Brazilian Journal of Microbiology (2003) 34:267-272
ISSN 1517-8382

EXTRA AND INTRACELULAR ACTIVITIES OF CARBONIC ANHYDRASE OF THE MARINE MICROALGA *TETRASELMIS GRACILIS* (CHLOROPHYTA)

Marilda Rigobello-Masini[1]*; Elizabeth Aidar[2]; Jorge C. Masini[3]

[1]Instituto de Biociências, Universidade de São Paulo, São Paulo, SP, Brasil. [2]Instituto Oceanográfico, Universidade de São Paulo, São Paulo, SP, Brasil. [3]Instituto de Química, Universidade de São Paulo, São Paulo, SP, Brasil.

Submitted: October 17, 2002; Returned to Authors: February 10, 2003; Approved: June 26, 2003

ABSTRACT

The activities of extra and intracellular carbonic anhydrases (CA) were studied in the microalgae *Tetraselmis gracilis* (Kylin) Butcher (Chlorophyta, Prasinophyceae) growing in laboratory cultivation. During ten days of batch cultivation, daily determinations of pH, cell number, enzymatic activity, and total dissolved inorganic carbon (DIC), as well as its main species, CO_2 and HCO_3^-, were performed. Enzymatic activity increased as the growing cell population depleted inorganic carbon from the medium. Carbon dioxide concentration decreased quickly, especially in the third day of cultivation, when a significant increase of the intracellular enzymatic activity was observed. Bicarbonate concentration had its largest decrease in the cultivation medium in the fourth day, when the activity of the extracellular enzyme had its largest increase, suggesting its use by the alga through CA activity. After the fourth cultivation day, half of the cultures were aerated with CO_2-free atmospheric air, which caused an increase in the total and external activity of the enzyme, although, in this condition, the stationary growth phase began earlier than in cultures aerated with atmospheric air. The pH of the media was measured daily, increasing from the first to the fourth day, and remaining almost constant until the end of the cultivation. Algal material transferred to the dark lost all enzymatic activity.

Key words: algal photosynthesis, carbon concentrating mechanism, carbonic anhydrase, inorganic carbon uptake, *Tetraselmis gracilis*.

INTRODUCTION

Marine phytoplankton, particularly when living in coastal areas, may be submitted to frequent environmental variations of pH, temperature and salinity, which affect the distribution of the inorganic carbon species dissolved in seawater (32). The major species of dissolved inorganic carbon at the pH of seawater is the bicarbonate ion, which must be converted by the enzyme carbonic anhydrase (CA) to CO_2, the substrate for the enzyme RUBISCO in photosynthesis (2,8,23). CA is a metalloenzyme, found in many organisms, and contains one atom of zinc essential for activity in its active site (34). Although not found in all microalgae, the extracellular CA occurs in the plasmamembrane and converts HCO_3^- to CO_2, which is a planar and apolar molecule that can pass freely through the lipid bilayer of the cellular membrane (4). Internally, CA may be located in the cytoplasm, mitochondria and mainly in the chloroplast (1). In the cytoplasm, the internal enzyme acts converting CO_2 into HCO_3^- to prevent the leaking of CO_2 from the cell (21,22). In the chloroplast HCO_3^- is converted to CO_2 by another CA, concentrating CO_2 around RUBISCO, helping to overcome the low affinity that this enzyme has for CO_2 (5) and thus, being part of the Carbon Concentrating Mechanism that is found mainly in microalgae and cyanobacteria (7,16,22).

Several factors regulate CA activity and the induction of the enzyme molecule; the most important are the availability of inorganic carbon and nitrogen species, light, photosynthesis and metabolites of respiratory path, such as glycolate and also the pH of the media (10,11,12,18,20,25).

The purpose of this work was to find how some environmental factors like inorganic carbon and its species, pH and light affect the activity of intra and extracellular CA, and how this activity

*Corresponding author. Mailing address: Instituto de Biociências, Universidade de São Paulo. Caixa Postal 11461. 05422-970, São Paulo, SP, Brasil. Telephone (+5511) 3091-7547. E-mail: rmmarilda@bol.com.br

affects carbon acquisition by the Prasinophycean microalga *Tetraselmis gracilis* growing in laboratory cultivation. This alga has been choosen for this research because it can grow near estuaries or even in dense populations (26), conditions in which the concentration and the distribution of inorganic carbon species may suffer frequent variations and, also because data on Prasinophyceae regarding to this subject are still lacking. As the atmospheric levels of carbon dioxide are continuously increasing in the atmosphere, it is interesting to know what effects this changes can bring for organisms that posseses mechanisms of concentrating carbon in their cells, especially because marine phytoplankton is responsible for almost 40% of the planetary photosynthesis (28).

MATERIALS AND METHODS

Culture of the alga *Tetraselmis gracilis*

The alga *Tetraselmis gracilis*, proceeding from the algal culture collection of the Oceanographic Institute of the University of São Paulo, was cultivated in unialgal cultures in a thermostatized chamber at 20ºC using filtered and sterilized seawater, with the addition of Guillard f/2 medium (13). For most of the experiments the alga was cultivated in 6 L flasks, in triplicate, under an irradiance of 350 µE M^{-2} s^{-1}. Atmospheric air was supplied continuously to the cultures during all the experiment (ten days). After the fourth day the cultures were divided into two flasks; one of them continued to be aerated with atmospheric air, while in the other, the aeration was switched to CO_2 free atmospheric air obtained with air pumped through a column filled with soda lime.

Cell counting: The algal cells were counted daily in an optic microscope, using a Hemocytometer-type chamber.

Determination of the activity of Carbonic Anhydrase (CA)

Extracellular CA: A volume of culture containing the adequate number of cells (10^6 cells) was centrifuged at 2000 x g and washed twice with 0.010 M Tris buffer (pH 8.3) containing 1 mM dithiothreitol (DTT) and 1 mM EDTA. The pellet consisting of intact cells, whose integrity was checked through the microscope, was resuspended with the same buffer, in which the measurements of enzymatic activity were made immediately after the ressuspension of the cells.

Total CA : A culture volume containing 10^6 cells was filtered using cellulose acetate membranes (0.45 µM pore size diameter, from Micron Separations Inc.). After removal of the membrane, the algal material was ground in a mortar in presence of liquid nitrogen to a fine powder and resuspended in 0.01 M Tris buffer containing 1 mM DTT and 1 mM EDTA (pH 8.3). The total extract was used to measure the enzymatic activity. Some extracts were checked at the microscope and no intact algal cells were found after this treatment.

Measurements of enzymatic activity: Measurements of enzymatic activity were made using the potentiometric method of Wilbur and Anderson (37), with modifications: 1.5 mL of deionized water, saturated with CO_2 at 0ºC, were added to a volume of 3 mL of algal material resuspended in the buffer (*Ta*), or buffer without alga (*Tb*). This reaction was performed in a closed flask kept at a temperature between 0 and 2ºC. The time necessary for the CO_2-saturated deionized water to lower one unit of pH of both solutions was measured and the enzymatic activity was calculated using the equation:

$$\frac{Tb}{Ta} - 1 = UAx10^6 \qquad (1)$$

Equation 1 expresses the enzymatic activity as Units of activity (*UA*) per cell, since 10^6 cells were used per measurement of activity.

Determination of concentration of dissolved inorganic carbon

Dissolved inorganic carbon (DIC) was determined by potentiometric titration of the cultivation medium with standard HCl. Titration data was treated by the derivative method (14). This computation was performed with the measured potential or pH values using the Microcal Origin 4.3 software (Microcal Software, Inc., Northampton, MA, USA). From the sample pH and DIC, the distribution of inorganic carbonic species was computed from ionization constants of carbonic acid (19,30).

RESULTS

Enzymatic activity and composition of the cultivation media - DIC concentration and pH

Figs. 1a and 1b show the extracellular and total (intracellular plus extracellular) CA activities, respectively. Extracellular CA was found in significant amount after the fourth day of cultivation, reaching a maximum in the fifth cultivation day and remaining active, but with oscillations, until the tenth day in the cultures submitted to aeration with atmospheric air (Fig. 1a). Under this aeration condition, the cultures continued to grow until the tenth day (Fig. 2). In the cultures where the aeration was switched to CO_2 free atmospheric air in the fourth day, the activity of the external enzyme, which was high since the fifth day, remained high through the rest of the cultivation. The cells entered stationary growth phase one day after the switch of the aeration system (Fig. 2).

Total enzymatic activity measured in crude extracts, therefore containing the extracellular enzyme as well as the enzyme of cytoplasm and organelles, was detected after the second day of cultivation (Fig. 1b). The total activity reached a maximum in the fifth day, remaining high and with small variation, for both aeration conditions, during the following cultivation days.

The pH of the medium (Fig. 3) increased from 7.7 in the first cultivation day to 9.3 in the fourth day, remaining close to this value until the tenth day for both aeration conditions. DIC

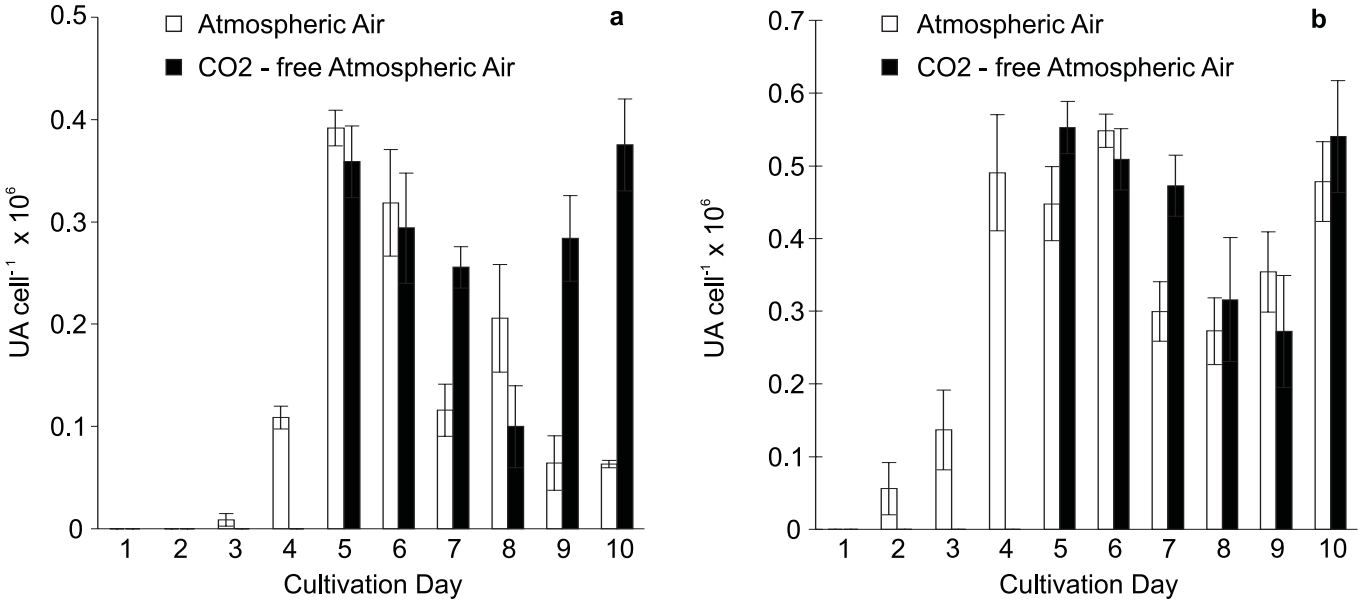

Figure 1. External (a) and total (b) Carbonic Anhydrase activity of *Tetraselmis gracilis* growing during ten days of batch cultivation. After the 4th day the cultures were divided in two flasks; one of them continued to be aerated with atmospheric air, while in the other, the aeration was switched to CO_2-free atmospheric air. Results correspond to mean values obtained from triplicate of cultivation. Vertical bars represent the standard errors of the mean.

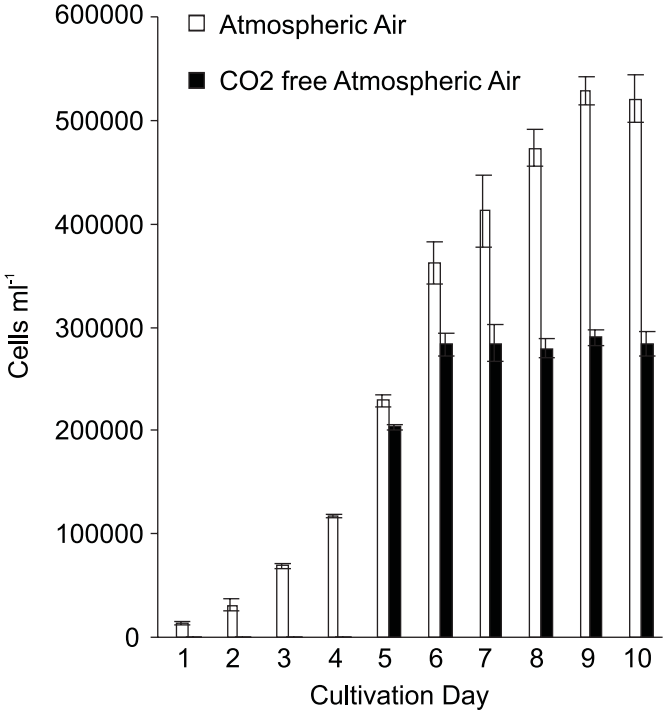

Figure 2. Growth curve of *Tetraselmis gracilis* during ten days of batch cultivation submitted to different conditions of aeration after the 4th day. Experimental conditions and statistical parameters are the same as described in Fig. 1.

concentrations dropped from 2 mM in the first cultivation day, to about 0.75 mM in the fourth day (Fig. 4a), the steepest drop occurring between the third and fourth cultivation days, coincident with the rise in the activity of intracellular CA enzyme (Fig. 1b). Fig. 4b shows that CO_2 was the first species of inorganic carbon to have its concentration decreased, during the second day of cultivation, dropping from 33 µM in the first day to 3 µM in the third day. From the fourth to the tenth day of cultivation, CO_2(aq) concentration remained below 0.2 µM (Fig. 4b, Insert). The concentration of bicarbonate had a significant decrease between the third and fourth days, dropping from 1.38 mM to 0.4 mM (Fig. 4c) simultaneous to the increase of the extracellular CA activity (Fig. 1a).

Influence of light: The influence of light in the enzymatic activity was investigated with cultures growing under atmospheric air and illumination, transferred from the light to the dark. Five hours after this transfer, 50% of the extracellular enzymatic activity was lost. After 25 h of this treatment no more activity of the external enzyme was detected in the alga (Fig. 5).

Sulfanilamide: Preincubation of crude extracts for 1h in sulfanilamide caused an inhibition of 40% of the enzymatic activity previously found (Table 1). This test was repeated for most of the crude extracts obtained in all cultivation days. No significant differences in the percentage of enzymatic inhibition caused by sulfanilamide were found among the tested culture samples.

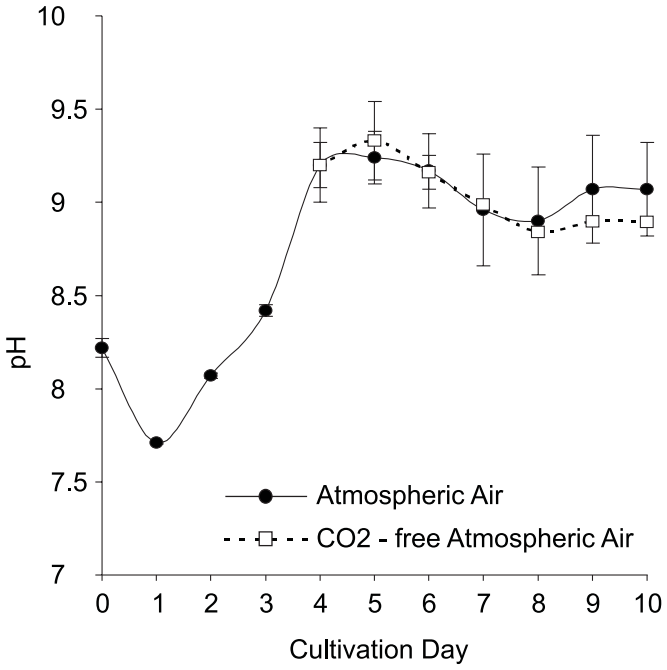

Figure 3. pH of the cultivation media of *Tetraselmis gracilis* submitted to different conditions of aeration after the 4th day. Experimental conditions and statistical parameters are the same as described in Fig. 1.

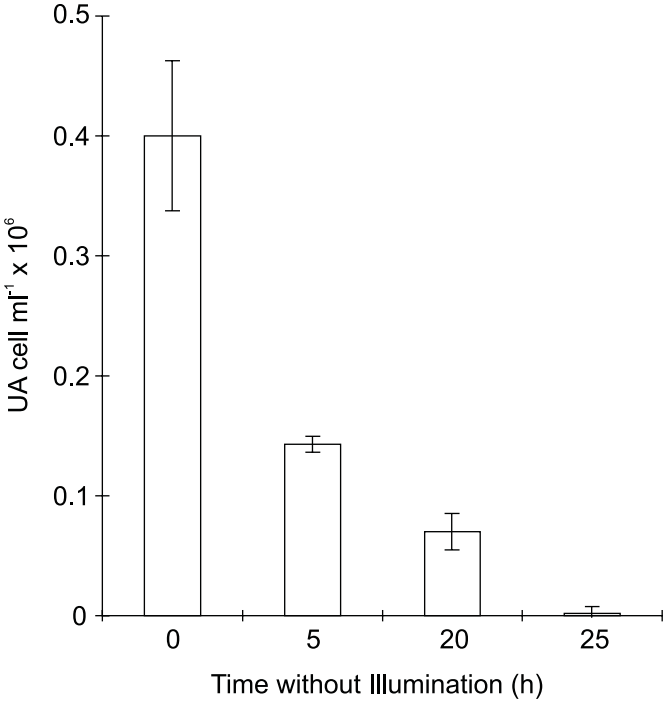

Figure 5. Inhibitory effect of dark on the activity of extracellular Carbonic Anhydrase of *Tetraselmis gracilis.* Results are average of three batch cultivation (n = 3).

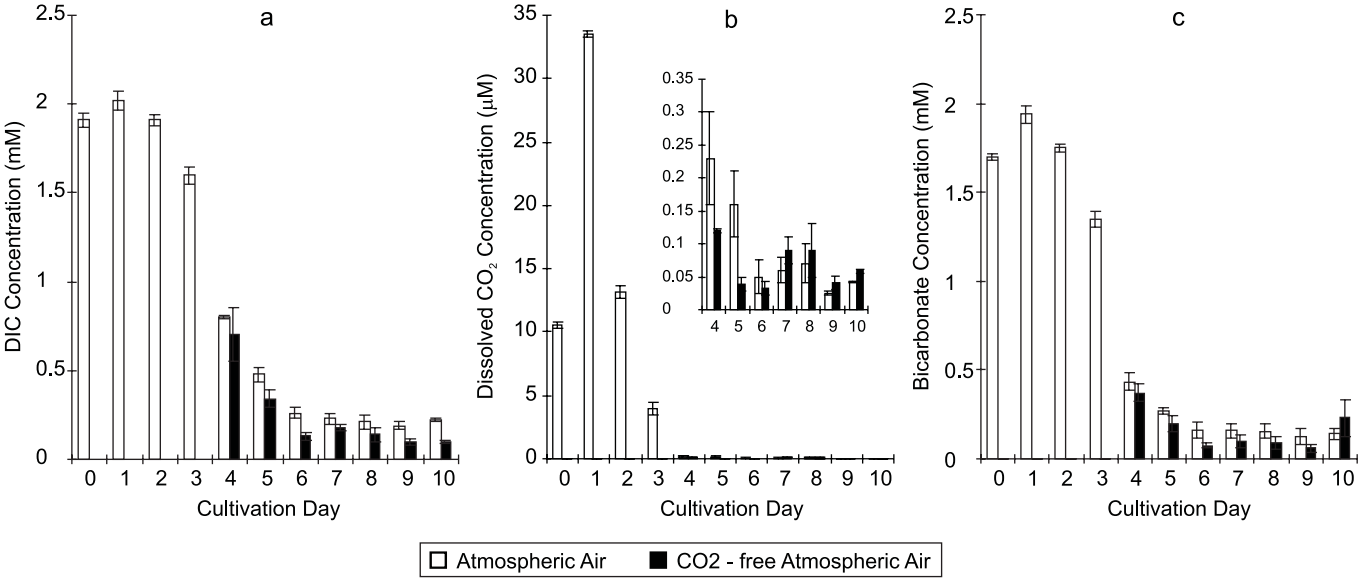

Figure 4. Concentrations of DIC (a), disssolved CO_2 (b) and bicarbonate (c) in the cultivation media of *Tetraselmis gracilis* submitted to different conditions of aeration after the 4*th* day. Results in Day zero correspond to concentrations in the media before culture inoculation and starting of aeration. White columns- Atmospheric air; Black columns - CO_2 free Atmospheric air. Experimental conditions and statistical parameters are the same as described in Fig. 1

Table 1. Activity of CA (UA 10^6 $cell^{-1}$) of *Tetraselmis gracilis*, intact and in crude extracts, preincubated for 1h with sulfanilamide. The control shows the enzymatic activities when sulfanilamide was not added during the preincubation. Data are mean values for n = 3.

Enzyme	Control	Sulfanilamide
Total AC	0.58 ± 0.06	0.35 ± 0.03
Extracellular AC	0.240 ± 0.001	N.D.

DISCUSSION

The regulation of activity of CA is correlated to several factors and does not follow the same pattern for all the microalgae studied (1). Although total inorganic carbon and CO_2 concentration are considered the most important factors inducing CA activity (10,17), alkalization of the growing media also plays an important role. One of the consequences of this alkalization, caused mainly by the photosynthetic process (3) and nitrate uptake (29,31) is the shift of the equilibrium of inorganic carbon species toward HCO_3^-, thus lowering the CO_2 concentration. Besides, the high pH of the media can cause a decrease of the photosynthetic rates by altering the internal pH of the cells with negative effects for the algal growth rate. The increase in the activity of the external and the total CA of *Tetraselmis gracilis* occurred parallel to both, the alkalization and the decrease in the inorganic carbon of the growing media (Figs. 1a, 1b, 3 and 4). In some *Chlorella* species CA activity is induced only at alkaline pH even at low CO_2 concentration, although this effect was not found for CA from *Chlorococcum* and *Stichococcus* (9,33). From the fourth to the tenth cultivation day, CO_2 concentration remained below 0.2 µM (Fig. 4c), which is much smaller than the Km for RUBISCO of most C3 plants (5). Thus, the activity of the internal enzyme was increased as a mechanism to increase CO_2 concentration around RUBISCO (Fig. 1b), whereas extracellular activity increased as a means of uptaking the bicarbonate ion, which became the major source of inorganic carbon as a result of alkalization. Similar profiles of extracellular CA activity along ten days of laboratory cultivation were observed for the microalgae *Micromonas pusilla* and *Prorocentrum minimum* (15,23,24).

The effect of light in CA induction may be dependent or independent of the photosynthetic process, and this effect may occur at the post-transcriptional or transcriptional step (6,36). Blue light caused a twofold increase of CA activity in *Chlamydomonas reinhardtii* (11,35). Spencer *et al.* (35) demonstrated that *Chlamydomonas reinhardtii* CA activity could not be induced without light, even at low CO_2 concentration. The literature results discussed above describe a situation where light was tested during the induction of CA, while in our work (Fig. 5) CA was already fully induced provided that the algae used in the experiment were in the sixth cultivation day (Figs. 1 and 2). Soon after the cultures were put in the dark, the enzymatic activity begun to fall. One model that explains the activation of CA by light proposes that the photosynthetic electron transport causes the formation of alkaline pockets around CA, in the thylakoid or in the cytoplasmatic membrane and, as a consequence, the generation of HCO_3^- from CO_2 is accelerated (17,27). Sulfanilamide binds to the Zn atom in the active site of the enzyme, causing its inhibition and it is widely used in the studies with Carbonic Anhydrase. The percentage of inhibition obtained for CA of *Tetraselmis* is in agreement with the data obtained for other organisms (1,34).

Conclusions and perspectives: The activity of the enzyme is induced by alkaline pH, low concentration of CO_2 and inorganic carbon in the media. Light is important for keeping the CA activity. According to the results obtained so far, some of the further studies on the regulation of CA activity of *Tetraselmis gracilis* should emphasize the search for a positive correlation between this activity and the photosynthetic rates of the algae submitted to low concentration of inorganic carbon, and the consequent participation of the enzyme in the microalgal Carbon Concentrating Mechanism. It is interesting to know if this mechanism may allow some phytoplanktonic species to work as a source, and not as a sink for carbon, in species where the AC is not inhibited by, otherwise, elevated environmental concentration of carbon dioxide.

ACKNOWLEDGMENTS

Authors acknowledge FAPESP, CNPq and CAPES for financial support and are grateful to Tomás Édison da Silva for his technical assistance.

RESUMO

Atividade extra e intracelular da Anidrase Carbônica na microalga marinha *Tetraselmis gracilis (Chlorophyta)*

As atividades da Anidrase Carbônica (AC) extra e intracelular foram estudadas na microalga marinha *Tetraselmis gracilis* (Kylin) Butcher (Chlorophyta, Prasinophyceae) crescendo em cultivos laboratoriais. Durante dez dias de cultivo, determinações diárias do pH, número de células, atividades enzimáticas, carbono inorgânico total dissolvido (CID) e suas principais espécies CO_2 e HCO_3^- foram feitas. A atividade enzimática aumentou na medida em que a população celular em crescimento retirava carbono inorgânico do meio de cultivo. A concentração de dióxido de carbono decresceu rapidamente, especialmente no terceiro dia do cultivo, quando um significante aumento na atividade enzimática intracelular foi observado. A concentração de bicarbonato teve seu maior decréscimo no meio de cultivo no quarto dia, quando a atividade da enzima extracelular teve

seu maior aumento, sugerindo seu uso pela alga através da atividade da AC. Após o quarto dia de cultivo, metade das culturas passou a ser aerada com ar atmosférico sem CO_2, o que causou um aumento na atividade total e externa da enzima, fazendo com que esses cultivos entrassem na fase estacionária do crescimento antes que aqueles aerados com ar atmosférico normal. O pH do meio foi medido diariamente, aumentando desde o primeiro até o quarto dia e permanecendo quase constante até o fim do cultivo. Material algal transferido para o escuro perdeu toda a atividade enzimática.

Palavras-chave: fotossintese algal, mecanismo de concentração de carbono, anidrase carbônica, *Tetraselmis gracilis*.

REFERENCES

1. Aizawa. K.; Miyachi, S. Carbonic anhydrase and CO_2 concentrating mechanisms in microalgae and cyanobacteria. *FEMS Microbiol. Rev.*, 39: 215-233, 1986.
2. Aizawa, K.; Tsuzuki, M.; Miyachi, S. Kinetic studies on the active species of inorganic carbon absorbed by cells of *Dunaliella tertiolecta*. *Plant Cell Physiol*., 27:37-49, 1986.
3. Axelsson, L. Changes in pH as a measure of photosynthesis by marine macroalgae. *Mar. Biol*., 97:287-294, 1988.
4. Axelsson, L.; Ryberg, H.; Beer, S. Two modes of bicarbonate utilization in the marine green macroalga *Ulva lactuca*. *Plant Cell Environ.*, 18:439-445. 1995.
5. Badger, M.R.; Price, G.D. The role of carbonic anhydrase in photosynthesis. *Ann. Rev. Plant Physiol. Mol. Biol.*, 45:369-392, 1994.
6. Baily, J.; Coleman, J.R. Effect of CO_2 concentration on protein biosynthesis and Carbonic anhydrase expression in *Chlamydomonas reinhardtii*. *Plant Physiol.*, 87:833-840, 1988.
7. Beardall, J. Carbon isotope discrimination on the CO_2 accumulating mechanism in *Chlorella emersonii*. *J. Exp. Bot*., 33:729-737, 1982.
8. Beardall, J.; Beer, S.; Raven, J.A. Biodiversity of marine plants in an era of climate change. Some predictions based on physiological performance. *Bot. Mar.*, 41:113-123, 1998.
9. Beauf, L.; Kurano, N.; Miyachi, S. Effect of external pH on inorganic carbon assimilation in unicellular marine green algae. *Phycol. Res*., 48:47-54, 2000.
10. Bozzo, G.L., Colman, B.; Matsuda, Y. Activity transport of CO_2 and bicarbonate is induced in response to external CO_2 concentration in the green alga *Chlorella kessleri*. *J. Exp. Bot.*, 51:1341-1348, 2000.
11. Dionisio, M.L.; Tsuzuki, M.; Miyachi, S. Blue light induction of carbonic anhydrase activity in *Chlamydomonas reinhardtii*. *Plant Cell Physiol.*, 30:215-219, 1989.
12. Dionisio-Sese, M.L.; Fukuzawa, H.; Miyachi, S. Light induced carbonic anhydrase expression in *Chlamydomonas reinhardtii*. *Plant Physiol*., 94:1103-1110, 1990.
13. Guillard, R.R.L.; Ryther J.H. Studies of marine plankton diatoms. *Canadian J. Microbiol.*, 8:229-239, 1962.
14. Hernández-Ayón, J.M.; Belli, S.L.; Zirino, A. pH, alkalinity and total CO_2 in coastal seawater by potentiometric titration with a difference derivative readout. *Anal. Chim. Acta.*, 394:101-108, 1999.
15. Iglesias-Rodrigues, M.D.; Nimer, N.A.; Merrett, M.F. Carbon dioxide-concentrating mechanism and the development of extracelular carbonic anhydrase in the marine picoeukaryote *Micromonas pusilla*. *New Phytol.*, 140:685-90, 1998.
16. Kaplan, A.; Ronen-Tarazi, M.; Zer, H.; Schwarz, R.; Tchernov, D.; Bonfil, D.J.; Schatz, D.; Vardi, A.; Hassidim, M.; Reinhold, L. The inorganic carbon concentrating mechanism in cyanobacteria: induction and ecological significance. *Can. J. Bot.*, 76:917-924, 1998.
17. Kaplan, A.; Reinhold, L. CO_2 concentrating mechanisms in photosynthetic microorganims. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 50:539-570, 1999.
18. Marcus, Y.; Harel, E.; Kaplan, A. Adaptation of the cyanobacterium *Anabaena variabilis* to low CO_2 concentration in their environment. *Plant Physiol.*, 71:208-210, 1983.
19. Masini, J.C. The use of linear potentiometric titration curves in the determination of alkalinity and acid-base properties of diluted solutions of humic substances. *Talanta*., 41:1383-1389, 1994.
20. Matsuda, Y.; Colman, B. A new screening method for algal photosynthesis mutants. CO_2 insensitive mutants of the green alga *Chlorella ellipsoidea*. *Plant Physiol*., 110:1283-1291, 1996.
21. Merrett, M.J.; Nimer, N.A.; Dong, L.F. The utilization of bicarbonate ions by the marine microalga *Nannochloropsis oculata* (Droop) Hibberd. *Plant Cell Environ.*, 19:478-484, 1996.
22. Moroney, J.M.; Somanchi, A. How do algae concentrate CO_2 to increase the eficiency of photosynthetic carbon fixation? *Plant Physiol.*, 119:9-16, 1999.
23. Nimer, N.A.; Iglesias-Rodrigues, M.D.; Merrett, M. Bicarbonate utilization by marine phytoplankton species. *J. Phycol.*, 33:625-631, 1997.
24. Nimer, N.A.; Warren, M.; Merrett M.J. The regulation of photosynthetic rate and activation of extracellular carbonic anhydrase under CO_2-limiting conditions in the marine diatom *Skeletonema costatum*. *Plant Cell Environ*., 21:805-812, 1998.
25. Nimer, N.A.; Ling, M.X.; Brownlee, C.; Merrett, M.J. Inorganic Carbon limitation, exofacial carbonic anhydrase activity, and plasma membrane redox activity in marine phyroplankton species. *J. Phycol*., 35:1200-1205, 1999.
26. Norris, R.E.; Hori, T.; Chihara, M. Revision of the genus *Tetraselmis* (Class Prasinophiceae). *Botanical Magazine (Tokyo)*, 93:317-399, 1980.
27. Price, G.D.; Sultemeyer, D.; Klughammer, B.; Ludwig, M.; Badger, M.R. The functioning of the CO_2 concentrating mechanism in several cyanobacterial strains; a review of general physiological characteristics, genes, proteins and recent advances. *Can. J. Bot.*, 76:973-1002, 1998.
28. Raven, J.A. Physiology of inorganic C acquisition and implications for resource use efficiency by marine phytoplankton: relation to increased CO_2 and temperature. *Plant Cell Environ*., 14:779-794, 1991.
29. Raven, J.A.; De Michelis, M.I. Acid-base regulation during nitrate assimilation in *Hydrodictyon africanum*. *Plant Cell Environ*., 2:245-257, 1979.
30. Rigobello-Masini M.; Masini J.C. Aplication of modified Gran functions and derivative methods on data of potentiometric acid titration to study the distribution of inorganic carbon species in cultivation medium of marine microalgae. *Anal. Chim. Acta.*, 448:239-250, 2001.
31. Rigobello-Masini, M. *Influência do carbono inorgânico na atividade da nitrato redutase e da anidrase carbônica na microalga marinha* Tetraselmis gracilis. São Paulo, 2001, 119p. (PhD Thesis, Instituto de Biociências. Universidade de São Paulo)
32. Schmid, R. Photosynthesis of *Ectocarpus siliculosus* in red light and after pulses of blue light at high pH – evidence for bicarbonate uptake. *Plant Cell Environ.*, 21:523-529, 1998.
33. Shiraiwa, Y.; Yokoyama, S.; Satoh, A. pH-dependent regulation of carbonic anhydrase induction and change in photosynthesis during adaptation of *Chlorella* cells to low CO_2. *Jpn. J. Phycol. (Sôrui)*, 39:355-362, 1991.
34. Smith, K.S.; Ferry, J.G. Prokaryotic carbonic anhydrases. *FEMS Microbiol. Rev.*, 24:335-366, 2000.
35. Spencer, K.G.; Kimpel, D.L.; Fisher, M.L.; Togasaki, R.K.; Miyashi, S. Carbonic anhydrase induction in *Chlamydomonas reinhardtii* II. Requeriments for carbonic anhydrase induction. *Plant Cell Physiol.*, 24:301-304, 1983.
36. Umino, Y.; Satoh, A.; Shiraiwa, Y. Factors controlling induction of external carbonic anhydrase and change in K1/2 (CO_2) of photosynthesis in *Chorella regularis*. *Plant Cell Physiol*., 32:379-384, 1991.
37. Wilbur, K.M.; Anderson, N.G. Eletrometric and colorimetric determination of carbonic anhydrase. *J. Biol. Chem.*, 176:147-154, 1948.