MELISSA

TN 12

MELISSA

ESTEC/Contract 8125/88/NL/FG

Technical note 12

Table of Contents P.

TN 12.1 AND TN 12.2: PHYSIOLOGICAL EFFECTS OF NITROGEN AND SULFUR LIMITATIONS DURING BATCH CULTURES OF THE CYANOBACTERIUM *SPIRULINA PLATENSIS*.

ABBREVIATIONS

Chl: chlorophyll; NNR: nitrogen recovery ratio; PC: phycocyanin; SRR: sulfur recovery ratio.

ABSTRACT

Depletion of nitrogen (N) or sulfur (S) principally results in proteolysis of phycocyanins (PC) and in a concomitant large accumulation of sulfated sugars including glycogen and exopolysaccharides. However, proteins other than PC continue to accumulate during N deprivation while they are degraded during S limitation. The recovery of supplied N or S into synthesized biomass is analyzed.

KEY WORDS

Cyanobacteria; *Spirulina*; mineral limitation; nitrogen; sulfur; batch culture.

INTRODUCTION

Control and mathematical modelling of microorganism cultures require preliminary knowledge of the effects of mineral limitations. Moreover, such limitations may result in modifications of the quality of the biomass produced as source of food.

Initial studies on the cyanobacterium *Anacystis nidulans* (Allen and Smith 1969) showed that during nitrate depletion, growth stops, apoproteins of phycocyanins (PC, the main light-harvesting pigment-protein) are degraded without significant changes in chlorophyll (Lau et al. 1977; Yamanaka and Glazer 1980), and carotenoid and intracellular glycogen concomitantly accumulate (Lehman and Wober 1976). Readdition of nitrates to deficient cultures reverses these trends. These observations were confirmed in *Synechocystis* (Allen and Hutchinson 1980) and *Agmenellum quadruplicatum* (Stevens et al. 1981). Allen et al. (1990) recently suggested that some inactivation of photosystem 2 could result in a decrease of the photosynthetic efficiency.

The cyanobacterium *Spirulina* that is analyzed in this report seems to follow this general response to nitrogen (N) deprivation (Boussiba and Richmond 1980), although carbohydrate accumulation has not been demonstrated. Few informations are available on intracellular glycogen (Sekharam et al. 1989) and exocellular polysaccharides (preliminary unpublished results) which both are sulfated.

Much less is known about sulfur (S) limitation, which also was reported by Cohen-Bazire and Bryant (1982) to impair phycobiliprotein content and composition in cyanobacteria.

In the present work, comparative effects of N and S limitation and replenishment on main nitrogen and sulfur containing molecular components are analysed during growth of *Spirulina platensis*. For each experiment, the recovery of the supplied N and S into the overall biomass and in its main molecular components is measured.

MATERIAL AND METHODS

Culture conditions:

Spirulina platensis 8005 (Institut Pasteur) were axenically grown in the Zarrouk (1966) medium modified as follow: NaCl 1g, $CaCl_2$ 0.03 g, K_2SO_4 1g, $MgSO_4,7H_2O$ 0.1g, KH_2PO_4 0.5g, $NaNO_3$ 2.5g, $NaHCO_3$ 10.5 g, Na_2CO3 7.6 g, EDTA 0.08g, $FeSO_4,7H_2O$ 0.005g and Arnon A_5B_6 solution 1ml, per liter. Nitrates and sulfates concentrations used for limitation studies are given in the figures. Cultures in flat Roux flasks were continuously mixed by magnetic stirring and by bubbling air-CO_2 (~0.5%, 1.5.10-5 $Nm^3.s^{-1}$) which allowed to maintain pH at 9.45$\pm$0.06. Temperature (36°C$\pm$1) was that of the thermostated room. Cultures were also performed in a 7l cylindrical bioreactor (Applikon) connected to a bioprocessor (Applikon ADI 1020) allowing to adjust temperature at 36°C$\pm$0.1 and to regulate pH at 9.5$\pm$0.01 by 1N HCl addition. The cultures were mechanically stirred (300 rpm) and aerated (air flow = $1.78.10^{-5}$ $Nm^3.s^{-1}$). For both culture conditions, loss of water by

evaporation was limited by a refrigerated condenser and unidirectional illumination was obtained by covering the culture with a black paper at the opposite side of the light sources (for Roux flasks: 4 Mazdafluor, 20W fluorescent tubes, 19 $W.m^{-2}$ and for the bioreactor, 2 Philips R7S, 500W halogen lamps, 100 $W.m^{-2}$ at the shortest distance).

Analytical methods:

Biomass was spectrophotometrically measured using a preestablished relation between dry weight and optical density at 750nm of culture samples. Total proteins were quantified with the BCA test (Pierce) on sonically disrupted cells. Total sugars were determined on filtered culture according to Herbert et al. (1971). Nitrates present in the culture medium were measured according to Cawse (1967). Sulfate were determined with the rhodizonate method (Terho and Hartiala 1971); disturbing effects of carbonates and bicarbonates were eliminated by lowering pH to 4 with acetic acid.

Chlorophyll *a* (Chl*a*) and phycocyanins were spectrophotometrically measured on sonically disrupted cells using the following equations:

$$Chla(kg.m^{-3}) = 1.04.10^{-2}\ A_{678} - 4.09.10^{-3}\ A_{620}$$

$$PC\ (kg.m^{-3}) = 0.297\ A_{620} - 0.076\ A_{678}$$

These equations were established from the relations between the concentrations of extracted chl*a* and PC, (calculated according to Myers et al. (1955) and to Bennett and Bogorad (1977) respectively), and the absorbances of chl*a* (A_{678}) and PC (A_{620}) in the disrupted cell suspension. A_{678} and A_{620} were obtained by correcting measurements of OD_{678} and OD_{620} from light scattering in the samples, according to the following relation

$$A_{678} = OD_{678} - 1.17\ OD_{750}$$

$$A_{620} = OD_{620} - 1.31\ OD_{750}$$

Photosynthetic oxygen evolution was measured at 35°C with an Hansatech Clark type oxygen electrode.

RESULTS AND DISCUSSION

1) Nitrogen limitation:

Effects of nitrate limitation are very similar whether batch cultures were performed in Roux flasks or in the bioreactor. Growth and accumulation of main cellular components in the presence of nitrates are initially exponential and rapidly become linear when light limitation is introduced by increasing shadowing (fig.1). As soon as nitrates are exhausted, total amount of proteins and of chl*a* in the culture stabilize to steady state levels. As expected, PC begin to be degraded and are used as N reserve for the continued synthesis of other proteins, since the amount of total proteins remains constant (fig.1a, see also fig.4). An

important residual biomass accumulation originates in a large synthesis of intracellular glycogen (Lehmann and Wober 1976, Allen and Hutchinson 1980, Stevens et al. 1981, Allen et al. 1990) which is probably sulfated (Sekharam et al. 1989). This intracellular glycogen accumulation is accompanied by an important excretion of exocellular sulfated polysaccharides (fig.1b).

Nitrate replenishment induces a rapid restoration of high levels of total proteins, which originates in a very important and rapid increase of PC, followed, to a lesser extent, by other cellular proteins (see also fig. 4). This does not lead to any important biomass accumulation since the level of total sugars correlatively falls down in consequence of metabolization of accumulated glycogen for rapid protein synthesis.

2) Sulfur limitation:

Effects of S (Fig 2) and N limitations are very similar. Phycocyanins are degraded while carbohydrates largely accumulate, leading to an important residual biomass formation. The main difference lies in the fact that the level of total proteins decreases, as the result of the degradation of phycocyanins, but also of other proteins (as determined by calculation, also shown in fig.5). The majority of cellular proteins thus appears to be used as S reserves for the synthesis and accumulation of sulfated glycogen and exopolysaccharides. Figure 2 also shows that after nitrate readdition, phycocyanins are synthesized more slowly than other proteins, suggesting that essential proteins were degraded during S limitation and are reconstituted in priority.

3) Photosynthetic oxygen evolution:

Measurements of photosynthetic oxygen evolution were performed on culture samples adjusted to the same concentration of the main light harvesting PC, and therefore absorbing similar amount of light quanta per time unit. Light saturation curves of oxygen evolution on a PC basis were measured during the time course of nitrate deprivation, on cells therefore containing decreasing proportions of PC (fig.3). The very similar initial slopes and saturation levels measured under low and high light intensities demonstrate that those of incident photons that were absorbed in the samples were used for photosynthesis with the same efficiency, for each light intensity up to 400 $W.m^{-2}$ (higher light intensities induce photoinhibition in PC rich culture samples).

When transposed to the culture conditions, where light is rapidly limiting (all or most of incident photons being absorbed), these results show that the amount of CO2 photosynthetically absorbed within the cultures is not significantly altered by the loss of PC subsequent to nitrate (and probably of sulfate)

deprivation, and they therefore account for the observed continued carbohydrate synthesis and accumulation.

4) Nitrogen and sulfur recovery in the synthesized biomass:

Mathematical modelling of the culture implies that equilibrated stoechiometric equations and mass conversion yields of N or S into biomass are determined. It is therefore important to verify that all N or S supplied to the culture as growth substrates are recovered in the synthesized biomass and that all main classes of N or S containing biological molecules involved in the biomass constitution are correctly identified and measured.

This can be qualitatively done by comparing the decrease of N or S in growth substrates to the increase of N or S in the measured total biomass or in the main classes of N or S containing synthesized molecules. Figure 4 shows that, at any stage of growth and limitation, N consumed from nitrates is entirely recovered in the synthesized biomass, and in great majority in proteins. Similarly, S from sulfate is totally recovered in the biomass (fig.5), and is shared between proteins and other molecules (sulfated glycogen and exopolysaccharides, which have not been presented because their N or S content is not known). One has to notice that calculations of N or S recovery in the biomass are only valid during the growth phase, since our spectrophotometric estimation of biomass does not take into account the sulfated exopolysaccharides synthesized in excess as secondary metabolite during the limitation phase.

A more quantitative approach consists in calculating the recovery ratio of N or S in the synthesized material. This ratio can be calculated by two methods. A global one just refers to the overall *Spirulina* biomass before limitation, the C molar formula of which has been determined to correspond to:

$$C\ H_{1.65}\ O_{0.53}\ N_{0.17}\ S_{0.0074}\ P_{0.0056}$$

with a C molar mass of 24.9 $kg.kmole^{-1}$. A more detailed expression of this ratio refers to the main biomolecules forming the biomass, knowing that N represents 16% (w/w) of proteins, 6.3% of chla and 16.8% of nucleic acids (nitrogen of nucleic acids corresponds to ~0.7% of *Spirulina* biomass).

The two expressions of the nitrogen recovery ratio (NRR) may be proposed since all major N containing molecules present in the synthesized biomass have been identified:

$$NRR1 = \frac{0.0956.Biomass}{0.226.Nitrates}$$

(0.0956 and 0.226 represent the weight proportions of N in the biomass and in nitrates respectively).

$$NNR2 = \frac{0.16.Proteins + 0.063.Chla + 0.007.Biomass}{0.226.Nitrates}$$

For sulfur, the first expression only is available since the proportion of S in total sugars is still unknown:

$$SRR = \frac{0.0095.Biomass}{0.333.Sulfates}$$

(0.0095 and 0.333 represent the weight proportion of S in the biomass and in sulfates respectively).

Average values of NRR1, NRR2 and SRR have been calculated (table 1) for different stages of the linear growth phase, during which we did not detect any excretion of secondary metabolites (such as sulfated exopolysaccharides), which would have escape to our determinations of synthesized biomass or main biomolecules classes.

The high values obtained, very close to 1 are consistent with figs.3 and 4 and confirm: 1) the validity of the experimental determinations, and 2) that all main N or S containing main molecular components have been identified. In addition, higher values obtained for NRR2 show that recovery ratios would be best expressed when referring to the main molecules constituting the biomass.

CONCLUSION

The good measured recovery ratios authorize to use the obtained experimental results in view of mathematical modelling.

The observed changes in the biomass composition during N or S limitation allow to calculate the resulting new stoechiometries during growth. Moreover, such changes open the possibility to adjust the carbohydrate to protein ratio of the produced *Spirulina* biomass to the desired value. This last point could be important for further studies of the photosynthetic compartment of a controlled ecological life support system (CELSS) designed for the production of food for a spatial crew.

REFERENCES

Allen MM. Hutchinson F (1980) Nitrogen limitation and recovery in the cyanobacterium *Aphanocapsa*6308. Arch Microbiol 128:1-7

Allen MM. Law A and Evans EH (1990) Control of photosynthesis during nitrogen depletion and recovery in a non-nitrogen fixing cyanobacterium. Arch Microbiol 153:428-431

Allen MM. Smith AJ (1969) Nitrogen chlorosis in blue green algae. Arch Mikrobiol 69:114-120

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

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

Cawse PA (1967) The determination of nitrate in soil solutions by ultraviolet spectrophotometry Analyst 92:311-315

Cohen-Bazire G, Bryant DA (1982) Phycobilisomes: composition and structure. In:Carr NG, Whitton BA (eds) The biology of Cyanobacteria. Blackwell Scientific Publications. Botanical Monographs 19:143-190

Herbert D, Phillips PJ, Strange RE (1971) Chemical analysis of microbial cells. In:Norris JP, Ribbons DW (eds) Methods in microbiology. Academic Press, 5b:209-344

Lau RH, Mac Kenzie MM, Doolittle FW (1977) Phycocyanin synthesis and degradation in the blue green bacterium *Anacystis nidulans*. J Bacteriol 132:771-778

Lehmann M, Wober G (1976) Accumulation, mobilization and turn-over of glycogen in the blue green bacterium *Anacystis nidulans*. Arch Microbiol 111:93-97

Myers J, Kratz KA (1955) Relation between pigment content and photosynthetic characteristics in blue green alga. J Gen Physiol 39:11-22

Sekharam KM, Venkataraman LV, Salimath PV (1989) Structural studies of a glucan isolated from blue green alga *Spirulina platensis* Food Chemistry 31:85-91

Stevens SE, Balkwill DL, Paone DAM (1981) The effects of nitrogen limitation on the ultrastructure of the Cyanobacterium *Agmenellum quadruplicatum*. Arch Microbiol 130:204-212

Terho TT, Hartiala K (1971) Method for determination of the sulfate content of glycosaminoglycans. Anal biochem 41:417-476

Yamanaka G, Glazer AN (1980) Dynamic aspects of phycobilisome structure. Phycobilisome turnover during nitrogen starvation in *Synechococcus sp.* Arch Microbiol 124:39-47

Zarrouk C (1966) Contribution à l'étude d'une cyanophycée. Influence de divers facteurs physiques et chimiques sur la croissance et la photosynthèse de *Spirulina maxima* (Setch et Gardner) Geitler. Thesis, Paris

Table 1: Recovery ratios of N and S (NRR and SRR) in the synthesized biomass (NRR1, SRR) or main biomolecules (NRR2).

N recovery				S recovery
Roux flasks		Bioreactor		Roux Flask
NRR1	NRR2	NRR1	NRR2	SRR
0.96	0.97	0.80	0.88	0.91

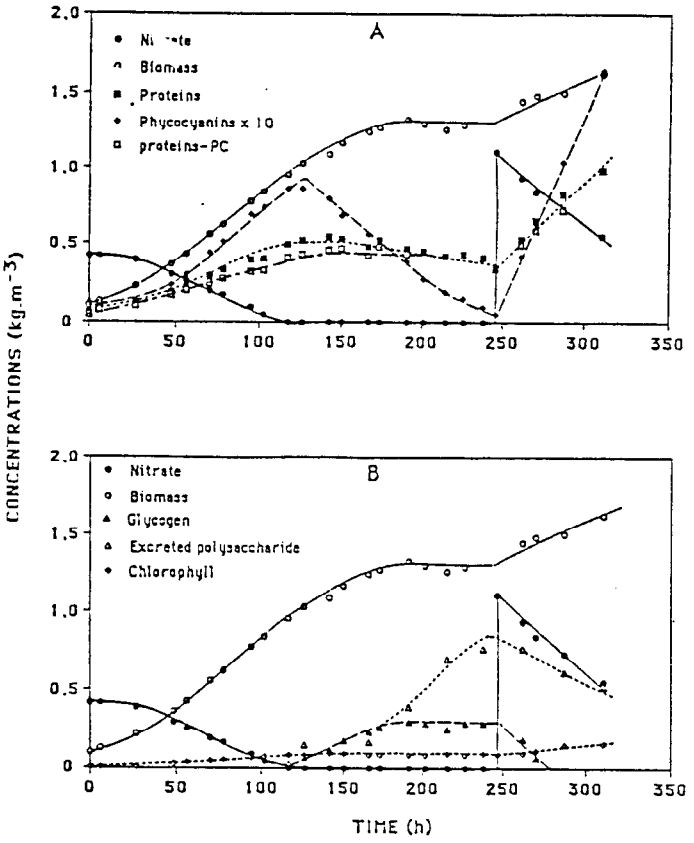

Figure 1 - Nitrate limitation and readdition during batch cultures of *Spirulina Platensis* in a bioreactor. Effects on the biomass and on the concentration of main molecules; A: Biomass and proteins, B: Biomass, carbohydrates and chlorophyll.

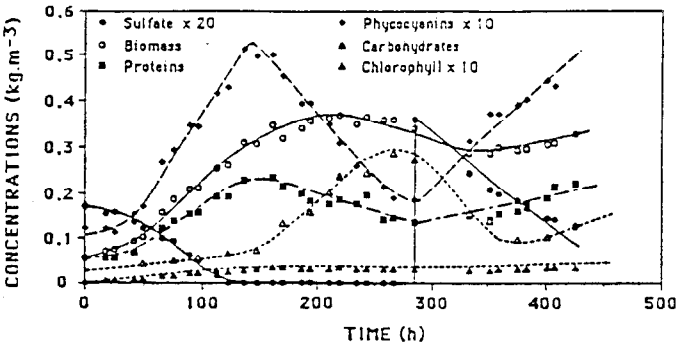

Figure 2 - Sulfate limitation and readdition during batch cultures of *Spirulina platensis* in a Roux flask. Effects on the biomass and on the concentration of main molecules.

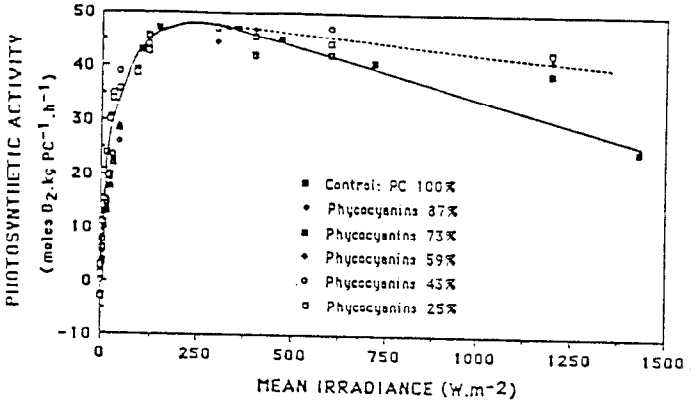

Figure 3 - Light saturation curves of photosynthetic oxygen evolution by *Spirulina* cells containing decreasing proportions of phycocyanins, as nitrate starvation proceeded.

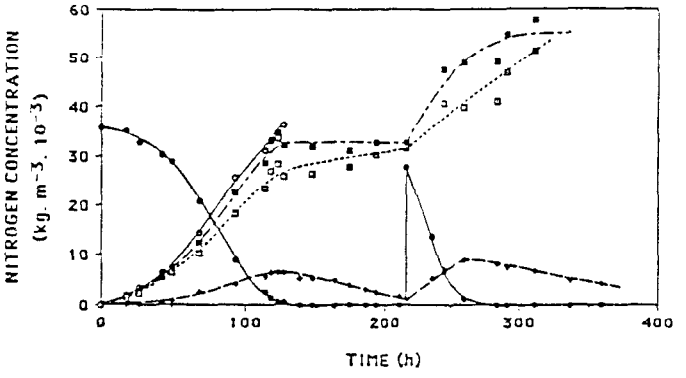

Figure 4 - Evolution of nitrogen distribution among main nitrogen containing components of the culture during growth, limitation and replenishment. N supplied as nitrates ● ; N recovered in the biomass ○ ; in total proteins ■ ; in phycocyanins ◆ ; in proteins other than phycocyanins ◘ .

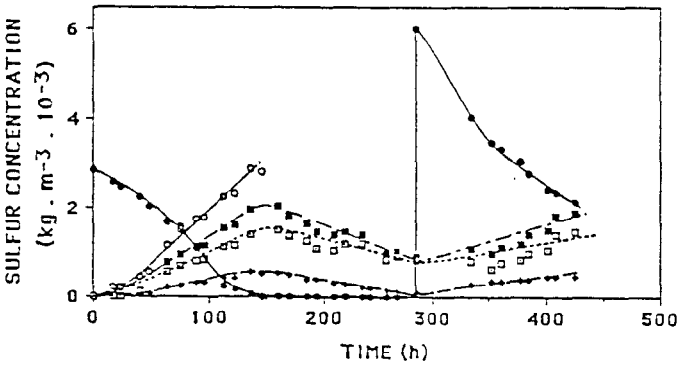

Figure 5 - Evolution of sulfur distribution among main sulfur containing components of the culture during growth, limitation and replenishment. S supplied as sulfate ● ; S recovered in the biomass ○ ; in total proteins ■ ; in phycocyanins ◆ ; in proteins other than phycocyanins ◘.

TN 12.3: EFFECTS OF IONIC STRENGHT ON THE PHYSIOLOGY AND GROWTH OF *Spirulina platensis*.

1- PHYSIOLOGICAL EFFECTS:

The cyanobacterium *Spirulina platensis* was grown in four media of different ionic strength, in which proportions between the different components were maintained. It appeared that such modifications of culture conditions affect the composition of the produced biomass, principally by modifying the mass fraction of phycocyanins (PC). This chromoprotein constitutes the main light-harvesting pigment of cyanobacteria and is principally associated to photosystem II, which is responsible for H_2O photolysis. Table I shows that the mass fraction of this protein increases when the ionic strengh of the medium decreases. These variations in PC content only result in variations in the amount of total cell proteins.

It should be noted that this adaptative process to low ionic strength conditions (increase in PC content) is slow and requires a long time (2 or 3 succesive cultures), while the decrease in PC after returning cells to high ionic strenght conditions requires only few hours.

2- KINETIC EFFECTS:

Phototosynthetic measurements were performed with a Clark type O_2 electrode, on samples of different PC contents, diluted in order that light was not limiting. Photosynthetic rates appeared to be proportional to the PC content in the cells.

Nevertheless, within the bioreactor culture, where self shadowing rapidly creates light limiting conditions, and in which growth rate is governed by the available radiant light energy, different ionic strenght conditions did not induced any change in the observed global kinetic (figure 1), and therefore just resulted in modifications of the biomass elemental formula .

Table I : Effects of different ionic strengths for the culture medium of Spirulina platensis.

Ionic strength (Mole / litre)	Phycocyanins content (% of dry weight)	Proteins content (% of dry weight)	Chlorophyll content (% of dry weigth)
0.84	11	66	1
0.42 *	15	70	1
0.21	18	74	1
0.105	23	78	1

(*) ZARROUK medium used for culture of Spirulina platensis

FIG.1 EFFECT OF IONIC STRENGHT ON S.platensis GROWTH KINETICS.