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MELiSSA

Growth capability of *Spirulina* on several nitrogen sources in the photosynthetic compartment of MELiSSA

Technical Note 33.1

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INTRODUCTION

The cyanobacterium Spirulina (*Arthrospira platensis*) is usually grown on nitrates because this nitrogen source do not set technical problems with culture mediums and is well accepted by cells.

In a previous contract (ESTEC 8125/88/NL/FG), optimal culture conditions on nitrates were determined for modelling growth in the photosynthetic compartment, and since 1992, modelling of the whole closed ecosystem MELiSSA continues in the Laboratoire de Génie Chimique Biologique at Clermont-Ferrand.

In this ecosystem, the crew and the liquefying compartments produce urea and ammonium that are oxidised in the nitrifying compartment to nitrite then nitrate before being used as nitrogen source by Spirulina in the photosynthetic compartment. It would therefore be interesting to determine to which extent Spirulina cells are able to directly use urea, ammonium or other nitrogen compounds produced by the preceding compartments in order to eventually introduce some flexibility in the MELiSSA loop or even to reduce the nitrifying compartment.

This work analyses the capability of Spirulina (*A. platensis*) cells to grow on several nitrogen sources, particularly on nitrate, urea and ammonium, supplied to the cultures individually or in combination by two or by three.

This Technical Note is organised as follow : first, a bibliographic study on assimilation of nitrogen by filamentous non heterocystous cyanobacteria and particularly *Spirulina* ; second, technical aspects of our work.

1. - ASSIMILATION OF NITROGEN BY CYANOBACTERIA

Cyanobacteria preferentially use inorganic nitrogen for growth, and particularly nitrates, nitrites and ammonium, but some of them are able to use organic nitrogen (Fogg *et al.*, 1973). Nitrate assimilation by cyanobacteria involves a transport system into the cells where it is sequentially reduced to ammonium by nitrate and nitrite reductases. Ammonium is then incorporated by the glutamine synthetase/glutamate synthase system.

1.1 - Inorganic nitrogen

Filamentous non-heterocystous cyanobacteria can use inorganic species, nitrate, nitrite or ammonium, as sole nitrogen source for growth, and nitrate is widely used in culture mediums. When these different nitrogen sources are simultaneously available, cells utilise ammonium, nitrate or nitrite in decreasing order of preference (Guerrero and Lara, 1987).

The reduction to ammonium of these inorganic nitrogen sources occurs in cyanobacteria on same pathways as in others organisms able to utilise inorganic nitrogen (Guerrero and Lara, 1987). These processes require ATP and a source of reducing power, which under autotrophic conditions are provided by the photosynthetic electron transport system. In cyanobacteria, ferredoxin (or flavodoxin under iron-depleted conditions) is the main physiological electron donor for all the enzymes concerned. Assimilation of ammonium necessitates, in addition, a source of fixed carbon.

The mechanisms by which the uptake of these different inorganic nitrogen sources and the activities of the enzymes involved are controlled are still controversial and may vary among different cyanobacteria species.

1.1.1. - *Nitrate and nitrite utilisation*

Nitrate assimilation involves nitrate uptake followed by its sequential intracellular reduction to nitrite by nitrate reductase and to ammonium by nitrite reductase.

Nitrate utilisation appears to be sensitive to ammonium ; Guerrero and Lara (1987) and Bisen and Shanthy (1991) found that ammonium reduces or even suppress the systems involved in

nitrate uptake and reduction, and Rodriguez *et al.* (1994) described an active transport system for nitrate and nitrite that is sensitive to ammonium. In addition to these transport systems, a passive diffusion of nitrous acid, insensitive to ammonium, would contribute to nitrite uptake (Flores *et al.*, 1987 ; Tapia *et al.*, 1995).

Several genes, which may provide a clearer understanding of the regulatory mechanisms that control nitrate utilisation in cyanobacteria have been identified in different species (Tandeau de Marsac and Houmard, 1993)

A. platensis use nitrate as principal nitrogen source (Zarrouk, 1966).

1.1.2. - *Ammonium utilisation*

Two mechanisms have been proposed for the uptake and accumulation of ammonium in cyanobacteria (Boussiba, 1990). In species growing in at pH 7-8, ammonium ions would cross the plasma membrane by means of an active transport system while in alkalophilic species growing at pH>10, such as *A. platensis*, ammonia would enter the cells by diffusion and would subsequently be protonated in the more acidic cell compartment. Whether ammonium is exogenously supplied by one of these two possible mechanisms or intracellularly produced, the GS/GOGAT pathway (glutamine synthetase/glutamate 2-oxoglutarate amino-transferase) is quantitatively the most important enzyme system for the assimilation of this cation (Meeks *et al.*, 1978). The glutamate synthase (GOGAT) has been poorly studied so far. Most of the work performed concerns glutamine synthetase (GS) that catalyses the ATP-dependent synthesis of glutamine from glutamate and ammonium ions. It represents the connecting step between the carbon and nitrogen metabolisms.

The regulation of the synthesis and activity of GS remains unclear. Different compounds have been shown to modulate the activity of this enzyme in some cyanobacteria (Stacey *et al.*, 1979 ; Tapia *et al.*, 1995). A repression of GS synthesis by ammonium has been observed in many species (Tandeau de Marsac and Houmard, 1993). Different studies suggest that the regulatory mechanisms of GS activity might depend on the nitrogen sources provided to the growing cells before their transfer on a medium lacking combined nitrogen (Tandeau de Marsac and Houmard, 1993).

In enteric bacteria, the PII protein regulates the activity and the synthesis of GS, and it has recently been demonstrated that cyanobacteria do synthesise a PII protein (Tsinoremas *et al.*, 1991). In cyanobacteria, this system responds to the activity of ammonium assimilation via the GS/GOGAT pathway and to the state of CO_2 fixation. Forchhammer and Tandeau de Marsac (1995) suggested that the phosphorylation and dephosphorylation of PII are part of a complex signal transduction network involved in global nitrogen control in cyanobacteria. In this regulatory process, PII might be involved in mediating the tight coordination between carbon and nitrogen assimilation.

Richmond (1988) shows that a concentration of 100 $mg.l^{-1}$ of ammonium do not inhibit growth of Spirulina.

1.1.3. - *Assimilation of mixed inorganic nitrogen sources*

Filamentous non-heterocystous cyanobacteria usually use nitrate as preferential nitrogen source. However, several nitrogen compounds may be simultaneously available in the environment, and in the particular case of nitrate and ammonium, cells first use ammonium then nitrate. Moreover, it has been shown that the presence of ammonium decreases or even inhibits the utilisation of nitrate by several cyanobacteria (Guerrero and Lara, 1987).

These characteristics are poorly documented for cyanobacteria and particularly for *A. platensis*. From experiments with *A. platensis* grown under non axenic conditions, Soyer (1992) concluded that in the binary nitrate + ammonium combination, ammonium is preferentially incorporated, but exerts an inhibitory effect on growth, that increases with ammonium concentration. He also showed that in binary combinations of nitrate (constant concentration of 420 mg/l) with nitrite (variable concentrations), concentrations of nitrite lower than 18 mg/l did not exert any effect while a toxicity appeared for concentrations higher than 59 mg/l. No informations have been published concerning growth in the presence of ternary combinations of nitrogen sources.

1.2. - Organic nitrogen

Cyanobacteria preferentially use inorganic nitrogen for growth, and particularly nitrates, nitrites and ammonium, but some are able to grow on organic nitrogen sources, and particularly on aminoacids (Fogg *et al.*, 1973).

Most attention has been paid to glutamine owing to its key role in nitrogen fixation and also in global nitrogen control (Tapia *et al.*, 1996). Labarre *et al.* (1987) have reported the existence of three different active transport systems (permeases), one for arginine, lysine, histidine and glutamine, another for glutamine and glutamate and a third one for all other amino acid. Thus, with the exception of glutamine, each amino acid would be transported into *Synechocystis* PCC 6803 cells by only one major transport system. The situation might in fact be more complex and depend on cyanobacteria species (Tandeau de Marsac and Houmard, 1993).

2. MATERIAL AND METHODS

2.1 - *Culture conditions*

Culture conditions are those provided by a 139 l orbital shaker (Gallenkamp), with the following parameters : agitation : 110 rpm ; temperature : 35°C ; continuous light : ~ 2890 lux (~8 $Wm.^{-2}$) ; atmosphere : ~ 210 $l.h^{-1}$ air + 3% CO2. The evaporation of the culture mediums, that has been measured under these conditions, corresponds to about 1 ml/day and 0.5 ml/day in 1000 and 100 ml erlenmeyers respectively.

These culture conditions, that allow a good control of culture parameters, were preferred to air - CO2 bubbling in the culture flasks for several reasons : they allow the simultaneous study of numerous cultures because easier to implement, and they limit the loss of gaseous NH3 that originate from $NH4^+$ at high pH, and that would then be lost for the cultures

2.2 - *Arthrospira platensis* strain and culture mediums

The strain *Arthrospira platensis* PCC 8005 was provided by Institut Pasteur and was initially adapted to the Zarrouk (1966) culture medium (pH 9.5) generally used to grow spirulina cells and modified by Cornet (1992). In this study, the number of nitrogen sources and their amount, as well Na2CO3/NaHCO3 buffer of this culture medium was modified as indicated in the text (T.N. 33.2)

2.3. - *Inoculation of cultures*

Inoculums were aseptically washed according the following standard procedure to avoid contaminations of the culture medium by the nitrogen sources present in the precultures.

0 - Absorbance measurements of the preculture for biomass estimations ;
1 - Centrifugation 30 min at 20000 rpm, 4°C, of 200 ml preculture ;
2 - Elimination of the surpernatant ;
3 - Wash of the pellet with fresh medium devoid of nitrogen ;
4 - Centrifugation 20000 rpm, 20 mn, 4°C ;
5 - Elimination of the supernatant ;
6 - Repeat of points 3, 4, 5, 3 ;
7 - Absorbance measurements and biomass estimation ;
8 - Inoculation of cultures
9 - Start of culture

2.4. - Measurement of growth parameters

- Biomass : by turbidimetry, as in Cornet (1992).
- Proteins : by spectrophotometry, using the Biorad Protein Assay kit.
- Nitrates : by UV absorbance measurements (Cornet, 1992).
- Ammonium : by spectrophotometry, using the Sigma n° 640A kit.
- Urea : by spectrophotometry, using the Sigma n°535 kit.

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