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Characterization of nitrate reductase activity *in vitro* in *Gracilaria caudata* J. Agardh (Rhodophyta, Gracilariales)

FUNGYI CHOW[1,2], FERNANDA V. CAPOCIAMA[1], RENATA FARIA[1] and MARIANA C. DE OLIVEIRA[1]

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ABSTRACT – (Characterization of nitrate reductase activity *in vitro* in *Gracilaria caudata* J. Agardh (Rhodophyta, Gracilariales)). The marine red alga *Gracilaria caudata* J. Agardh has been used in Brazil for agar extraction, mainly in the northeast region of the country. Nitrogen availability is the most important abiotic factor in seawater that limits the growth of seaweeds. The enzyme nitrate reductase (NR) is the key regulatory point in the nitrogen assimilation in photosynthetic organisms. This study describes an *in vitro* assay, characterizing the enzymatic activity of NR in terms of kinetic constants and stability, its oscillation during the day and glucose effect on NR modulation. Maximal peaks of NR activity were recorded at 20 °C and pH 8.0. The enzymatic stability in crude extracts stored at 3 ± 1 °C decreased significantly after 48 hours. Apparent Michaelis-Menten constants (K_M) for NADH and nitrate were 22 µM and 3.95 mM, respectively. *Gracilaria caudata* NR activity showed an oscillation under light:dark photoperiod (14:10 hours LD) with 3-fold higher activity during the light phase, peaking after 10 hours of light. Under optimal assay conditions, the maximal activity was 92.9 10^{-3} U g^{-1}. The addition of glucose induced the enzymatic activity during the light and dark phase, evidencing a possible modulation of this enzyme by the photosynthesis. This relationship can be explained by the need of carbon skeletons, produced by the photosynthetic process, to incorporate the intermediary metabolites of nitrate assimilatory pathway, avoiding the toxic intracellular accumulation of nitrite and ammonium. The optimization of enzymatic assay protocols for NR is essential to establish appropriate conditions to study nutritional behaviour, compare different taxonomic groups and to understand its regulatory mechanism.

Key words - agarophyte, *Gracilaria caudata*, nitrate reductase, nitrogen metabolism

RESUMO – (Caracterização da atividade *in vitro* da enzima nitrato redutase em *Gracilaria caudata* J. Agardh (Gracilariales, Rhodophyta)). A alga vermelha *Gracilaria caudata* J. Agardh tem sido utilizada no Brasil para a extração de ágar, principalmente no Nordeste do país. Na água do mar, a disponibilidade de nitrogênio é o principal fator abiótico que limita o crescimento de macroalgas. A enzima nitrato redutase (NR) é o ponto chave na regulação da assimilação do nitrogênio em organismos fotossintetizantes. Este estudo descreve o ensaio *in vitro* para caracterizar a atividade enzimática da NR em termos de constantes cinéticas e estabilidade, avalia sua oscilação ao longo do dia e a influência da glicose na modulação dessa enzima. Picos máximos de atividade da NR foram observados a 20 °C e pH 8,0. A estabilidade enzimática do extrato bruto armazenado a 3 ± 1 °C foi significativamente reduzida após 48 horas. As constantes aparentes de Michaelis-Menten (K_M) para NADH e nitrato foram 22 µM e 3,95 mM, respectivamente. A atividade da NR apresentou oscilação sob fotoperíodo de 14 horas de luz e10 horas de escuro com três vezes mais atividade durante a fase de luz, apresentando seu máximo após 10 horas de luz. Sob condições ótimas de ensaio, a atividade máxima foi de 92,9 10^{-3} U g^{-1}. A adição de glicose induziu a atividade da NR durante as fases de luz e de escuro, evidenciando uma via de modulação dessa enzima pela fotossíntese. Essa relação pode ser explicada pela necessidade de esqueletos de carbono, fornecidos pela fotossíntese, para incorporar os metabólitos intermediários da via de assimilação do nitrato, evitando assim a toxicidade intracelular pela acumulação de nitrito e amônio. A otimização dos protocolos de ensaio enzimático para NR é essencial para estabelecer condições apropriadas para estudos nutricionais, comparar diferentes grupos taxonômicos, e entender os mecanismos de sua regulação.

Palavras-chave - agarófita, *Gracilaria caudata*, metabolismo do nitrogênio, nitrato redutase

Introduction

Nitrogen is the most important abiotic factor that limits the algal growth in the marine environment (Lobban & Harrison 1994, Oliveira & Plastino 1994) and its main available source is in the form of nitrate (NO_3^-) (Chapman & Harrison 1988). The assimilation of nitrate involves its cytoplasmatic reduction to nitrite (NO_2^-), catalyzed by the enzyme nitrate reductase (NR), using NAD(P)H as electron donor. In the chloroplasts, nitrite is quickly reduced to ammonium (NH_4^+) by the enzyme nitrite reductase (NiR) that uses ferredoxine as electron donor, and ammonium is then incorporated to nitrogen molecules as amino acids, purines, pirimidines and amines (Lea & Leegood 1995).

1. Universidade de São Paulo, Instituto de Biociências, Caixa Postal 11461, 05422-970 São Paulo, SP, Brazil.
2. Corresponding author: fchow@ib.usp.br

The enzyme NR is a flavoprotein of high molecular weight, composed by identical subunits containing three prosthetic groups: flavin adenine dinucleotide (FAD), cytochrome b557 (heme) and molybdenum cofactor (MoCo) (Lea & Leegood 1995). There are three forms of NR in eukaryotes: (a) NADH-specific, in most of the plants and some algae; (b) bispecific for NAD(P)H, in some plants (usually monocotyledons), some algae and fungi; and (c) NADPH-specific, in fungi (Campbell 1999).

The NR activity is regulated by several environmental and intracellular factors, such as light quantity and quality, concentration of nitrogen compounds, CO_2, molybdenum, iron, biological clock, phytohormones, plastidic factors, and carbon metabolites (Crawford & Arst Junior1993, Lea & Leegood 1995). Many studies have demonstrated that NR activity can vary with light regimes, showing circadian fluctuations with diurnal assimilatory peaks (Ramus 1981, Edmunds Junior 1988, Lopes *et al.* 1997, Rossa 1999, Chow *et al.* 2004). The regulation of this enzyme involves protein synthesis and degradation cycles (Solomonson & Barber 1990), control by redox and allosteric modulation (Kaiser & Brendle-Behnisch 1991), and phosphorylation and dephosphorylation processes (Huber *et al.* 1992). The intermediaries of nitrogen assimilatory pathway, nitrite and ammonium, are toxic and mutagenic. Therefore, they must be quickly incorporated to carbon skeletons provided by the photosynthetic process to avoid their accumulation into the cells (Huppe & Turpin 1994, Chow 2002). This complex regulation serves to integrate nitrate assimilation with photosynthesis and carbon metabolism (Crawford & Arst Junior1993).

The genus *Gracilaria* Greville (Rhodophyta, Gracilariales) includes about 100 species widely distributed in the world (Oliveira & Plastino 1994) and *Gracilaria caudata* J. Agardh is one of the most common species in tropical and subtropical Atlantic Ocean. This species is exploited as an important agarophyte in northeastern Brazil (Oliveira & Alveal 1990). Therefore, there is a great interest to study the nutritional and growth mechanisms of this seaweed.

This study describes an *in vitro* assay method to quantify NR activity in the agarophyte *G. caudata*. The optimized assay was used to characterize the enzymatic activity in terms of kinetic constants and stability. Activity oscillation and glucose effect on NR modulation were also evaluated.

Material and methods

Culture – *Gracilaria caudata* was collected in Martim de Sá Beach (Caraguatatuba, São Paulo, Brazil) and cultivated under unialgal conditions in sterile seawater enriched with 50% von Stosch (VS) medium (Edwards 1970) at 25 ± 1 °C, 65 ± 6 µmol photons m^{-2} s^{-1}, light:dark (LD) photoperiod of 14:10 hours, salinity of 32 g L^{-1}, and intermittent air bubbling 30:30 min (on:off). The renewal of culture medium and algal cleaning were performed once a week. Contamination by cyanobacteria and other bacteria were controlled by addition of potassic G penicillin (Fluka, Buchs) at 25 mg L^{-1} (Hoshaw & Rodowski 1973) or amplicillin (Sigma, St. Louis, MO, USA) at 50 mg L^{-1} for 48 hours (Oliveira *et al.* 1995). Germanium dioxide (Merck, Frankfurt) at 2 mg L^{-1} was used to control diatom contamination (Lewin 1966).

Enzymatic assay of nitrate reductase – All experiments were performed with unbranched tips of 1.5 cm of length of algae previously grown in 50% VS for 24 hours. The samples were frozen in liquid nitrogen and stored at -80 °C until the *in vitro* enzymatic assay (maximum 1 month). The samples were ground in liquid nitrogen to a fine powder and suspended in the standard extraction buffer (0.2 M phosphate buffer, pH 8.0; 5 mM EDTA; 1 mM DTT and 0.3% w/v BSA) at a concentration of 1 g wet weight per 5 mL of buffer. Cell debris was removed by centrifugation at 12,000 *g* for 15 minutes at 4 °C. The supernatant (crude extract) was recovered and kept on ice until the NR activity was assayed (maximum 1 hour, except for the stability experiment). Nitrate reductase activity was determined by pre-incubating the crude extracts in the reaction mixture (0.2 M phosphate buffer, pH 8.0; 6 mM KNO_3 and 0.5 mM $MgSO_4$) at 20 °C for 10 minutes. The mixture was then incubated for another 5 minutes after addition of 0.02 mM NADH. Controls without NADH were prepared for each experiment. The enzymatic reaction was interrupted by adding 1.4 mM $ZnSO_4$ and 43% v/v ethanol. Precipitates were removed by centrifugation at 12,000 g for 10 minutes at 20 °C. Nitrite concentrations were determined by measuring absorption at 543 nm after the addition of 9.6 mM sulphanilamide and 0.7 mM *n*-(1-naphtyl)ethylenediamine dihydrochloride. One unit of NR (U) is the enzymatic activity producing 1 µmol of nitrite per minute at 20 °C (Chapman & Harrison 1988).

The *in vitro* NR assay method for *G. caudata* was standardized by testing several parameters. (a) methods to interrupt the enzymatic reaction (1.4 mM $ZnSO_4$ and 43% v/v ethanol; 1.4 mM $ZnSO_4$ and 3 minutes boiling; 14 mM $ZnSO_4$; 1.4 mM $ZnSO_4$ and 3 minutes boiling); (b) assay incubation time (5, 10 and 15 minutes); (c) apical (1.5 cm) and basal (1.5 cm immediately below) segments; (d) NADH (0-250 µM) and nitrate (0-40 mM) concentrations; (e) optimal pH and temperature; and (f) stability of NR activity by storing the crude extract at 3 ± 1 °C for up to three days. The effect of nitrate on NR activity was performed in dialysed crude

extracts. K_M values for NADH and nitrate were calculated using the software ENZFITTER v.1.05 described by Leatherbarrow (1990) and using the Lineweaver-Burk plots.

Oscillation of NR activity during 24 hours was evaluated from apical segments kept in a LD cycle (14:10 hours) under the standard culture conditions described above. Light was turned on at time zero (T0). Samples of 70 mg were collected every 2 hours and immediately frozen in liquid N_2. Nitrate reductase assay was performed as described above. Glucose (2% w/v) in VS medium was added to unbranched tips of *G. caudata* either 1 hour or 24 hours before the samples of 70 mg were collected during the light (T8) and dark phase (T18). Samples were immediately frozen in liquid N_2 and nitrate reductase assay was performed as described above.

Statistical significances were analysed by Student test and one-way ANOVA with a probability of 95% (Zar 1999). When necessary, an *a posteriori* Newman-Keuls test was performed. The means ± standard deviations (SD) were based on triplicate measurements from one crude extract. The inference on pseudoreplicate data is usual in biochemistry studies and present an estimate of the variation related the methodology (Hurlbert 1984).

Results

The addition of 1.4 mM $ZnSO_4$ and 43% v/v ethanol to the *in vitro* NR assay was the most efficient procedure to interrupt the nitrate reduction (data not shown). An incubation period of up to 10 minutes during the enzymatic assay was proportional and linear to nitrite production (figure 1). Basal segments of the thalli showed only 72% of activity compared to apical segments. Maximal NR activities were observed at pH 8.0 (24.21 ± 3.14 10^{-3} U g^{-1}; figure 2A) and 20 °C (38.65 ± 4.16 10^{-3} U g^{-1}; figure 2B). Storing crude extracts at 3 ± 1 °C decreased the enzymatic activity significantly by approximately 50% after 24 hours and only 16% activity remained after 48 hours (data not shown). The apparent K_M value for NADH was 22 µM, and for nitrate was 3.95 mM (table 1). The addition of concentrations higher than 80 µM NADH inhibited the NR activity (figure 3A) and 30 mM nitrate reduced the NR activity (figure 3B). Lower activity levels of 7% and 20% in relation to the controls were observed when NADPH or Mg^{2+} was added, respectively. The highest NR activity (92.9 10^{-3} U g^{-1}; table 1) was registered when 50% VS was added 24 hours before the extraction assay under standard culture conditions and optimal assay conditions (see materials and methods).

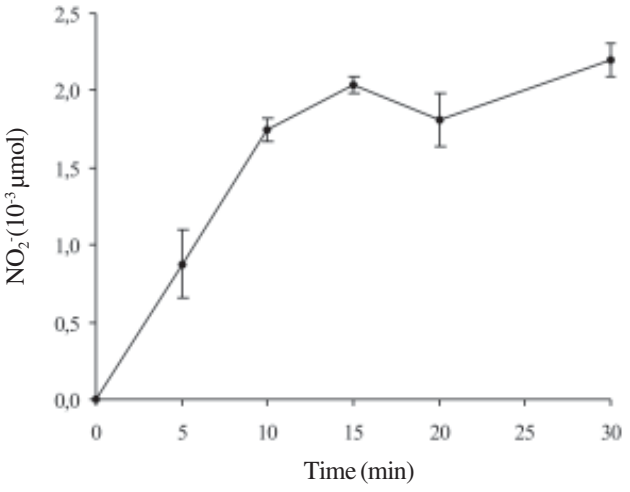

Figure 1. Nitrite concentration produced in the *in vitro* reduction of nitrate by nitrate reductase (NR) activity. Means ± SD (three measurements from the same crude extract).

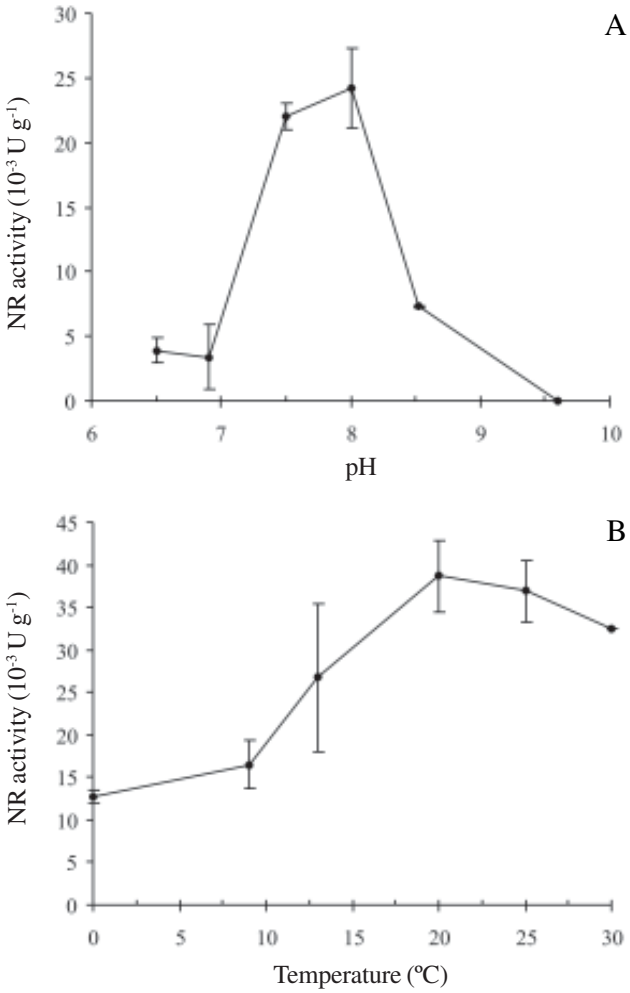

Figure 2. Nitrate reductase (NR) activity under different levels of (A) pH and (B) temperature in the red seaweed *Gracilaria caudate*. Means ± SD (three measurements from the same crude extract).

Table 1. Characterization of optimal conditions for in vitro nitrate reductase assay and apparent Michaelis-Menten constants (KM) for NADH and nitrate for different species of red seaweeds. (n.d. = no data).

Species	Biomass/volume rate of extraction buffer (g mL^{-1})	Volume of crude extract (µL)	Optimal pH	K_M NADH (µM)	K_M nitrate (mM)	Maximal NR activity (10^{-3} U g^{-1})	References
Gracilaria tikvahiae McLachlan	n.d.	n.d.	n.d.	n.d.	n.d.	43.3	Hwang *et al.* (1987)
Gracilaria tenuistipitata var. *liui* Zhang & Xia	0.08	100	8.0	95.0	0.197	43.0	Lopes *et al.* (1997)
Gracilariopsis tenuifrons (Bird & Oliveira) Fredericq & Hommersand	0.20	100	8.0	12.2	0.031	n.d.	Rossa (1999)
Gracilaria chilensis Bird, McLachlan & Oliveira	0.10	50	8.0	8.0	0.68	253.2	Chow *et al.* (2004)
Gracilaria caudata J. Agardh	0.20	50	8.0	22.0	3.95	92.9	Present study
Porphyra perforata J. Agardh	n.d.	n.d.	8.5	32.0	0.03	n.d.	Thomas & Harrison (1988)
*Porphyra yezoensis** Ueda	n.d.	n.d	8.5	23.0	0.06	n.d.	Nakamura & Ikawa (1993)

*Nitrate reductase activity from purified extract.

Algae grown under LD (14:10 hours) photoperiod showed NR activity oscillation with lower levels of activity during the dark phase. The maximal NR activity peak was observed after 10 hours of light (T10) and was three times higher than the activity observed during the dark (figure 4). The addition of glucose one hour before NR assay showed no influence on NR activity (data not shown), but the addition of glucose 24 hours before NR assay induced the enzymatic activity both in light and dark phases by 43% and 55%, respectively (figure 5).

Discussion

Despite the importance of nitrogen and presence of the enzyme NR in seaweeds, little is know about its

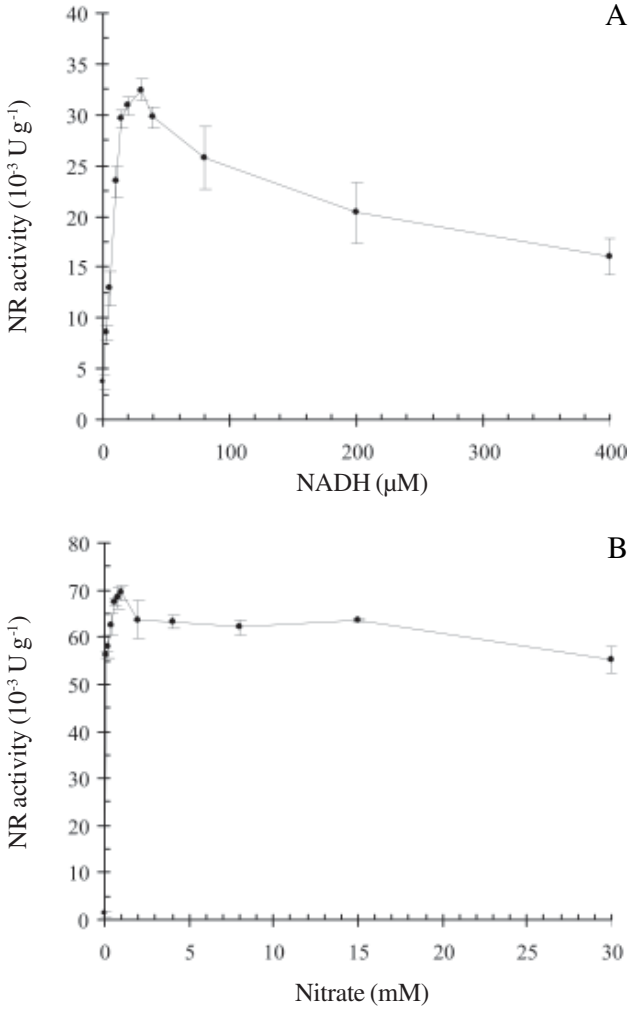

Figure 3. Effect of different concentrations of (A) NADH and (B) nitrate on nitrate reductase (NR) activity in the red seaweed *Gracilaria caudate*. Means ± SD (three measurements from the same crude extract).

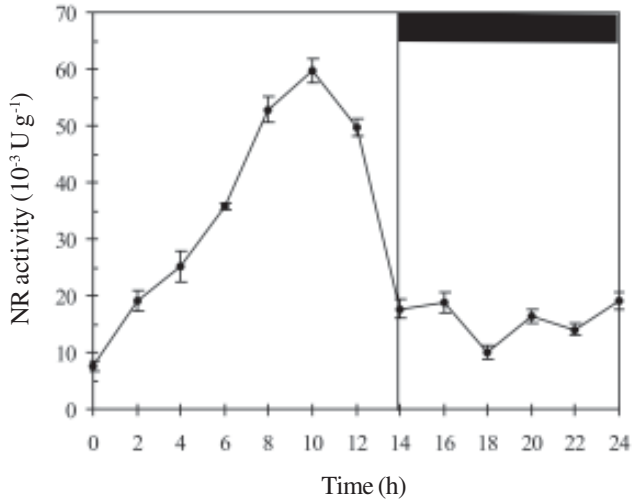

Figure 4. Oscillation of *in vitro* nitrate reductase (NR) activity under light:dark photoperiod (14:10 h) in the red seaweed *Gracilaria caudate*. Black bar represents dark phase. Means ± SD (three measurements from the same crude extract).

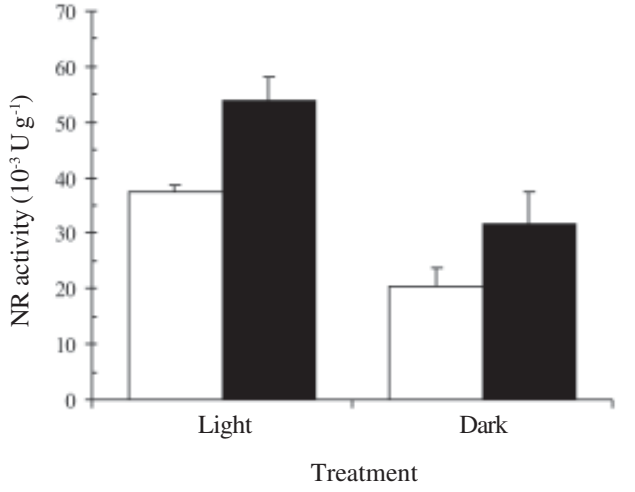

Figure 5. Effect of glucose addition (2% w/v) during the light and dark phases on nitrate reductase (NR) activity in the red seaweed *Gracilaria caudate*. White bars represent controls without glucose addition, and black bars with glucose addition. Means ± SD (three measurements from the same crude extract).

metabolism, when compared to the extensive information available for higher plants and microalgae. Nitrate reductase shows great variations among photosynthetic organisms evidenced by different levels of requested substrate (nitrate and NADH) to reach the maximal activity and the effect of many regulating factors, such as light quantity and quality. Therefore, different extraction protocols result in a wide variation of enzymatic activities (Berges & Harrison 1995), being essential to establish optimal assay conditions for each species (Thomas & Harrison 1988, Hurd *et al.* 1995, Chow *et al.* 2004). To optimize an assay method for *Gracilaria caudata*, variations in the methodology described by Thomas & Harrison (1988) and Lopes *et al.* (1997) were tested.

The high NR activity recorded for *G. caudata*, compared to some other species (table 1), evidences the optimized protocol for this species. Key steps in the protocol optimization were a extensive extraction of the enzyme by grinding the samples in liquid nitrogen and the preservation of its activity by addition of compounds that minimize protein degradation (*e.g.* EDTA, DTT, and BSA). The high NR activity is also due to the addition of NADH and nitrate in saturating amounts, avoiding sub-optimal or inhibitory conditions for the assay. The main modifications in the protocol registered for different species of *Gracilaria* are: (a) the rate of fresh weight per volume of extraction buffer, and (b) volume of crude extract used for each incubation (table 1). These differences indicate the variable physiological state of each species determined by the amount of internal stored nitrogen (*e.g.* proteins, amino acids, phycobilins and Rubisco) and, therefore, affecting the level of available NR enzyme.

The optimal pH for *G. caudata* NR was 8.0, the same found for other species of seaweed, higher than found in higher plants (pH 7.5), but similar to seawater pH (8.2). *Gracilaria caudata* NR is active in a wide range of temperatures (from 0 to 30 °C), similar to observed for *G. tenuistipitata* var. *liui* (Lopes *et al.* 1997). These species are tropical and subtropical, therefore, their tolerance to both pH and temperature ranges may be related to their natural geographic distribution.

According to Berges & Harrison (1995), there are species-specific differences on kinetic parameters for NADH and nitrate. The apparent K_M for NADH in *G. caudata* (22 ± 7 μM) is closer to those of *Porphyra perforata* (32 ± 9 μM; Thomas & Harrison 1988) and *P. yezoensis* (23 μM; Nakamura & Ikawa 1993). On the other hand, the value for nitrate K_M was much higher for *G. caudata* than for other tested red seaweeds. The differences in the K_M values for NADH and nitrate are related to the particular characteristics of metabolic capacity of each species, and associated to laboratory culture conditions that determine the nutrient availability (stocked into the vacuoles or as organic molecules).

The low NR activity registered with NADPH as electron donor did not agree to the data in the literature for other red seaweeds that showed no activity when NADPH was added (Nakamura & Ikawa 1993, Lopes *et al.* 1997). This NADPH-activity could be explained by the presence of another NADPH-activated isoenzyme, by dephosphorylation of NADPH to NADH, or conversion of NADPH in NADH by a transhydrogenase (Jackson 1991). Berges & Harrison (1995) described that this transhydrogenase could be present in crude extracts, and NADPH would be converted to NADH and then used by a NADH-specific NR. However, it is more common in homogenates that are not centrifuged, which is not the case for *G. caudata*, once the crude extract was centrifuged.

The maximal NR activity (92.9 10^{-3} U g^{-1}) for *G. caudata* was higher than the activity observed for *G. tikvahiae* (43.3 10^{-3} U g^{-1}; Hwang *et al.* 1987) and *G. tenuistipitata* (43.0 10^{-3} U g^{-1}; Lopes *et al.* 1997) and lower than that of *G. chilensis* (253.2 10^{-3} U g^{-1}; Chow *et al.* 2004). All differences found between the species reinforce the need of a previous standardized protocol and characterization of NR activity for each studied species.

The NR activity of *G. caudata* showed an oscillation during 24 hours, with maximal peak after 10 hours of light (60 ± 1.33 10^{-3} U g^{-1}), coinciding with maximal irradiances observed in natural environments between 12:00 to 15:00 pm. This peak during the light phase evidences a light-dependent activation/inactivation response as have been reported by Lillo *et al.* (2004) for land plants.

Our results showed that *G. caudata* NR is active mainly during the light phase of the 24 hours cycle. In higher plants, light has been showed to induce NR activity in two ways, (1) rapid (minutes) induction, modulating the activation of pre-existing protein, and (2) slow (hours, days), inducing NR gene expression, in this case, the effect of the light could be replaced by glucose (Lillo *et al.* 2004). Based on what is known for higher plants, glucose was added to the growing media to see its effect on NR activity. Fries (1963) showed for red algae (including six species) that glucose and other sugars have an effect on algal growth. Yokoya (1996) showed that an increment in growth and morphogenesis occurred in *Gracilariopsis tenuifrons* and *Grateloupia dichotoma* with the addition of 1% sucrose, although its presence in the culture media was not essential. When glucose was added to *G. caudata* cultures 1 hour before the NR assay, no difference in enzymatic activity was observed, indicating that glucose does not affect directly the modulation of the NR enzyme. When glucose was added 24 hours before the NR assay, it was observed an increase in the enzymatic activity indicating that glucose is activating the NR through a slow response mechanism (*i.e.* gene expression and/or new synthesis of the protein). These results indicate that light is one of the major regulating factors of NR activity and that the photosynthesis could be modulating NR gene expression through glucose production.

Glucose mimics the effect of light on NR, inducing its activity both during the light and dark phases. This could be explained by inducing protein dephosphorylation, as evidenced by Iglesias-Bartolomé *et al.* (2004). Reports on the mechanisms involved in sugar regulation of phosphorylation state on NR are scarce for macroalgae. Regulatory mechanisms of the enzymatic activity include complex modulator processes to optimize nitrogen assimilation and prevent the formation of toxic products. The post-translational control of NR by phosphorylation is the most important controlling mechanism to avoid this deleterious accumulation (Lillo *et al.* 2004), but there are many aspects still to be solved, opening an interesting research area to elucidate the signalling mechanism that modulates the nitrogen assimilatory pathway in macroalgae.

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