Enhancement of carotenoid production in *Nannochloropsis* by phosphate and sulphur limitation

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Because of its value as antioxidants, food-supplements and in animal-feeding, carotenoids are among the most attractive target compounds of marine microalgae. In this contribution the influence of essential nutrients (N, S and P) limitation on the accumulation of carotenoids in the marine microalga *Nannochloropsis gaditana* has been studied. High and rapid biomass production, which depends on growth conditions, is the first step in a bioproduction process. In our work was observed that *Nannochloropsis gaditana* growth was significantly faster when using 5 % (v/v) CO_2 in air instead air or HCO_3^- as carbon source, at two different pH values assayed (7.2 and 8.2), as revealed from cell chlorophyll content, nitrate consumption rates and light-dependent oxygen production. Afterwards, cells grown to exponential phase were incubated in culture media containing none or limiting concentrations of either nitrate, sulphate or phosphate. The results showed that both starvation and low concentrations of nutrients (0.005 mM P, 0.2 mM N, $1.1 \cdot 10^{-5}$ mM S) drove to increases of carotenoids/chlorophyll ratios, specially for sulphur limitation. In a general view, zeaxanthin and violaxanthin content increased in all limiting conditions tested, the increase of violaxanthin content being less pronounced.

Keywords *Nannochloropsis*; carotenoids; nutritional stress

1. Introduction

Microalgal cultivation has been investigated for production of fine chemicals and health foods [1,2]. Carotenoids show growing evidence of benefits to human health. Recent studies have suggested that carotenoids can prevent or delay cancer and degenerative diseases in human and animals by contributing to antioxidant defenses against metabolic oxidative byproducts [3,4].

Many scientific papers that focus on influence of growth conditions on intracellular accumulation of certain products in microalgae have been published, most of them paying major attention to specific stress conditions including both absence and excess of a given substrate molecule [5,6,7,8]. Either lack or low level of certain nutrients including nitrogen act like a metabolic sign for microalgae to induce a fast physiological response which is the trigger of secondary biosynthetic pathways [9,10]. Moreover, many biotechnological applications are based on transients of cells from full nutrient to nutrient deficient culture media and then back to full nutrient media to recover cells from stress. The kind of nutrients (e.g. C and N) can also have noticeable effect on growth and biomolecule profile of the microorganisms studied, affecting parameters like growth rate, protein content, pigment content and biological activity, therefore affecting the biochemical composition of the microalgae and the quality and quantity of valuable compounds from the microalga metabolism [5,6,11].

Nannochloropsis gaditana is a microalgae that belongs to the class Eustigmatophycea. This alga is used in aquaculture for the cultivation of fish, either directly or via rotifers [12], as *N. gaditana* has a very stable behaviour during the cultivation process. The alga has adapted to the climatic conditions of the Bay of Cádiz (Spain), possesses a good nutritional profile [13,14], it is source for commercially valuable compounds as extensively described [15] and is also recognized as a good potential source of EPA (20:5v3), an important polyunsaturated fatty acid for human consumption for prevention of several diseases [16,17]. This marine microalgae presents only chlorophyll a [18] and the main accesory pigments, violaxanthin and vaucheriaxanthin esters play a major role in light harvesting [19]. Some

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minor xanthophylls (canthaxanthin, anteraxanthin, zeaxanthin) and carotenes (β-carotene) are also present in much lower amounts [20].

In this paper, nutrient deficiency has been found to increase intracellular accumulation of specific carotenoids in *N. gaditana*. Many scientific papers in the literature have dealt with the stimulation of carotenoid biosynthesis by cell growth in culture media with low concentrations of inorganic nitrogen (nitrate or ammonium), nitrogen starvation or even an imbalance in the N/C ratio in the culture medium [37]. However much less information is available on the effect of nutrient limitation other than nitrogen on carotenoid biosynthesis. Even no information is available from the literature regarding the cell content of specific carotenoids of *N. gaditana* growing in culture media limited in the main essential nutrients.

2. Materials and Methods

2.1 Microorganism and culture conditions

Nannochloropsis gaditana was kindly provided by ICMAN-CSIC, Cádiz. Standard cultures were grown with F/2 medium [21] modified with double nitrate and phosphate concentrations to avoid nutrient limitation at 25ºC, bubbled with air containing 5% (v/v) CO_2 and continuously illuminated with fluorescent lamps (200 $\mu E\ m^{-2}\ s^{-1}$, at the surface of the flasks). For carotenogenesis induction *N. gaditana* cells were cultured in the same liquid medium without nitrate, phosphate and sulphate source as indicated.

2.2 Oxygen evolution

The biological activity used to test cell viability was the photosynthetic activity. For photosynthetic activity determinations 1 ml cell culture of the microalgae was placed in a Clark-type electrode to measure O_2-evolution. Measurements were made at 25º C under saturating white light (1500 $\mu E\ m^{-2}\ s^{-1}$) or darkness (endogenous respiration).

2.3 Spectrophotometric determinations

Aliquots (5 ml) of the cultures were spinned down and the pellet obtained was freeze-dried and resuspended in 2.5 ml of absolute methanol to extract pigments. Chlorophyll and total carotenoids concentrations in the supernatant were determined either spectrophotometrically using the equations proposed by Wellburn [22] or by HPLC analysis for specific carotenoids (see below). Protein content was determined following the method described by Bradford [23]. The cell concentration was determined by measuring the optical density of the culture at 680 nm in a spectrophotometer. Nitrate was determined spectrophotometrically as described by Cawse [24].

2.4 HPLC analysis of carotenoids

Separation and chromatographic analysis of pigments was performed in a Merck Hitachi HPLC equipped with a UV-Vis detector as described by Young [25], using a RP-18 column and a flow rate of 1 ml min^{-1}. The mobile phase consisted on: solvent A, ethyl acetate; solvent B, acetonitrile/water (9:1, v/v) and the gradient programme applied was: 0-16 min, 0-60% A; 16-30 min, 60% A; 30-35 min, 100%. Pigments detection was carried out at 450 nm, and their identification and quantification was achieved by injecting known amounts of pigment standards. Chlorophyll *a* and β-carotene were purchased from Sigma Chemical Co. (St. Louis, MO), all other standards were obtained from the Water Quality Institute VKI (Horsholm, Denmark). A typical chromatogram of the pigment profile of *N. gaditana* cells grown with F/2 medium is shown in Figure 1.

2.5. Cell counting

The number of cells was determined by counting *N. gaditana* cells in a Neubauer chamber using an Olympus microscope model CX41.
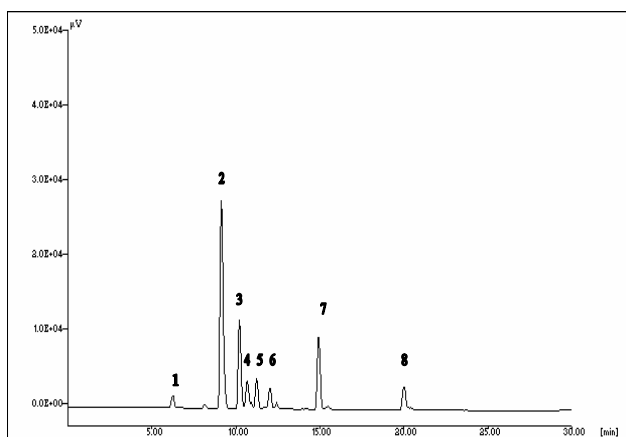
Fig. 1 Typical profile of carotenoids from *N. gaditana*. Chromatogram of control culture was obtained following the HPLC procedure described in Materials and Methods. Peaks were identified as: 1 Neoxanthin; 2 Violaxanthin; 3 Anteraxanthin; 4 Zeaxanthin; 5 No identified; 6 Canthaxanthin; 7 Chlorophyll a; 8 β-carotene

3. Results and Discussion

3.1 Influence of the carbon source and pH on cell growth of *N. gaditana*

The production of high biomass concentrations is the first step in bioproduction processes of commercial value compounds from microorganisms. The growth of *N. gaditana* is rather dependent on the carbon source than on the pH of the culture medium. According to the results in Figure 2, the growth pattern of *N. gaditana* in buffered culture mediums at pH 7.2 was very similar to that at pH 8.2, in terms of number of cells, chlorophyll content and light-dependent oxygen production. It was only after one week of cell growth that the culture at pH 7.2 showed a slightly faster growth than the one at pH 8.2 and reached the stationary growth phase earlier. With regard to the carbon source, typical growth parameters of photosynthetic cell growth (Figure 2) showed a significantly faster growth in *N.gaditana* cell cultures growing with 5 % (v/v) CO_2 in air with respect to those growing in the presence of only air. The light-dependent oxygen production data increased in parallel to number of cells in those cultures growing with CO_2, as a sign of cell viability of the produced biomass.

It has been previously described that *N. gaditana* can grow with HCO_3^- as carbon source as efficiently as with CO_2, via an effective concentration mechanism which would be active where HCO_3^- is the predominant carbon form [29]. Our results are according to that, but moreover *N. gaditana* showed a faster growth with CO_2 as carbon source (Figure 2), even at that pH value (8.2) at which HCO_3^- is the predominant inorganic carbon form and at which the referred effective HCO_3^- concentration mechanism should have therefore been induced, then being active. The growth rate determined for *N. gaditana* al pH 7.2 was slightly higher than that at pH 8.2. Under continuous supplying of CO_2 as carbon source, there is a major carbon availability in the form of CO_2 at pH 7.2 than at pH 8.2. As a consequence, a slightly faster growth of *N. gaditana* at pH 7.2 means that the CO_2 transport at that pH is being as efficient as that of HCO_3^- at a higher pH, in coherence with the existence of effective transport mechanisms in microalgae for CO_2 as described previously [30].

In our experiments, all culture media were buffered in order to avoid pH changes which could produce continuous change of the CO_2/HCO_3^- ratio around the cells and, subsequently, the induction and action of the different carbon uptake mechanisms. Continuous supply of CO_2 as carbon source allows better control of the carbon concentration in the culture.
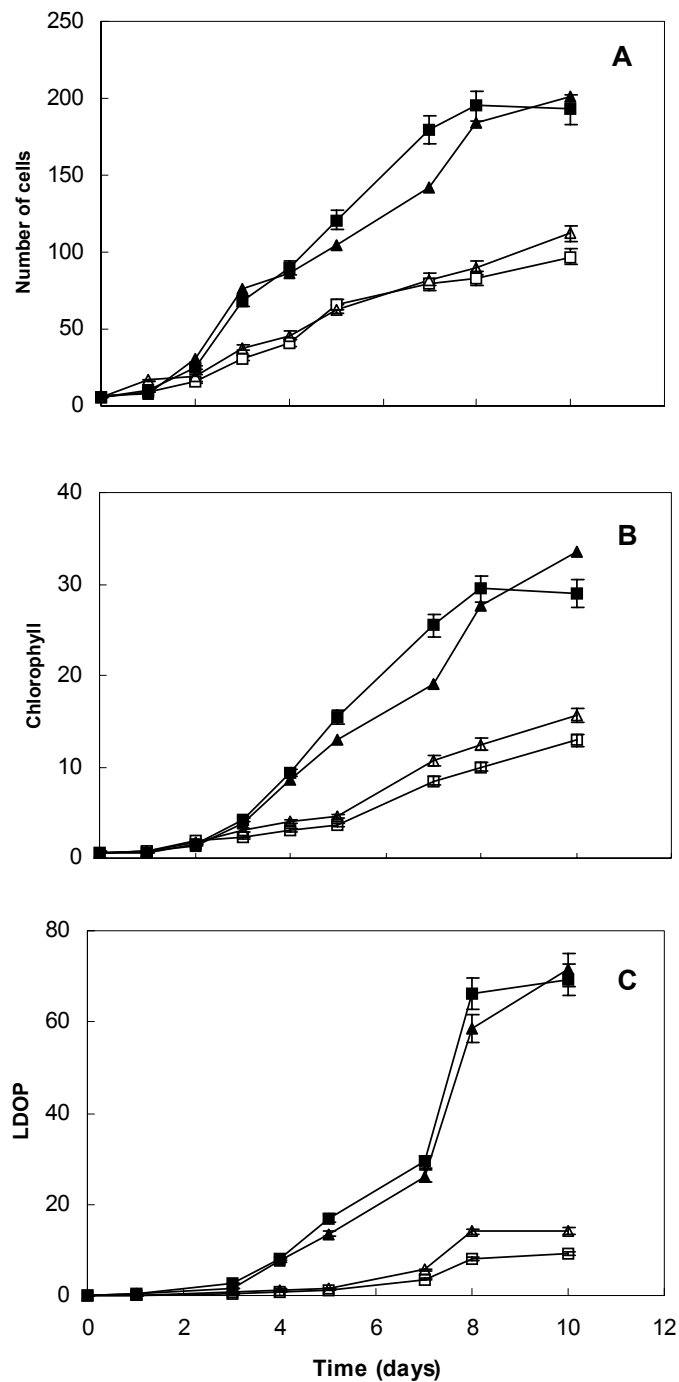
Fig. 2 Effect of carbon source and pH on *N. gaditana* growth. Cells of *N. gaditana* were grown in buffered culture mediums at pH 7.2 (□) and 8.2 (△) bubbled only with air (open symbols) or 5% (v/v) CO_2 in air (closed symbols) as carbon source. At the indicated times, number of cells (N 10^6 ml^{-1}) (A), chlorophyll (μg ml^{-1}) (B) and light dependent O_2 production (LDOP) (μmol O_2 mg^{-1}Chl.h^{-1}) (C) were determined.

3.2 Influence of nitrate, sulphate and phosphate limitation on cell growth and total carotenoids of *N. gaditana*

N. gaditana cells were grown in F/2 culture medium prepared as described in Materials and Methods and supplemented with different limiting concentrations (10% of non limiting conditions, [26]) or starvation of either nitrate, sulphate or phosphate as follows: N1, S1 and P1 contained 0.005 mM P, 0.2 mM N, 1.1 10^{-5} mM S and N2, S2 and P2 contained neither nitrate, sulphate nor phosphate. *N. gaditana* cells growing in a full nutrient culture medium were used as control cells. Cell growth was followed during 5 days (Figure 3A). Number of cells of each one of the cultures was counted and the specific growth rates at the applied stress conditions were calculated. After five days in nutrient deficient culture media the growth rates of *Nannochloropsis gaditana* cells decreased under all nutrient conditions tested. After five days those cultures growing in N-limiting conditions showed the minimum growth rate (almost zero).

The total carotenoid content of *N. gaditana* was also studied under the nutritional conditions previously described (Figure 3B). The content of carotenoids per biomass unit (10^6 cells) of *N. gaditana* was similar in all of the cultures incubated in nutrient-limiting conditions with the exception of those growing in either nitrogen limitation or lack of nitrogen, where the content of carotenoids per biomass unit accounted for about 50 % less. On the opposite, the total carotenoids to chlorophyll ratio was significantly higher in those *N. gaditana* cells incubated in either nitrogen defficient or lacking culture medium, as it can be observed from the data in Figure 3C.

It has been widely reported that cell growth of microalgae is affected by either limitation or starvation of essential nutrients [31,32]. Significant differences in the growth of microalgae cells have been observed depending on the limiting nutrient [33,34]. From our results it can be induced that the growth of those cell cultures incubated in either nitrogen limitation or nitrogen starvation resulted more affected than those incubated under either phosphorous or sulphur limitation (Figure 3). Particularly, the chlorophyll content of cells growing in nitrogen deficient culture media decreases with time. This is a clear sign of nitrogen starvation, where neither proteins nor the nitrogen-containing chlorophyll molecules can be synthesized and the light transduction photosynthetic process is not active. Although after five days of incubation in nutrient-deficient culture media some signs of cell growth were observed, the light-dependent oxygen production data (Figure 3) revealed that only control cells were viable. This is in agreement with other results reported in literature [35].

3.3 Profile of the main carotenoids of *Nannochloropsis gaditana* grown in the presence of different nitrate, sulphate and phosphate concentrations

The profile of the main carotenoids was obtained by HPLC on *N. gaditana* methanol extracts as described in Materials and Methods. Pigment type and concentration were determined in *N. gaditana* cell cultures grown to exponential phase in full nutrient culture media with different sulphate and phosphate concentrations. Since N limitation resulted in a clear decrease of carotenoid content (Figure 3), the analysis of specific carotenoids is shown only for those cells cultivated in either phosphate or sulphate limitation. The results were compared to those obtained from control cells growing in full nutrient culture media. In a first general view, cantaxanthin content and β-carotene content were found to decrease with time course under either sulphate or phosphate limitation, whereas zeaxanthin and violaxanthin content increased in all limiting conditions tested, the increase of violaxanthin content being less pronounced.

The growth of *N. gaditana* cells in culture media with either phosphorus limitation or lack of phosphorus (Figure 4) resulted in a different quantitative pigment pattern. As shown in Figure 4, with the exception of β-carotene, the content of pigments increased in the tested nutrient limiting conditions, when compared to control cultures. With respect to control cells, the trend of zeaxanthin to accumulate in *N. gaditana* cells incubated in such those limiting conditions was specially noticeable. In addition to nutrient limitation, the modulated use of light intensity could improve the xanthophylls production [14,18,36,38].

A

B

C
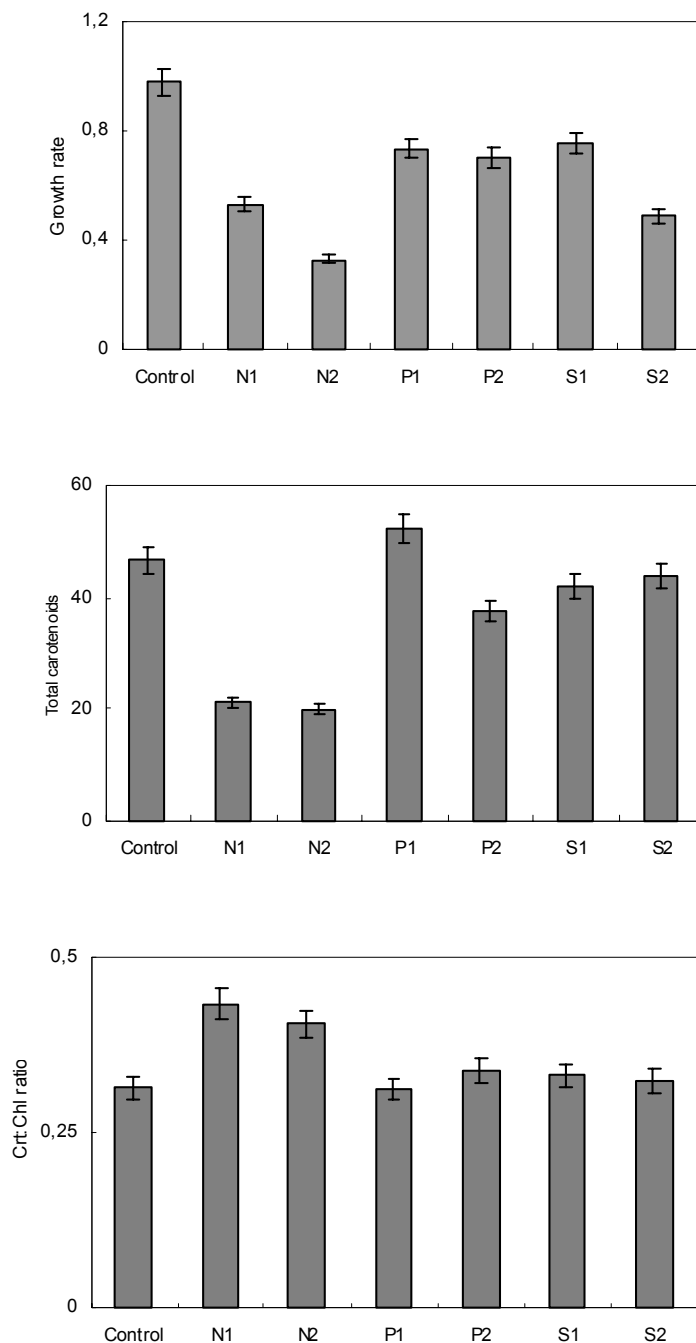
Fig. 3 Growth rate, total carotenoids and chlorophyll to carotenoids ratio of *N. gaditana* cells *after 5 days of incubation under different nutrient conditions.* Cells grown under standard culture conditions to exponential phase were harvested, washed and resuspended in culture mediums with different nutrient conditions. N1, S1 and P1 contained 0.005 mM phosphate, 0.2 mM nitrate, $1.1 \ 10^{-5}$ mM sulphate and N2, S2 and P2 contained neither nitrate, sulphate nor phosphate At the indicated times, growth rate (days^{-1}) (A), total carotenoids (ng 10^6 cell^{-1}) (B) and chloropyll (µg ml^{-1}) (C) were determined.
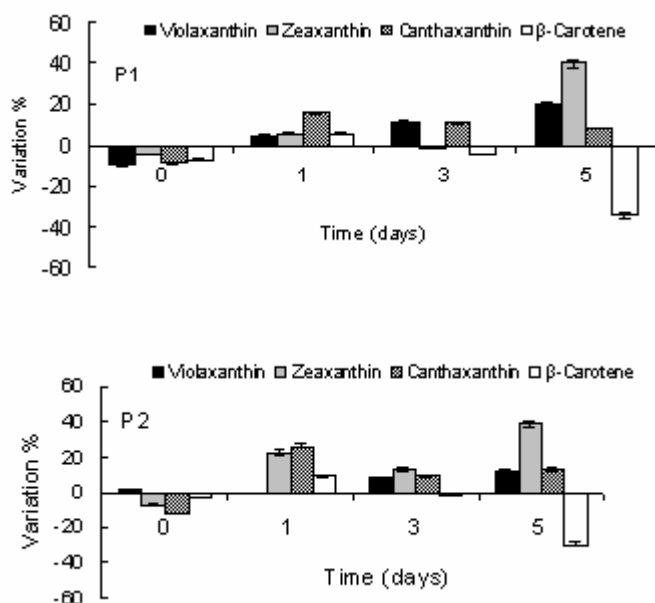
Fig. 4 Effect of several phosphate concentrations on carotenoid content of *N. gaditana*. Cells in exponential growth under standard culture conditions were harvested, washed and resuspended in culture medium with low concentrations of inorganic phosphorous (0.005 mM PO_4^-) (P1) and in phosphorous lacking culture medium (P2). In both cultures, the content of violaxanthin, zeaxanthin, cantaxanthin and β-carotene was determined by HPLC, at the indicated times. 100% violaxanthin = 35.6 ng 10^{-6} cells, 100% zeaxanthin = 1.15 ng 10^{-6} cells, 100% cantaxanthin = 7.7 ng 10^{-6} cells, 100% β-carotene = 16.9 ng 10^{-6} cells

When the culture media were either deprived from sulphur or limited in its concentration (Figure 5), the obtained results were rather similar to those previous ones of *N. gaditana* cells growing in culture media in either absence or limitation of phosphorus (Figure 4). Specifically, zeaxanthin content increased with respect to control cells growing in full nutrient culture media whereas the content of violaxanthin, cantaxanthin and β-carotene did not change.

S-limitation drove the rapid inhibition of PSII specially affecting the primary products from photosynthesis (NADPH and Fd_{red}) [39,40]. From a long time cell exposure to S-defficiency, ascorbate pool should be significantly affected due to the minor sulphur assimilatory reduction. According to the regulation of the xanthophyll cycle. The decrease of ascorbate supply to the cycle should result in the reduction of xanthophyll biosynthesis, which is in agreement with the results obtained when *N. gaditana* was grown in S-deficient culture media (Figure 5).

Our results suggest that sulphur and phosphate limitation could be applied to enhance the production of commercially valuable carotenoids, specially the xanthophylls violaxanthin and zeaxanthin.
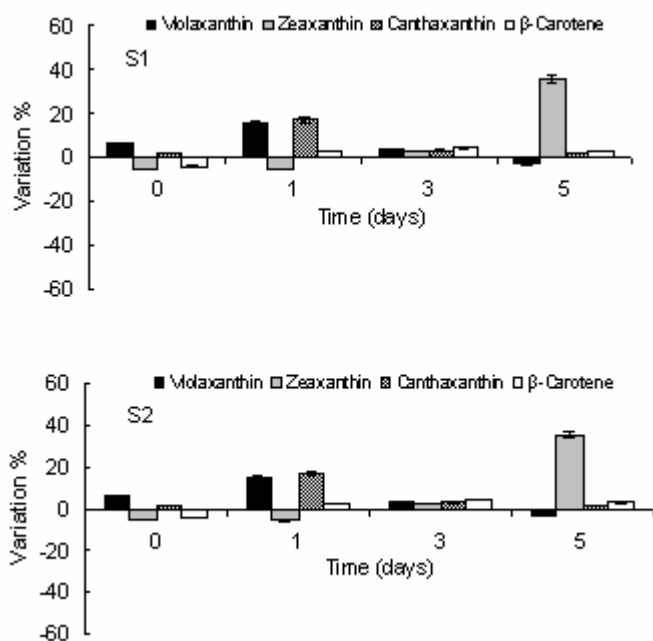
Fig. 5 Effect of several sulphate concentrations on carotenoid content of *N. gaditana*. . Cells in exponential growth under standard culture conditions were harvested, washed and resuspended in a culture medium with low concentration of inorganic sulphur (1.1 10^{-5} mM SO_4^-) (S1) and in a sulphur lacking culture medium (S2). In both cultures the content of violaxanthin, zeaxanthin, cantaxanthin and β-carotene was determined by HPLC, at the indicated times. 100% violaxanthin = 35.6 ng 10^{-6} cells, 100% zeaxanthin = 1.15 ng 10^{-6} cells, 100% cantaxanthin = 7.7 ng 10^{-6} cells, 100% β-carotene = 16.9 ng 10^{-6} cells

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