanning spectrophotometer.

Enzyme assays

Nitrogenase activity in laboratory grown cultures was estimated by the acetylene reduction method (Stewart *et al.*, 1967). Samples of 4.6 ml, washed in fresh BG_{11}-N medium, were placed in 25 ml Wheaton bottles sealed with a flanged rubber septum. The Wheaton bottles were placed on a rotary shaker and illuminated at a quantum flux of 75 μmol photon $m^{-2} s^{-1}$ during the assay. The cell suspensions were allowed acclimate for 10 min before injection of C_2H_2. For the estimation of nitrogenase activity in outdoor cultures, the Wheaton bottles containing the algal culture (resuspended in fresh medium) were either allowed to float in the culture medium or fixed at the surface of the culture (data for light intensity and temperature during nitrogenase assays in the outdoor cultures are given in Fig. 3). All assays were carried out for 1 h. The ethylene evolved was analyzed by a HP 5890 gas chromatograph using a stainless steel column packed with Poropak-N (0.2 cm i.d., 265 cm length). Nitrogenase activity was expressed as μmol C_2H_4 produced per mg chlorophyll per hour. Data of Fig. 5 are from one representative day of Summer 1988 (usually about 100 days a year with the same average temperature and light irradiance).

Results

Optimization of growth under laboratory conditions

G. natans was capable of growing under a wide range of growth conditions of CO_2 enrichment, pH, temperature, and NaCl concentration (Table 1). Growth was not enhanced by the addition of CO_2 or of $NaHCO_3$. Under optimal growth conditions (38 °C, pH 8.0 and no carbon enrichment) the specific growth rate (μmax) attained was 0.076 h^{-1}, corresponding to a doubling time of 9.1 h.

Effect of CO_2 supplement on phycobiliproteins

The growth rate of cultures grown with 1.5% CO_2 was similar to that of cultures not supplied with CO_2 (Fig. 1). However, a large difference was observed in the colour of the cultures, i.e., greenish-brown (with CO_2) vs. brownish-black (without CO_2) due to the lower proportion of both the red and blue phycobiliprotein pigments to chlorophyll in the former (Table 2), i.e., at 96 h of growth 8.6 : 1 of total protein with CO_2 vs. 19.9 : 1 without CO_2 and at 156 h of growth 11.3 : 1 and 16.7 : 1 respectively. The colour change is due to quantitative change in the pigments and not qualitative, as their absorption profile is the same under both conditions. The possibility that the pH of the medium, which was 7.0 with 1.5% CO_2 and 9.0 without CO_2 supplement, was responsible for these pigment differences was verified by the use

Table 1. Optimum growth conditions for *Gloeotrichia natans* grown on $BG\text{-}11_0$.

Variable	Range permitting growth	Optimum value*
CO_2 (%) in air	0.03–1.5	0.03
$NaHCO_3$ ($g\,l^{-1}$)	0–1.5	0
pH	6.5–9.5	8
Temperature (°C)	25–44	38
NaCl ($g\,l^{-1}$)	0–15	0

* A value leading to a doubling time of 9.1 h.

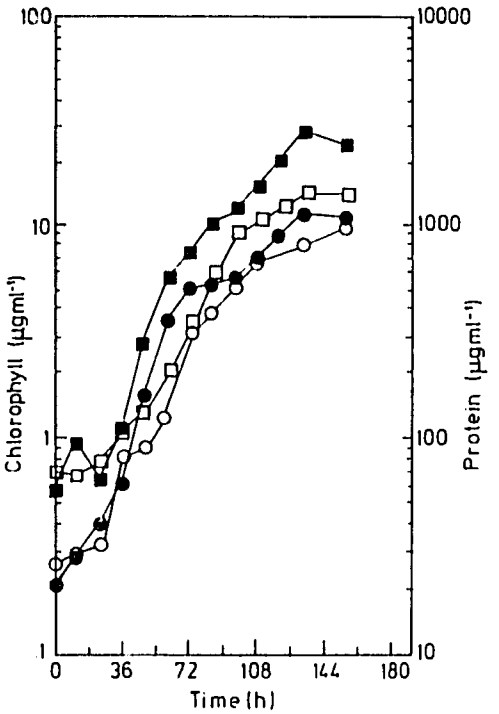

Fig. 1. Growth of *G. natans* with 1.5% CO_2 (closed symbols) and without CO_2 enrichment (open symbols). ● ○ chlorophyll; □ ■ protein The pH of the medium was 6.5–7.0 with CO_2 and 8.5–9.0 without CO_2.

of media buffered between 6.5 and 9.0 for cultures agitated on the gyratory shaker (inocula were taken from cultures grown with continuous CO_2 supply). The change in the colour of the culture was observed on the 3rd day after inoculation. At a pH above 8.0 the colour of the cultures gradually changed from green to black (data not shown).

Table 2. Effect of CO_2 enrichment on pigment content (% of protein) of *G. natans* cultivated in the laboratory.

Pigment	$-CO_2$		$+CO_2$ (1.5%)	
	96 h	156 h	96 h	156 h
Chlorophyll *a*	1.4	1.1	1.1	1.0
Phycocyanin	14.9	9.2	5.3	6.8
Phycoerythrin	13.0	9.2	4.2	4.5

Growth of outdoor cultures

The specific growth rate of outdoor cultures was related directly to the rate of turbulence and related inversely to cell density (Fig. 2). At a turbulence of 30 rpm, the growth rate was 26 and 33% higher at cell densities of 1.15 and 1.5 g l^{-1} than that at a culture density of 0.7 g l^{-1}. At a turbulence of 15 rpm the growth rate at a cell density of 1.5 g l^{-1} was 50% lower than that at 0.7 g l^{-1}. At a stirring rate of 30 rpm, daily production of outdoor cultures harvested to maintain cell densities of 0.7, 1.15 and 1.5 g l^{-1} were 14.7, 17.1 and 18.1 g m^{-2} d^{-1}, respectively. This rate of production was maintained for more than 45 days. During that period, daily microscopic inspection of the different cultures revealed that there was no contamination with other microalgae. The major cell constituents were proteins (42% of DW), carbohydrates (~33% of DW) and lipids (8% of DW). Phycobiliproteins constituted 14% of total dry weight.

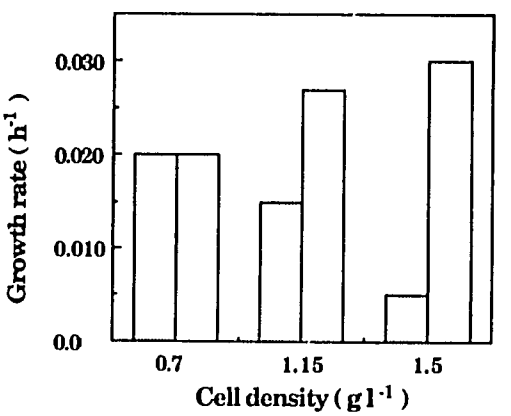

Fig. 2. Effect of rate of turbulence on the specific growth rate of *G. natans* in outdoor cultures as a function of cell density. □ culture stirred at 15 rpm; ■ culture stirred at 30 rpm.

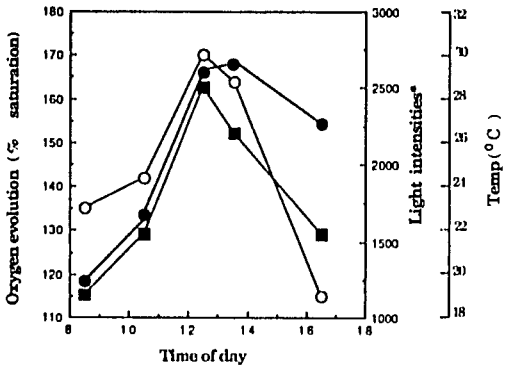

Fig. 3. Light intensity (μmol photon $m^{-2} s^{-1}$) (■), temperature (●) and oxygen evolution (○) during the day.

Temperature, rather than light, was the major limiting factor for photosynthetic activity, as determined by O_2 evolution (Fig. 3). Although light intensity at 0900 was sufficient for photosynthesis, O_2 evolution rate was low at that time, since the water temperature was 7 to 9 °C below air temperature and 14 to 17 °C below the optimum required for growth. Water temperature had still to rise to 30 °C or higher for optimal photosynthetic activity. This behaviour was observed at all cell densities tested.

Unlike the photosynthesis process, nitrogenase activity seemed to be affected mainly by light intensity and not by temperature. At 0010, nitrogenase activity was near its optimal value riched at noon time (Fig. 4), while temperature was far from optimum (Fig. 3). Nitrogenase activity assayed in the pond in non-agitated cultures was relatively low as compared with that in agitated flasks (Fig. 4). These differences were attributed mainly to the effect of light, since cultures of *G. natans* sampled in the pond during the day and assayed under controlled conditions in the laboratory yielded the same value of activity (Fig. 4), which was lower than the values obtained outside. The difference in the level of activity indoors and outdoors is attributed to the high light intensity outdoors (Fig. 3).

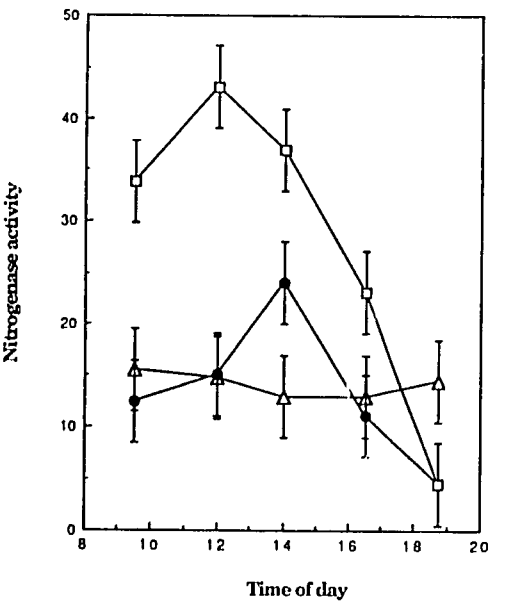

Fig. 4. Nitrogenase activity of *G. natans* under different environmental conditions. △, controlled conditions indoors; □ outdoors with mixing; ● outdoors without mixing. Nitrogenase activity is expressed as μmol C_2H_4 produced per mg chlorophyll per hour.

Discussion

One of the major problems still limiting widespread used of cyanobacteria as nitrogen fertilizers in rice fields is the difficulty of producing efficiently large quantities of monoalgal inoculum for commercial use. This situation is largely due to the lack of information on the growth characteristics of these microorganisms. The isolation of the main dominant strains that thrive in rice fields and characterization of their growth requirements are therefore essential for their utilisation. Since *G. natans* is found in many rice fields in the Philippines, we chose to study this species.

Until recently inocula of *G. natans* were obtained from natural blooms in non-lined ponds, which are an unreliable source. Hence, the ability to ensure a large and continuous supply of clean cultures of this cyanobacterium by the use of open raceways would be advantageous. In the present study we have succeeded in improving the growth

rate of *G. natans* to a considerable extent and in shortening its mean generation time to 48 h in outdoor cultures (calculated from Fig. 3) and to 9.1 h under laboratory conditions. In earlier investigations this isolate was found to divide in rice fields every 168–240 g, and in laboratory cultures every 72–120 h (Martinez *et al.*, 1981).

As has been found for other microalgae cultivated outdoors (Richmond, 1986) light and temperature are the main factors limiting growth of *G. natans*. The light regime to the algal cells could be improved by optimising both the cell density and the rate of stirring (Fig. 3). Since the optimal temperature could be maintained only for part of the day (Fig. 4), it seems that temperature is the most important factor limiting production in our system. We therefore expect that in the Philippines where the ambient temperature is higher than in Israel, a yield higher than 18 g m^{-2} d^{-1} may be obtained.

The maintenance of steady-state conditions also reduces the chances of contamination of the culture. We assume that the following conditions protect the cultures from contamination: relatively high cell density; clumps formation of the cultures (making grazing by predators difficult); and the absence of an external source of nitrogen (probably preventing the proliferation of nitrogen-consuming microorganisms).

G. natans did not respond to CO_2 supplement by increasing its growth rate, the concentration of this gas present in the air and water being apparently sufficient for its growth. However, CO_2 absence effected considerable increase in the phycobilins content as compared within that under CO_2 enrichment. Our data suggest that the pH of the culture regulates this characteristic. Such a phenomenon has not been reported for other cyanobacteria. Cyanobacteria usually possess either the blue (phycocyanin) or the red (phycoerythrin) phycobiliproteins as their major accessory pigment. The relative content of these pigments can be affected by the wavelength supplied during growth, a phenomenon called complementary chromatic adaptation (Bennet and Bogorad, 1973). This strain of *G. natans* is unusual in possessing the two phycobiliproteins in equal concentrations. It is noteworthy that our rice cyanobacterium isolate contains a phycocyanin having an absorption spectrum resembling that of the phycocyanin pigment present in red algae (O'heocha, 1965), while its phycoerythrin spectrum is typical of cyanobacteria (Rodriguez *et al.*, 1989).

The possibility of obtaining a uni-algal inoculum of *G. natans* at a reasonable rate of production will thus improve its efficiency as a nitrogen biofertilizer as well as facilitate the extraction of valuable products, such as phycobiliproteins. The red and blue phycobiliprotein pigments, which together constitute 14% of the algal dry weight may be used as colouring agents for food and cosmetics, and as immunofluorescence probes in medical research (due to their high fluorescence).

Acknowledgement

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References

Bennett A, Bogorad L (1973) Complementary chromatic adaptation in a filamentous blue-green algae. J. Cell Biol. 58: 419–435.

Boussiba S (1988) N_2-fixing cyanobacteria as nitrogen biofertilizer. A study with the isolate *Anabaena azollae*. Symbiosis 6: 129–138.

Boussiba S, Sandbank E, Shelef G, Cohen Z, Vonshak A, Ben-Amotz A, Arad S, Richmond A (1988) Outdoor cultivation of the marine microalga *Isochrysis galbana* in open reactors. Aquaculture 72: 247–253.

Cohen Z (1986) Products from Microalgae. In Richmond A (ed.), Handbook of Microalgal Mass Culture. CRC Press, Inc. Boca Raton, Florida PP, 421–454.

Fontes AG, Vargas MA, Moreno J, Guerrero MG, Losada M (1987) Factors affecting the production of biomass by a nitrogen-fixing blue-green alga in outdoor culture. Biomass 13: 33–43.

Hassid WZ, Abraham S (1957) Chemical procedures for analysing of polysaccharides. In Colowick SP, Kaplan NO (eds), Methods in Enzymology, vol 3. Academic Press, New York, 34–50.

Lowry OH, Rosenbrough NJ, Far AL, Randall RJ (1951)

Protein measurements with the folin phenol reagent. J. Biol. Chem. 193: 265-277.

Martinez MR, Pantastico JB, Cosico WC (1981) Blue-green algae and the fertility of lowland rice fields in the Philippines. In Wetselaar R, Simpson J, Rosswall T (eds), Nitrogen Cycling in South East Asia Wet Monsoonal Ecosystem. Austr. Acad. Sci., Canberra, 29–35.

O'heocha C (1965) Phycobilins. IN T. W. Goodwin (ed.), Chemystry and Biochemistry of Plant Pigments. Academic Press, New York, 175–196.

Richmond A (1986) Microalgaculture. CRC Critical Reviews in Biotechnology 4(4): 369–438.

Rippka R, Deruelles J, Waterbury JB, Herdman M, Stanier RY (1979) Generic assignments, strain histories and properties of pure culture of cyanobacteria. J. Gen Microbiol. 111: 1–61.

Rodriguez H, Rivas J, Guerrero MG, Losada M (1989) Nitrogen-fixing cyanobacterium with a high phycoerythrin content. Appl. Environ. Microbiol. 55(3): 758–760.

Roger PA, Kulasooriya SA (1980) Blue-green algae and Rice. International Rice Research Institute, Los Bãnos, Laguna, Philippines, 112 pp.

Stewart WDP, Fitzgerald GP, Burris RM (1967) *In situ* studies on N_2-fixation using the acetylene reduction technique. Proc. Natl. Acad. Sci. 58: 2071–2078.

Venkataraman GS (1969). The Cultivation of Algae. Indian Council of Agricultural Research, New Delhi, 317 pp.

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Ammonium Excretion by a Mutant of the Nitrogen-Fixing Cyanobacterium *Anabaena siamensis*

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Abstract

Anabaena siamensis *isolated from rice fields in Thailand is a fast growing cyanobacterium with a high nitrogen-fixing activity. Mutant strains resistant to the* L*-glutamate analogue,* L*-methionine sulfoximine (MSX) were isolated by ethyl methanesulfonate mutagenesis. A stable mutant named* A. siamensis *SS1, which released ammonium to the medium, was studied further. In batch cultures the rate of ammonium production peaked at the early log phase and gradually decreased until the 4th day of growth when the cultures reached a density of 90 μg chl ml^{-1}. To obtain constant release of ammonium by SS1, continuous culture experiments were performed at a cell density of 5 μg chl ml^{-1} and the following results were obtained: (1) growth rate as the parent (μ: 0·123 h^{-1}) in the presence and absence of 500 μM MSX; (2) 48% GS transferase activity when compared with the parent; (3) ammonium excretion at a rate of 8 μmol (mg chl)$^{-1}$ h^{-1} as measured up to 20 generations (120 h); (4) derepressed nitrogenase activity; and (5) 30% higher nitrogenase activity than that of the parent. SS1 immobilized in alginate beads (5 μg chl ml^{-1}) exhibited values of glutamine synthetase and nitrogenase activity similar to those of free cells. However, ammonium excretion at the rate of 11·61 μmol (mg chl)$^{-1}$ h^{-1} was obtained only up to 20 h after loading in bioreactors, due to the fast growth of SS1 as also occurred in batch cultures.*

Key words: Anabaena siamensis, mutagenesis, amino acid analogue resistance, nitrogen fixation, ammonium excretion, glutamine synthetase, rice fields.

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INTRODUCTION

The heterocystous cyanobacteria can utilize light energy to support both carbon dioxide fixation and nitrogen fixation under aerobic conditions (Stewart, 1980). This allows them to produce fertilizer nitrogen that can contribute to the growth of plants. The agronomic significance of cyanobacteria, either free-living or in symbiotic association with the aquatic fern, *Azolla*, has long been recognized (Moore, 1969; Venkataraman, 1975). The application of nitrogen-fixing cyanobacteria in the Orient replaces 20–30% of the chemical nitrogen fertilizer demand, increasing the natural fertility of the paddy-field soils (Venkataraman, 1975).

However, the inoculation of non-indigenous cyanobacterial strains is found to be not totally successful due to the failures to overcome the interspecific competitions and environmental constraints (Grant *et al.*, 1985). There is hence a need to identify ideal strains from the indigenous population of a particular location of interest for the production of inoculum for algalization, and to assess its effects upon rice yields. In this respect, *Anabaena siamensis* isolated from Thai rice fields appears to be a promising organism for algalization, due to its fast growth rate and adaptation to wide temperature (25–42°C) and salinity (1–2%) ranges (Antarikanonda, 1982*a*; Antarikanonda & Lorenzen, 1982).

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The nitrogen fixed by cyanobacteria is made available to the environment mainly by autolysis and mineralization after death (Martinez, 1984). Free-living cyanobacteria release ammonium in insignificant quantities during growth but can excrete it in high amounts when treated with MSX, a highly specific, irreversible inhibitor of glutamine synthetase (Ronzio *et al.*, 1969). However, in symbiotic association, cyanobacteria such as *A. azollae* and *Nostoc* sp. 7801, release fixed nitrogen to the host's nitrogen requirements in the form of ammonium due to an inhibition of GS activity under symbiotic conditions (Orr & Haselkorn, 1982; Joseph & Meeks, 1987). Ammonium excretion in cyanobacteria seems to be dependent upon GS rather than on nitrogenase activity. Actually, mutations in the GS structural gene, *glnA*, causing reduction of GS activity result in excretion of ammonium (Polukhina *et al.*, 1982; Hien *et al.*, 1988). Based on this principle, an ammonium excreting mutant of *A. variabilis* has been found to be effective as a supplier of nitrogen fertilizer to rice plants in laboratory experiments (Lattore *et al.*, 1986). However, growth of these strains can be very slow when compared to their parent strains, which in turn are not indigenous organisms. Hence, we have selected a rice field isolate (*A. siamensis*) which is already marketed as algal biofertilizer for rice crops and claimed to increase the growth and yield of rice plants due to its high nitrogen-fixing capacity (Antarikanonda, 1982*b*, *c*).

In this paper we describe the isolation and characterization of an ammonium excreting mutant of *A. siamensis*.

METHODS

Organism and growth medium

The nitrogen-fixing cyanobacterium, *Anabaena siamensis* used in this study is an isolate from Thailand (Antarikanonda, 1985) and has been deposited in the Sammlung von Algenkulturen, Pflanzenphysiologisches Institute, Universität Göttigen, Federal Republic of Germany under the signature *Anabaena* spec. B 11.82. The components of growth medium (Antarikanonda, 1982*a*) (AS medium) contained per liter: 0·20 g $K_2HPO_4.3H_2O$, 0·25 g $MgSO_4.7H_2O$, 0·056 g $CaCl_2$, 0·23 g NaCl, 0·025 g $FeSO_4.7H_2O$, 0·027 g Titriplex III, 0·002 g $MnCl_2.4H_2O$, 0·0015 g $NaMoO_4.2H_2O$, 0·0002 g $ZnSO_4$, 0·00008 g $CuSO_4.5H_2O$, 0·00002 g $CoCl_2.6H_2O$ and 0·003 g H_3BO_3. After autoclaving the pH was adjusted to pH 7·5 and the phosphate solution was autoclaved separately.

Growth conditions

A. siamensis was cultivated in 500-ml sterilized glass columns placed in a transparent plexiglass circulating water bath. The water temperature was maintained at 42°C. A constant photon flux of 175 μE m^{-2} s^{-1} at the surface of the growth vessel was supplied laterally by a battery of eight cool-white fluorescent lamps. Continuous aeration was provided by bubbling filtered air containing 1·5% CO_2. The pH was thus maintained at 7·0–7·2. Unless otherwise stated, cultures were sampled during the logarithmic growth phase for use in the different experiments.

Continuous culture experiments were carried out as described previously (Boussiba & Richmond, 1980). The cultures were maintained at 5 μg chl ml^{-1}. Cells were harvested up to 20 generations (120 h) and used for different experiments.

Immobilization of cyanobacteria was carried out by using the alginate entrapment method (Kerby *et al.*, 1986) with a few modifications. Cyanobacterial cell suspensions at 10 μg ml^{-1} concentration of chlorophyll were mixed with equal volumes of 3% sodium alginate solution and dropped in 0·1 N $CaCl_2$ solution through a capillary tube to form beads of 2–5 mm diameter. The alginate-entrapped cyanobacteria were collected after 2 h and kept at 4°C for 12 h. The beads were loaded in packed-bed reactors. The flow rate of the culture medium was 0·2 h^{-1}.

Growth determination

Growth was followed by means of chlorophyll determination, starting from an initial inoculum containing 1 μg chl ml^{-1}. Chlorophyll *a* was determined colorimetrically in methanol extracts (MacKinney, 1941). Protein was determined colorimetrically after digestion with 0·5 N NaOH by Lowry's method (Lowry *et al.*, 1951).

Mutagenesis

Mutagenesis with ethyl methanesulfonate was carried out as reported by Spiller *et al.* (1986) with a few modifications. Mutants resistant to L-methionine-DL-sulfoximine (MSX) were selected on agar plates containing 500 μM MSX. Ammonium-excreting mutants were selected on solid media containing the pH indicator phenol red. One of the mutants, which induced the most marked

colour change in the plates (from red to pink), was used for further studies and designated as *A. siamensis* SS1.

A filament of *A. siamensis* generally consists of 8–10 vegetative cells between two terminal heterocysts. Before treating with the mutagen the filament length was reduced to 1–3 cells by providing mild sonication for 3 s in a sonicator bath.

Ammonium uptake

Ammonium uptake was followed for 20 min starting from an initial concentration of 100 μM, as described previously (Zimmerman & Boussiba, 1987).

Ammonium determination

Filtrates from cyanobacterial cultures were collected during growth at different time intervals to estimate the ammonium released into the medium. Ammonium concentration in the medium was measured by Solorzano's phenol-hypochlorite method (Solorzano, 1969).

Enzyme assays

Nitrogenase activity was estimated in intact filaments by the acetylene reduction assay (ARA) (Stewart *et al.*, 1967). Samples of 4·6 ml of cyanobacterial culture were washed in fresh AS medium and placed in a 25-ml Wheaton bottle sealed with a flanged rubber septum. The Wheaton bottles were placed on a rotary shaker (100 rpm) while being illuminated with a quantum flux of 75 μE m^{-2} s^{-1} during the assay. The filaments were allowed to stand for 10 min before injection of acetylene. Ethylene was analyzed with an HP 5890 gas chromatograph (California, USA) using a stainless steel column packed with Porapack-N (0·2 cm id, 265 cm length). The nitrogenase activity was expressed as μmol C_2H_4 produced $(mg\ chl)^{-1}\ h^{-1}$.

Nitrogenase activity in immobilized cyanobacteria was determined by incubating the alginate beads containing cyanobacteria (= 5 μg chl ml^{-1}) in 130-ml Wheaton bottles and assayed as described above.

Glutamine synthetase (GS) was assayed in concentrated suspensions (1 mg protein ml^{-1}). Cells were permeabilized with 2% toluene for 1 min and kept in ice for 15 min before the activity measurements. Activity was measured as transferase (Sampio *et al.*, 1979) and expressed in μmol γ-glutamyl hydroxamate formed $(mg\ protein)^{-1}\ min^{-1}$.

Chemicals

L-methionine-DL-sulfoximine (MSX) was purchased from Sigma Chemical Co., (St Louis, Missouri, USA) and other chemicals were from E. Merck AG, (Darmstadt, FRG). Sodium alginate was the product of Aldrich Chemical Co., USA.

RESULTS AND DISCUSSION

Under our standard conditions, *A. siamensis* exhibited a high growth rate (doubling time 5·6 h), higher than other rice-field nitrogen-fixing cyanobacterial strains reported in the literature. The nitrogenase activity was also high (350 nmol $(mg\ protein)^{-1}\ min^{-1}$). This strain has been reported to release amino acids to the environment (Antarikanonda, 1984). However, it did not excrete ammonium during active growth. On the other hand, if the GS inhibitor, L-methionine sulfoximine (MSX) was added to the cell suspensions, ammonium was released into the medium in significant quantities (data not shown). MSX concentrations above 100 μM inhibited the growth of *A. siamensis* significantly and 500 μM MSX was completely lethal in liquid as well as solid media (Fig. 1). Among the MSX-resistant strains selected, only a few were able to change the color of phenol red, orange to pink, in pH indicator plates. Another class of mutants grew well in the presence of 500 μM MSX, apparently because of an MSX-resistant GS, but they did not induce color change in pH indicator plates. A third class of MSX-resistant mutants were those which survived at 500 μM MSX in a solid medium, but not in a liquid medium. A fourth group comprised the MSX-resistant mutants which tolerated high concentrations (1000–1500 μM). Among the mutants belonging to the first group, a particular strain (designated SS1), which *promoted* a very marked change in the color of phenol red, was selected for a detailed study.

A. siamensis SS1 grew as the parent strain under all growth conditions tested (Table 1). In batch cultures the nitrogenase activity was high during the early log phase, with a maximum value (30% higher than the parent strain) after 12 h of inoculation, decreasing thereafter. The ammonium release followed a similar trend, being a maximum at 12 h and slowing down during the late log to be almost negligible at the stationary phase (Fig. 2). This behaviour can be explained, since when growth progressed the availability of light was progressively limited due to the high

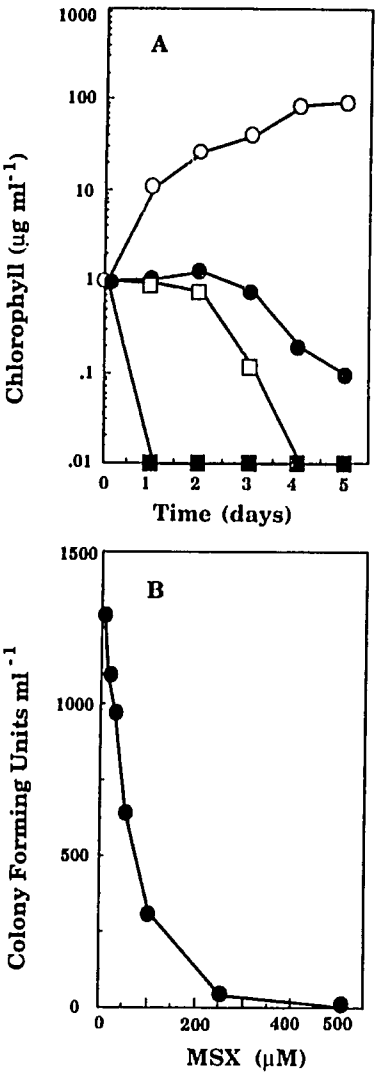

Fig. 1. Influence of MSX on the growth of *Anabaena siamensis* in liquid (A) (○—○, control; ●—●, 100 μM MSX; □—□, 250 μM MSX, and ■—■, 500 μM MSX) and solid (B) media.

levels of pigmentation, and nitrogen fixation was therefore lowered. In reflection of this, the rate of ammonium release was reduced significantly. In addition, part of the released ammonium was probably taken up by the cells since SS1 showed the same ammonium uptake rate in the presence (30·6 nmol (μg protein)$^{-1}$ min^{-1}) and absence (30·1 nmol (μg protein)$^{-1}$ min^{-1}) of MSX, at rate analogous to that of the parent in the absence of MSX (32·6 nmol (μg protein)$^{-1}$ min^{-1}). In order to improve ammonium release by SS1 the culture was diluted to 5 μg chlorophyll ml^{-1} and maintained in continuous culture. High ammonium excretion was observed for a prolonged period with a high nitrogenase activity. Immobilization of cells in alginate beads increased nitrogen fixation by 50% over that of the parent and increased the rate of ammonium excretion by 33% over the cell-free system. However, under the immobilized system ammonium release occurred only up to 20 h after loading in packed-bed reactors, when the initial cell concentration was of 5 μg chloro-

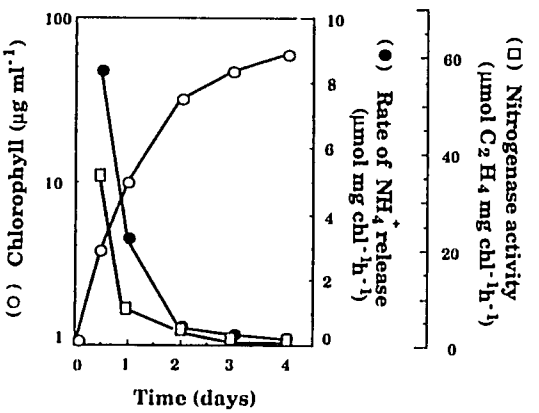

Fig. 2. Growth, nitrogenase activity and rate of ammonium excretion in batch cultures of *A. siamensis* mutant strain SS1.

Table 1. Ammonium release, growth rates and GS and nitrogenase activities for the parent strain of *A. siamensis* and the mutant SS1, under different growth conditions

Physiological parameter	*Batch culture*		*Continuous culture*		*Immobilized cells*	
	Parent	*SS1*	*Parent*	*SS1*	*Parent*	*SS1*
μ (h^{-1})	0·123	0·123	0·123	0·123	0·123	0·123
Nitrogenase activity (μmol C_2H_4 (mg chl)$^{-1}$ h^{-1})	21·0	35·5[a]	24·5	35·3	20·5	41·3
GS activity (μmol γ-glu. (mg protein^{-1} min^{-1})	3·6	1·3	3·0	1·4	3·1	1·2
Rate of NH_4^+ excretion (μmol (mg chl)$^{-1}$ h^{-1})	0·0	8·7*	0·0	8·7	0·0	11·6

[a]Measured after 12 h of inoculation.

phyll ml^{-1}. The growth on alginate beads was similar to that in batch cultures and the time course of ammonium excretion under immobilized conditions being also analogous. However, under all the conditions assayed, glutamine synthetase activity of the strain SS1 was less than 50% of that of the parent (Table 1). In order to check whether the MSX resistance and the reduced GS were due to a failure in transport of MSX into the mutants cells, GS activity was assayed at 500 μM MSX in the assay mixture. In the presence of MSX the parent's GS activity was completely inhibited whereas that of SS1 remained unaffected. Based on the above results, it is concluded that maintaining SS1 at a low cell density under continuous cultivation is a suitable condition for sustained excretion of ammonium by the cells.

Ammonium-excreting mutants of cyanobacteria are useful for many purposes. Mutants of *A. variabilis* have been used to study the mechanisms of ammonium uptake and assimilation and to understand the regulation of enzymes involved in amino-acid biosynthesis and nitrate assimilation (Spiller *et al.*, 1986). The mutant SA1 of *A. variabilis* has been proved to be an effective nitrogen-fertilizer source to contribute to the growth of rice plants (Lattore *et al.*, 1986). The fast growth rate of SS1 represents an advantage for the production of inoculum for algalization on a short-time basis. Field studies of SS1 in rice fields, in comparison with the parent strain, will provide information on the usefulness of SS1 in increasing the growth and grain yield of rice.

ACKNOWLEDGEMENT

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REFERENCES

Antarikanonda, P. (1982*a*). Effect of salinity on growth, nitrogen fixation and sodium uptake of rapidly growing N_2-fixing blue-green alga *Anabaena* sp. TA1. *Microbios*, **34**, 177–84.

Antarikanonda, P. (1982*b*). Influence of pretreating the seeds with extract of *Anabaena siamensis* on germination and growth of some rice varieties from Thailand. *FAO Int. Commission*, **31**, 37–9.

Antarikanonda, P. (1982*c*). Influence of nitrogen fixing blue green alga *Anabaena siamensis* on yield and protein quality of highly yielding rice cultivar (Khao Dowk Mali 4-2-105). German Res. Soc. Report No. 10680.

Antarikanonda, P. (1984). Production of extracellular free amino acids by cyanobacterium *Anabaena siamensis*. *Curr. Microbiol.*, **11**, 191–6.

Antarikanonda, P. (1985). A new species of the genus *Anabaena*: *Anabaena siamensis* sp. nov. (Cyanophyceae) from Thailand. *Nova. Hedwig*, **41**, 343–52.

Antarikanonda, P. & Lorenzen, H. (1982). N_2 fixing blue green algae (Cyanobacteria) of high efficiency from paddy soils of Bangkok, Thailand: Characterization of species and nitrogen fixing capacity in the laboratory. *Acta. Hydrobiol. Suppl.*, **63**, 53–70.

Boussiba, S. & Richmond, A. E. (1980). C-Phycocyanin as a storage protein in the blue-green alga, *Spirulina platensis*. *Arch. Microbiol.*, **125**, 143–7.

Grant, I. F., Roger, P. A. & Watanabe, I. (1985). Effect of grazer regulation and algal inoculation on photodependent nitrogen fixation in a wetland rice field. *Biol. Fert. Soils*, **1**, 61–72.

Hien, N. T., Kerby, N. W., Machray, G. C., Rowell, P. & Stewart, W. D. P. (1988). Expression of glutamine synthetase in mutant strains of the cyanobacterium *Anabaena variabilis* which liberate ammonia. *FEMS Microbiol. Lett.*, **56**, 337–42.

Joseph, C. M. & Meeks, J. C. (1987). Regulation of expression of glutamine synthetase in a symbiotic *Nostoc* strain associated with *Anthoceros punctatus*. *J. Bacteriol.*, **169**, 2471–5.

Kerby, N. W., Musgrave, S. C., Rowell, P., Shestakov, S. V. & Stewart, W. D. P. (1986). Photoproduction of ammonium by immobilized mutant strains of *Anabaena variabilis*. *Appl. Microbiol. Biotechnol.*, **24**, 42–6.

Lattore, C., Lee, J. H., Spiller, H. & Shanmugam, K. T. (1986). Ammonium ion-excreting cyanobacterial mutant as a source of nitrogen for growth of rice: a feasibility study. *Biotech. Lett.*, **8**, 507–12.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**, 265–75.

Mackinney, G. (1941). Absorption of light by chlorophyll solutions. *J. Biol. Chem.*, **140**, 315–22.

Martinez, M. R. (1984). Algae: biofertilizer for rice. *Philippines Council for Agriculture Research and Resources Development (PCARRD) Monitor*, **12**, 9–12.

Moore, A. W. (1969). *Azolla*: biology and agronomic significance. *Bot. Rev.*, **35**, 17–35.

Orr, J. & Haselkorn, R. (1982). Regulation of glutamine synthetase activity and synthesis in free living and symbiotic *Anabaena* sp. *J. Bacteriol.*, **152** 626–35.

Polukhina, L. E., Sakhurieva, G. N. & Shestakov, S. V. (1982). Ethylenediamine-resistant *Anabaena variabilis* mutants with derepressed nitrogen fixing system. *Microbiol.*, **51**, 90–5.

Ronzio, R., Rowe, W. & Meister, A. (1969). Studies on the mechanism of inhibition of glutamine synthetase by methionine sulfoximine. *Biochem.*, **8**, 1066–75.

Sampio, M. J. A. M., Rowell, P. & Stewart, W. D. P. (1979). Purification and some properties of glutamine synthetase from the nitrogen fixing cyanobacteria. *Anabaena cylindrica* and a *Nostoc* sp. *J. Gen. Microbiol.*, **111**, 181–91.

Solorzano, L. (1969). Determination of ammonia in natural waters by the phenolhypochlorite method. *Limnol. Oceanogr.*, **14**, 799–801.

Spiller, H., Latorre, C., Hassan, M. E. & Shanmugam, K. T. (1986). Isolation and characterization of nitrogenase derepressed mutant strains of cyanobacterium *Anabaena variabilis*. *J. Bacteriol.*, **165**, 412–19.

Stewart, W. D. P. (1980). Some aspects of structure and function in N_2-fixing cyanobacteria. *Ann. Rev. Microbiol.*, **34**, 497–536.

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Stewart, W. D. P., Fitgerald, G. P. & Burris, R. H. (1967). In situ studies on N_2 fixation using the acetylene reduction technique. *Proc. Natl. Acad. Sci. USA*, **58**, 2071–8.

Venkataraman, G. S. (1975). The role of blue green algae in tropical rice cultivation. In *Nitrogen Fixation by Free-living Microorganisms*, ed. W. D. P. Stewart. Cambridge University Press, London, pp. 207–18.

Zimmerman, W. J. & Boussiba, S. (1987). Ammonia assimilation and excretion in an asymbiotic strain of *Anabaena azollae* from *Azolla filiculoides*. *Lam. J. Plant. Physiol.*, **127**, 443–50.

Terminal Report
CDR Project No. C5-233
AID-CDR Grant No. DPE-5544-G-SS-6036-00

Title: Ammonium Translocation in Cyanobacteria and their Possible Use as Biofertilizers

Collaborating Investigator: Dr. Milagrosa R. Martinez
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I. Field Experiments

A. Soil-based ponds

A series of experiments were conducted on twenty seven 2.5 m^2 soil-based concrete ponds for biomass production of nitrogen fixing blue-green algae (BGA) in relation to rice production from January, 1987 to April, 1991. Lipa clay loam or Maahas clay [with original chemical properties as follows: 0.11% total nitrogen, 3.1 available phosphorus (Bray P_2), and 7.0 pH_7 was maintained at 50cm depth and flooded with 10-15 cm depth of tap water from soil surface.

For about four years prior to these experiments, i.e., before January, 1987, these ponds were used for various algalization and inoculation studies of BGA.

1. Open pond experiments (No rice plants) January, 1987-March, 1988

A 14 month study was conducted to determine the potentials of the ponds to produce BGA biomass by just modifying some agronomic practices for rice production. In this case we applied a weekly fertilization of phosphorus equivalent to 1 kg P.ha^{-1} (Solophos, 18% P) per pond without any inoculation of BGA.

Among the eighteen ponds studied, only 13 ponds produced appreciable algal harvest, i.e., algal growth that fully covered the surface area of the pond.

Among the bloom-forming BGA noted were the mucilaginous, colonial types: Gloeotrichia natans and Nostoc carneum whose yield ranged from 17.4 to 64.6 g, dry wt., $m^{-2}.wk^{-1}$. A non-colony forming, planktonic species of Anabaenopsis formed also a bloom that yielded a biomass from 0.4 g to 15.8 g, $dw.m^{-2}, wk^{-1}$.

There were 43 harvests that yielded appreciable algal biomass, from 18 ponds due to colony-forming BGA, while the rest were credited to the planktonic Anabaenopsis.

There was a total algal biomass of 985.5 g, $dw.m^{-2}$ throughout the study and 84% was due to the colony-forming BGA. A total of 9,855 kg, dry wt. was harvested. Assuming a conservative average of 2.6% N for the algae, we obtained a total yield of 256 kg $N.ha^{-1}$ out of an input of 56 kg $P.ha^{-1}$.

Among the six abiotic floodwater analyses studied, as: orthophosphates, ammonium-nitrogen, conductivity, dissolved oxygen, pH and temperature, only conductivity reading was found to be significantly related to algal biomass. Algal harvest were accompanied by a previous conductivity readings of 400-600 $umhos.cm^{-2}$ at 25°C at 8:00 A.M. and 700 $umhos.cm^{-2}$ at 11:00 A.M. Poor or no algal yield was noted in ponds with conductivity values below 400 umho at 8:00 A.M. Likewise, algal yield was accompanied by pH values of 7.0-7.5 and water temperatures of 28-35°C.

The limiting effect of rainfall on algal growth was alleviated with the use of fish nets (9 mm^2 mesh size) at the soil surface to support and keep the algae afloat despite the battering and fragmenting effects of heavy rainfall.

Protective sheds over each pond were put up either of the monitor or hemispherical type covered with heavy duty clear plastics that likewise prevented the physical destructive effect of rainfall on the colonial,

massive BGA. The condition under the sheds promoted the growth of the planktonic Anabaenopsis, from May 26 to November 12, 1987. This occurred despite the lower light intensity in these conditions as compared to the open ponds. Hence, thereafter, a large hemisphorical shed was constructed over the 18 ponds, but it was not effective in producing BGA with the plastic cover probably due to some structural defects.

2. Open pond experiments with rice plants

a. October, 1987 - January, 1988

A completely randomized experimental design was set up on 9 ponds planted with rice variety, IR-64, to test the effect of three levels of phosphorus fertilization with 3 replicates [i.e., 0, 60, and 90 kg. $P.ha^{-1}$] on algal and grain yield despite the absence of algal inoculation. No chemical nitrogen fertilizer was applied.

Algal biomass was positively correlated to phosphorus treatment but there was no significant difference among treatments. Of the plant parameters, only the number of filled grain showed significant positive relationship to algal biomass and phosphorus contents.

b. March-July, 1990

A completely randomized experimental design was set up on 18 ponds to determine the effects of chemical fertilization of nitrogen and phosphorus in ponds without and with rice plants (IR-70) and on algal and grain yield. No algal inoculation was done.

Indigenous nitrogen-fixing blue-green algae observed in the production ponds in their decreasing order of abundance and occurrence were: Gloeotrichia natans, Aphanothece pallida, Nostoc carneum and Nostoc commune.

The highest biomass obtained was about 120 g, dry $wt.m^{-2}$ per week in May, 1990 in open ponds but no appreciable biomass was obtained in ponds with rice. Of the 4 agro-meteorological parameters

examined (sunshine duration, radiation intensity, total rainfall and air temperature) algal biomass was positively correlated to solar radiation and sunshine duration. On the other hand, algal biomass was inversely related to dissolved oxygen, ammonium-nitrogen and orthophosphates of the floodwater but significantly positively correlated to conductivity, pH and water temperature. No significant effect on treatments were noted on rice yield.

c. January 25-April 11, 1991

The experimental set up in b (March-July, 1990) was repeated to determine the interrelationship between the planktonic algae and the colony forming algae in six different treatments with three replicates using IR-72 rice variety as needed. The effect of the season can also be implied in this study if we compare these results with that of b.

Four species of BGA constituted the floating mass of BGA in unplanted flooded ponds and without chemical nitrogen and phosphorus fertilizers. The dominant and frequently occurring species in decreasing order were: Gloeotrichia natans, Aphanothece pallida, Nostoc corneum and Nostoc commune. Algal biomass ranged from 1.0 to 2,589.64 g, fresh wt.m^{-2} per week. The highest biomass was equivalent to 26 g, dry wt., m^{-2} per week. No appreciable algal biomass was noted in planted ponds.

In the open ponds, the BGA showed positive correlation to the amount of ammonium-nitrogen and orthophosphates in the floodwater but showed an inverse relationship to the amount of rainfall. Biomass increased with time reaching its maximum near the end of the experiment.

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On the other hand, the planktonic algae in open ponds had bimodal peak, i.e., near the beginning and near the end of experiment. The dominant algae in the former were diatoms while at the latter period tney were blue-green algae but different from the colony-forming types. This bimodal peak of algal density was also noted in rice paddies and the dominant algae in the beginning of the crop cycle in decreasing order were: diatoms, green algae and euglenoids. At the end of the crop cycle the dominant planktors were the diatoms and the blue-green algae. The usual planktonic algae in decreasing order of abundance were: Oscillatoria, Merismopedia, Lyngbya and Anabaena. These genera were quite different from the bloom-forming colonial species.

There was no significant difference in rice yield in the different treatments.

B. Raceways Pond - Philippines

A $3m^2$ raceways pond was constructed that cost us ₱44,957.65 or $1,665.10 to grow "clean" unialgal cultures.

Initial growth studies on Gloeotrichia natans during the dry season when the sunshine duration and radiation intensity were at optimum values showed a doubling time of 0.0058 h^{-1}. This value is 1/30 of that obtained for the same species under Israeli condition (Palacpac, et. al., 1990).

Algal growth was limited during the dry season and once the wet season commenced the biomass further declined due to the chironomid larvae that fed on the colonial mass of algae.

II. Laboratory and Field Experiments - Israel condition

Gloeotrichia natans, a nitrogen fixing cyanobacterium common in rice fields in the Philippines, was used for studies to establish key features of its physiology and potential production in outdoor cultures. Under optimal growth conditions (38°C, pH 8.0, no carbon enrichment) the specific growth rate of the rice-field isolate was 0.076 h^{-1}. The pH of the medium (between 6.5 and 9.0) did not influence the growth rate, but it did affect phycobiliprotein content, as reflected by a change in colour. At pH 7.0 the culture was green-brown, with phycobiliproteins constituting up to 10% of the total protein, while at pH 9.0 the culture was brownish-black and the pigment content was as high as 28% of the total protein. In outdoor cultures the specific growth rate was related directly to cell density in the range of 0.7-1.5 g dry weight l^{-1} at a rate of stirring of 30 rpm, and inversely related to cell density at half this rate. At a stirring of 30 rpm, daily production of outdoor cultures harvested to maintain cell densities of 0.7, 1.15 and 1.5 g l^{-1} were 14.7, 17.1 and 18.1 g m^{-2} d^{-1}, respectively. This rate of production was maintained for more than 45 days. Phycobiliprotein content in the culture kept at a density of 1.5 g l^{-1} reached 14% of the total biomass.

III. Laboratory experiments

A. October, 1987-December, 1988

In vitro studies on the following nitrogen-fixing blue-green algae: Anabaena laxa, Hapalosiphon welwitschii, Tolypothrix tenuis and Nostoc commune indicated their ability

to grow in pH ranging from 6 to 10 but with optima at basic pH of 7-9. These algae could also grow up to 15 $g.l^{-1}$ NaCl added to the basal inorganic medium (BG-11 minus nitrogen).

Gloeotrichia natans favored growth in air (9-10 hr doubling time). It could also grow in 1% NaCl added to the basal inorganic medium but very slowly (96 h doubling time). Nitrogenase activity was already depressed at 0.5% NaCl concentration. The optimum temperatures for its growth ranged from 35-44°C while 9.0 was the optimum pH.

B. 1989-1990

Gloeotrichia natans was successfully cultured in the turbidostats (purchased from Israel) at ambient temperature of 29°C and an illumination of 3000 lux. Likewise, it grew well in 25 l demijohn in the basal inorganic medium but continuously aerated and with illuminance of 2,000 lux. Its doubling time ranged from 24.3 to 79 h. depending upon the cultural conditions.

Table 1 summarizes the doubling time of the various BGA studied. Anabaena azollae 7120 obtained from Israel did not show good growth under Philippine conditions.

Carotene contents of some floating mass of algae were determined and their importance in decreasing Order is as follows: Trentepohlia, Rhizoclonium, Cladophora, Spirogyra, Chara, Lyngbya, Gloeotrichia natans, Nostoc commune, Nostoc carneum and Aphanothece pallida. Hence, this indicates that the green algae have higher carotenoid composition than the blue-green algae.

Table 1. Doubling time of some blue-green algae in different culture vessels bubbled with air at 25°C in the laboratory.

BGA	DOUBLING TIME (h)				
	Turbidostat (500 ml)	Erlenmeyer flask (500 ml)			Demijohn
Anabaena laxa	41.2b	19.09	21.26	45.89	
Gloeotrichia natans	24.32	63.58	31.26	78.75	32.75
Hapalosiphon welwitschii	31.50	43.31	18.51	43.31	
Anabaena azollae 7120	26.65	58.80		62.60	
Tolypothrix tenuis			67.54		

OUTDOOR PRODUCTION OF NITROGEN-FIXING BLUE-GREEN ALGAE*

by

M.R. Martinez, V.M. Marcelino, N.Q. Palacpac
and Sammy Boussiba

A 14-month study was conducted to determine the potentials of soil-based concrete ponds (2.5 m^2) to produce indigenous nitrogen-fixing blue-green algae BGA by adding phosphorus equivalent to 1 kg P . ha^{-1} (Superphosphate, 18% P). No algal inoculation was done.

Of the 18 ponds studied, only 13 ponds yielded harvestable algae or algal bloom giving a total of 43 harvests. Among the bloom-forming BGA noted during the dry season were Gloeotrichia natans and Nostoc carneum. All of these were mucilaginous, colonial types. On the other hand, the non-colony forming planktonic species, Anabaenopsis sp. was observed during the wet season. The biomass of the colonial BGA ranged from .17.4 to 65 g dry wt . m^{-2} . wk^{-1} while the non-colonial species had a biomass of 0.4 to 16 g dw . m^{-2} . wk^{-1}.

The total algal yield was equivalent to 9,855. kg . ha^{-1}, and 84% of this was due to the colony-forming BGA. Assuming a conservative N content of 2.6% for the algae, a total N yield of 256 kg . ha^{-1} out of an input of 56 kg P . ha^{-1} in 14 months was obtained.

Conductivity readings were found to be significantly correlated to algal growth/bloom while rainfall limited the growth of the colony-forming BGA. However, Anabaenopsis sp., was induced to grow during the wet season housed under the plastic sheds.

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*Technical Paper no.1.

EFFECTS OF PHOSPHORUS ON ALGAE AND RICE GROWTH*

by

M.R. MARTINEZ, J.F. SARMIENTO, and S. BOUSSIBA

A completely randomized experimental field design was set up on nine plots planted with rice variety, IR-64, to test the effect of three levels of phosphorus fertilization (superphosphate, 18% P) with 3 replicates, i.e., 0, 60, and 90 kg· $P \cdot ha^{-1}$, on algal and grain yield. No algal inoculation was done nor nitrogen fertilizer was applied.

Algal growth/bloom was positively correlated to phosphorus treatment but not significantly different among treatments. Of the plant parameters, only the number of filled grain showed significant positive relationship to algal growth and phosphorus treatments.

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*Technical Paper no. 2.

To Dr. Sammy Doussiba

INTERRELATIONSHIP OF PLANKTONIC AND COLONNY FORMING ALGAE IN SOIL-BASED PONDS*

by

M.R. Martinez, D. Encio, P.P. Paje, H.T. Guevarra and Sammy Doussiba

The dominant colony-forming algae were different from the dominant planktonic algae in both planted and unplanted with rice (IR-72) in soil-based ponds regardless of chemical fertilizer (N and P) treatments.

The common and abundant colony-forming algae were the mucilaginous blue-green algae in this decreasing order: Gloeotrichia natans, Aphanothece pallida, Nostoc carneum and Nostoc commune.

Dominant planktonic algae varied with treatments and over time. Important factors that affected the two forms of algae (colony-forming and planktonic) were sunshine duration, solar radiation (inversely, amount of rainfall); water temperature, conductivity values, pH, ammonium-nitrogen and orthophosphates. The possible influence of these factor on algal bloom is discussed. The effect of treatments on Shannon's species diversity index and Simpson's dominance index are presented.

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*Technical Paper no.3.

PRODUCTION OF INDIGENOUS NITROGEN-FIXING BLUE-GREEN ALGAE IN PADDY FIELDS OF THE PHILIPPINES[1]

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ABSTRACT

Ten years of research on nitrogen-fixing blue-green algae (BGA) in Philippine paddy fields have laid down the foundation for BGA mass production in small-scale for farmers.

Focus were on 2 species observed in great abundance and of widespread occurrence which were Gloeotrichia natans and Nostoc commune.

Biomass productivity and inoculation technology are presented.

[1] Paper presented at the Research Seminar and Workshop on "Mass Cultures of Microalgae" held on November 18-23, 1991 at Silpkorn University, Nakorn Pathom 7300, Thailand.

INTRODUCTION

It has long been recognized that the natural fertility of flooded rice soils is due to the nitrogen-fixing blue-green algae (BGA) (De, 1939; Watanabe, et al., 1978; Martinez, et al., 1981). Their abundance in the tropics, especially in paddy fields, is well established (Roger and Kulasooriya, 1980). The Philippines is no exception to this fact.

Considerable progress have been made in developing appropriate biotechnology for making use of these indigenous BGA. However, despite the accumulated data supporting the beneficial effects of algalization to increase rice yield (Martinez and Palacpac, 1990; Pantastico and Gonzales, 1976), there has been no concerted effort to bring the technology any nearer to the village level or for commercial applications. This report attempts to assess the requirements and potentials for making this bictechnology economically viable.

RECORDS OF BGA ABUNDANCE

Studies on blue-green algae in the Philippines have recorded a wealth of species in the country (Martinez, 1981). Heterocystous species account for 34% of the total 361 species listed. Of these, Nostoc has the most number of species (20), followed by Anabaena (16), Calothrix (15), Scytonema (14), and Cylindrospermum (8)

Evidence of nitrogen fixation were also observed for some unicellular and non-heterocystous filamentous forms, such as: Aphanothece pallida, Gloeocapsa sp. and Oscillatoria limnetica (Querijero and Martinez, 1986)

In range of habitats, about 13% of the total freshwater species were recorded in rice paddies. This latter ecosystem seems to favor the occasional bloom of the colonial, mucilaginous species of BGA, such as Gloeotrichia natans, Aphanothece pallida, Nostoc carneum, N. linckia, and N. commune. A bloom in this case, is defined as an occurrence of floating mass of algae that cover at least 50% or more of at least 2.5 m^{-2} surface area of a paddy field.

QUANTITATIVE ESTIMATIONS OF BGA

Methodology

Direct and indirect measurements were done in estimating BGA biomass. Among the direct methods used were 1) biomass determinations, 2) cell enumeration, and 3) plating technique.

Biomass determination gives data in units of weight per unit volume or area with the use of a balance. This is one of the easiest direct way of obtaining biomass except that determination of species composition has to be done separately. Sampling technique has to be done carefully to give a good representation of the uneven spatial distribution of the algae.

Cell enumeration involves the direct observation of the algae under the microscope and enumeration is done with any calibrated slide, like the haemacytometer (Martinez, et al 1975). The method yields both qualitative and quantitative results. The results can be expressed in terms of biomass by determining the mean volume of each "count unit". The method, however, is usually more appropriate for unicellular, planktonic species than the mucilaginous, colonial types.

Plating technique provides also qualitative and quantitative data. It can also convert data into biomass. The accuracy of the results depends on the media used and in the dilution technique.

One of the indirect measurements is acetylene reduction assay (ARA) technique.

Acetylene reduction assay is a good method for analyzing the nitrogen fixing activity of the BGA but it is expensive and its accuracy is difficult to ascertain in situ.

Results

Biomass measurements indicate that the BGA can develop large biomass up to a few tons per hectare (Table 1). The shallow wetland ricefields yield the highest BGA biomass (Roger and Reynaud, 1979) among the cultivated soils. Among the common and abundant colony forming BGA species in decreasing order were:

Gloeotrichia natans, Nostoc commune, Aphanothece pallida, Nostoc carneum, and Anabaenopsis sp

Chemical composition of two of these species, N. commune and G. natans (Table 2), shows that they have sufficient nutritiona value either as N fertilizer source or protein source for food These algae could contribute between 0.4 to 83 kg N . ha^{-1} (Roger and Kulasooriya, 1980). The protein content of the edible species (N. commune) can be as much as 48%

Data on enumeration of planktonic nitrogen fixing blue-green algae are scanty. Table 3 presents the counts of Nostochopsis lobatus taken from two ricefields with different soil pH. On the average there was about 300 units . ml^{-1}.

The BGA were noted to be abundant in the upper layer of most Philippine soils. They were also noted to be more abundant in paddy fields of lower elevations (lowlands) than in upper elevations (uplands) (Table 4) (Roger, et al., 1986b)

Measurements of acetylene reduction activity show higher values from field-cultured BGA than laboratory cultured samples (Table 5).

INOCULATION TECHNOLOGY

as biofertilizer source.

Biomass production of BGA can be done in wetland ricefields using an inoculum rate of 10-20 kg dw.ha^{-1}, with supplementation of phosphorus at the rate of 30 to 60 kg P . ha^{-1} . $crop^{-1}$. The amount

of inoculum varies with soil type and the season, e.g., using more inoculum in poor N soil and in the wet season.

Algalization experiments in the dry and wet seasons using BGA inoculation rates of 10 and 20 kg. dw.ha^{-1} showed grain yield that are comparable to one another, although higher grain yield was obtained when BGA was supplemented with chemical N fertiliser (Martinez and Querijero, 1986a)

Inoculation of non-indigenous BGA, Gloeotrichia natans, either surface-applied or incorporated, as fresh or dried material did not dominate the algal flora in a soil with indigenous BGA. Indigenous BGA, Nostoc commune and N. carneum attained maximum biomass of 60-90 kg.dw.ha^{-1} in 50-60 days (Martinez and Querijero, 1986a). Addition of urea (30 kg N.ha^{-1}) suppressed the total algal biomass but its effect was only temporary (up to 7 days of fertilization)

Surface applied fresh BGA gave a higher grain N content over the dried BGA whether surface-applied or incorporated. Plants fertilized with urea at 30 kg. N.ha^{-1} together with BGA inoculation showed comparable grain yield to plants fertilized with 60 kg urea-N.ha^{-1}.

Inoculation was observed to be ineffective in acidic soil (pH 5.7) with poor available phosphorus (5.8 ppm, Olsen's method) (Reddy, et al., 1986).

As Biofertilizer and biomass sources.

When the BGA is used both as a N-biofertilizer and as biomass; the above rates are also recommended and are applied once during land preparation, preferably to be incorporated into th soil. Before maximum tillering, about 40 days after transplanting (DAT), the floating algal biomass are harvested by a weeder that passes in between rows of rice tillers. In this case, other weeds are also being harvested.

As biomass source.

If the BGA will be used mainly as biomass source, either for its food value or its other commercial products, then this could be done in fallowed fields (flooded soil-based ponds without rice plant).

Soils with total N_2-fixing BGA enumeration of 5.0×10^4 on the upper horizon usually do not need to be inoculated with BGA In this case, the usually recommended fertilisation is only the application of 1 kg $P.ha^{-1}$. wk^{-1}. The commercial source of phosphorus is superphosphate (18% P). Using this methodology, BGA yield can reach a maximum value of 4 tons dry matter in 60 days (Martinez, unpubl.)

STORAGE AND TRANSPORT

The alga can easily be stored by simple air-drying in a non umid place, or in a freezer.

Gloeotrichia natans, after storage for 24 h showed favorable recovery both in terms of growth and its nitrogen-fixing ability. Oven-dried samples (30-70°C) showed higher biomass recovery yield than air-dried lots (Martinez, et. al 1983). Earlier dried BGA have been demonstrated to be viable even after two years of storage in sand (Venkataraman, 1961) or in porous volcanic gravel (Watanabe, 1958)

In the dried form, the BGA can easily be transported in bulk for inoculation in the field

Another easy method of transporting BGA is by storing spores or akinetes instead of the colonial mass of filaments. Spores have other advantages of being more resistant to adverse conditions and having the ability to produce a greater bulk of biomass when germinated. Sporulation in Gloeotrichia natans was noted in low phosphorus media (less than 0.12 g P/l). On the other hand, supplementation of 3% glucose or fructose in inorganic media promoted spore formation in Nostoc commune (Quimado, et. al., 1988)

CONCLUSIONS

The paddy field ecosystem provides a favorable environment for the growth of nitrogen-fixing blue-green algae Among these algae that were observed to be of widespread occurrence and in great abundance were Gloeotrichia natans and Nostoc commune.

Among the edaphic factors, soil pH and available phosphorus are the two most important limiting factors affecting BGA biomass

Bulk production was successfully demonstrated in soil based ponds and when dually cultivated with rice

The inoculation technology developed is easy to follow and does not involve expensive infrastructure for its production.

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LITERATURE CITED

De, P.K. 1939. The role of blue-green algae in nitrogen fixation in ricefields. Proc. Roy. Soc. London B. 127: 121-139.

Kulasooriya, S. A., P.A. Roger, W.L. Barauqio and I. Watanabe. 1981. Epiphytic nitrogen fixation on weeds in a ricefield ecosystem. In. R. Wetselaar, et al., (eds.). Nitrogen Cycling in Wet Monsoonal Ecosystem, Canberra, Australia, Australian Acad. Sci. pp. 56-61.

Martines, M.R. 1964. A Checklist of Blue-green Algae of the Philippines. College, Laguna, Biotech, University of the Philippines at Los Banos, 96 p

_____. 1988. Nostoc commune Vauch., a nitrogen-fixing blue-green alga, as source of food in the Philippines. Phil. Agric. 71(3): 295-307

_____ and E.F. Aquino. 1986. Availability of blue-green algal nitrogen under flooded soil condition. Phil. Agric. 69: 567-582

Martinez M.R., R.P. Chakroff and J.B. Pantastico. 1975. Direct phytoplankton counting technique using the haemacytometer. Phil. Agric. 59: 43-50.

_____, N. Dulay-Leong, and R.F. Aquino. 1983. Resistance of Gloeotrichia natans to desiccation. Kalikasan Phil. J.Biol. 12: 182-186.

_____ and N.M.B.Q. Palacpac. 1990. Biotechnology and uses of nitrogen-fixing blue-green algae in the Philippines. In. Proceedings of the National Symposium on Bio-and Organic Fertilizers, held at UPLB on October 9-12,1989. UPLB National Azolla Action Program (NAAP). pp. 109-130.

_____, J.B. Pantastico, and W.C. Cosico. 1981. Blue-green algae and the fertility of lowland ricefields in the Philippines. In R. Wetselaar, et al., eds., Nitrogen Cycling in Southeast Asian Wet Monsoonal Ecosystem, Canberra, Australia. Australian Acad. Sci. pp. 29-35.

_____ and N.M.B.Querijero. 1986a. Fate of applied blue-green algae and their effects on the yield of wetland rice. Phil. Agric. 69 (4B): 611-627.

_____ and N.M.B. Querijero. 1986b. Macrocolony formation in the nitrogen-fixing blue-green alga, Nostoc commune Vauch. Phil. Agric. 69 (4B): 547-565.

Pantastico, J.B. and J.L. Gonsales. 1976. Culture and use of Nostoc commune as biofertiliser. Kalikasan, Phil. J. Biol. 5: 221-234.

Querijero, N.M.B. and M.R. Martinez. 1986. Acetylene reduction activity of some blue-green algae. Phil. Agric. 69 (4B): 593-603.

Quimado, M.O., M.R. Martinez, N.K. Torreta, and N.M.Q. Palacpac. 1988. Sporulation in the nitrogen-fixing blue-green alga, Nostoc commune Vauch. Phil. Agric. 71 (3): 309-316.

Reddy, P.M., P.A. Roger, M. Ventura, and I. Watanabe. 1986. Blue-green algal treatment and inoculation had no significant effect on rice yield in an acidic wetland soil. Phil Agr. 69 (4B): 629-632.

Roger, P.A. and S.A. Kulasooriya. 1980. Blue-green Algae and Rice. Los Banos, Laguna, International Rice Research Institute, 112 p.

Roger, P.A. and S.A. Kulasooriya, W.L. Baraquio, and I Watanabe. 1981. Epiphytic nitrogen fixation on lowland rice plants. In. R. Wetselaar, et al., (eds.). Nitrogen Cycling in Wet Monsoonal Ecosystem, Canberra, Australia, Australian Acad. Sci., pp. 62-66

_____and P. Reynaud. 1979. Ecology of blue-green algae in paddy fields. In Nitrogen and Rice. Los Banos, Laguna. Philippines, International Rice Research Institute. pp. 289-309.

_____, S. Santiago-Ardales, and I. Watanabe. 1986a. Nitrogen fixing blue-green algae in rice soils of Northern Luson (Philippines). Phil. Agri. 69 (4B): 589-598.

Roger, P.A., M. Voggesberger and J. Margraf. 1986b. Nitrogen-fixing phototrophs in the Ifugao rice terraces (Philippines). Phil. Agric. 69 (4B): 599-609.

Saito, M. and I. Watanabe. 1978. Organic matter production in rice field flood water. Soil Sci. Plant Nutr. 28(3): 427-440.

Venkataraman, G.S. 1981. A method of preserving blue-green algae for seeding purposes. J. Gen. Appl. Microbiol. 7(2): 96-98.

Watanabe, A. 1956. Some devices for preserving blue-green algae in viable state. J. Gen. Appl. Microbiol. 5: 153-157.

Watanabe, I., K.K. Lee, B.V. Alimagno, M. Sato, D.C. del Rosario, and M.P. de Guzman. 1977. Biological N_2-fixation in paddy field studied by in situ acetylene-reduction assays IRRI Res. Pap. Ser. 3:1-16.

_____. K.K. Lee and B.V. Alimagno. 1978. Seasonal change of N_2-fixing rate in rice field assayed by in situ acetylene reduction technique. I. Experiments in long-term fertility plots. Soil Sci. Plant Nutr. 24 (1): 1-13.

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Table 1. Biomass of field-cultured nitrogen-fixing blue-green algae.

Condition	Dominant BGA	Dry weight (Kg . ha^{-1})	Days of cultivation	Reference
Pot experiment	Gloeotrichia natans	20	70	Martinez & Querijero, 1986a
	Nostoc carneum	60-90	50-60	Martinez & Querijero, 1986a
	Nostoc commune	25	60	Martinez & Querijero, 1986a
		65	65	
On moist soil	N. commune	[illegible]	-	Martinez, 1988
Fallowed fields	Aphanothece pallida	617	7	Encio, unpubl.
	G. natans	87	70	Martinez, unpubl.
		4160	60	Martinez, unpubl.
		542	7	Encio, unpubl.
	N. carneum	19	7	Encio, unpubl.

Table 1 continued.

Condition	Dominant BGA	Dry weight (Kg . ha-1)	Days of cultivation	Reference
	N. commune	11	7	Encio, unpubl.
		75		Kulasooriya, et al 1981
Rice Paddy				
Free-floating	A. pallida	38	7	Encio, unpubl.
	G. natans	110-320	90	Martinez, et al., 1981
	G. natans	177	90	Watanabe, et al., 1977
	N. commune	200	90	Martinez, unpubl.
		2-114	90	Saito & Watanabe, 1978
		10-1,300	few days	Roger, et al., 1986b
Epiphytic on rice.		5.5-20	90	Roger, et al., 1981
on weeds		3(	90	Kulasooriya, et al., 1981

Table 2 Chemical composition of some field-cultured nitrogen-fixing blue-green algae.

Chemical composition	Gloeotrichia natans (%, dw)	Nostoc commune
C	38.3	30 - 43
N	1.6-2.5	3.8-6.02
P	0.32	0.36-0.42
Dry matter (%, fw)	0.10	3.0 -7.0

After: Martinez and Querijero, 1986a, b and Martinez and Aquino, 1986.

Table 3. Cell density of *Nostochopsis lobatus* and its percentage composition of the total phytoplankters in paddy fields with basic and acidic soils.

	Basic soil[1] 63 DAT[3]	Acidic soil[2] 70 DAT
units ml^{-1}	300	144
% of total phytoplankters	40	15

[1] Lipa clay loam, pH 6.0, Dry season, 1991

[2] Luisiana soil, pH 5.0, Dry season, 1991 (after: Martinez, unpubl.)

DAT = Days after transplanting

Table 4. Colony forming units (CFU) of nitrogen-fixing blue-green algae from top soil in different elevations of rice paddies around the Philippines.

	CFU cm^{-2} ($\times 10^5$)		
	Max	Min	Ave.
Lowlands (n=14)[1]	9.6	0.07	1.6
Uplands (n=12)[2]	11.7	0.03	3.21
Luisiana soil, pH 5.7; available P 5.6 ppm.[3]			0.16

1 After: Roger, et al., 1986 a

2 Data from Ifugao, Mountain Province, Dry season, 1983 (After: Roger, et al., 1986b)

3 After: Reddy, et al., 1986.

Table 5 Acetylene reduction activity of *in vivo* and in *vitro* cultures of blue-greenn algae.[1]

Species	Micromole C_2H_4 h^{-1} . g^{-1} dw *in vivo*[1]	*in vitro*[2]
Gloeotrichia natans	66 (no akinetes)	5
Anabaena cycadae	9	6

[1]Data in dry season 1987 (after: Martinez, unpubl.)

[2]After: Querijero and Martinez, 1986.