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Effect of organic carbon sources and Fe^{2+} ions on growth and β-carotene accumulation by *Dunaliella salina*

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Abstract

Effects of adding Fe^{2+} ions in photoheterotrophic cultivation of *Dunaliella salina* on growth and β-carotene synthesis were investigated. Different concentrations of Fe^{2+} ions and organic carbon source (acetate and malonate) were studied. A significant increase of cellular β-carotene content was observed, with a maximum value of 70 pg/cell in a culture enriched with 67.5 mM of acetate and 450 μM of $FeSO_4$ and 33 pg/cell in the case of 67.5 mM of malonate and 450 μM of $FeSO_4$. For comparison, the well-known nitrate starvation protocol was applied, and 35 pg/cell of β-carotene were obtained. By inducing an oxidative stress, Fe^{2+} ions revealed to stimulate β-carotene synthesis, especially when acting in the presence of the carbon source. Furthermore for some concentration of Fe^{2+} ions and organic carbon source, the loss in cells division was reduced compared to nitrate starvation.

Keywords: β-carotene; *Dunaliella salina*; Oxidative stress; Iron; Acetate; Malonate

1. Introduction

Microalgae are of increasing interest for research and industry. A major commercial application is the production of β-carotene from *Dunaliella salina* which has been developed first by Western biotechnology Ltd. and Betatene Ltd. in Australia at 1986, and more recently by other companies with production facilities in Israel, USA [1] and China [2]. The β-carotene is a lipophilic high-value compound [3]. It has been traditionally commercialized as food additives including colorants, antioxidants and vitamins [4], and is added to numerous cosmetic and body-care products as a harmless colorant to improve the attractiveness of the product [5]. Its protective ability against oxygen free radicals leads to therapeutic applications, as preventive in degenerative disease and anti-cancer agents [6]. It is also used in immune-system stimulators [7] which are implied more than 60 life-threatening diseases including various forms of cancer, coronary heart disease, premature aging and arthritis [8,9]. β-carotene can be produced from different natural sources such as carrots [10], fruit wastes [11,12] and microalgae such as *D. salina*.

D. salina is a halotolerant unicellular microalga. Under specific stress conditions it can produce and accumulate high concentrations of β-carotene in oil droplets in the cells up to a concentration of 10% of dry weight [13]. The stress conditions are various, such as high light intensity [14], high salt concentration [15,16], nitrogen starvation [17], or high temperature [18]. Actually, the production of β-carotene is mainly located in area presenting high solar irradiances using open-air outdoor cultivation systems [19]. Such a production is operated in batch mode, high salt concentration being, for example, naturally achieved by evaporation. In addition, nitrogen starvation resulting in an increase in β-carotene intracellular accumulation can be obtained by using a limited amount of nitrogen that is next consumed during growth. Despite these systems have shown their efficiency, especially for extensive production in semi-desert areas, it also appears interesting to investigate alternative protocols such a high light intensity applicable in closed intensified photobioreactors, more suitable for production in more temperate regions. Hejazi et al. [20] have recently shown feasibility and interest of continuous extraction protocol using a "milking" process. This opens new optimization possibilities, especially in the definition of stress conditions more adapted to continuous regime, where the β-carotene metabolism must be supported without too much deteriorating the growth rate of *D. salina* (as it is observed with nitrogen starvation).

Interest of enriching the growth medium with specific elements to enhance growth and β-carotene synthesis was investigated. Influence of adding an organic carbon source such as acetate or malonate has been especially considered. Previous studies on the nutrition of *Haematococcus pluvialis* and *Phaffia rhodozyma* have shown that acetate and malonate appear to be an interesting carbon sources, enhancing both growth and carotenogenesis [21–25]. This can be explained by the fact that acetic acid and mevalonic acid are a key carotenoid precursors [26].

In addition to the carbon source, interest of reinforcing the mineral composition of the basic growth medium with iron (Fe^{2+}) was also investigated. Indeed, iron is an essential element for most living organisms, being a co-factor in major processes like DNA synthesis, respiration and photosynthesis [27]. Kobayashi et al. [23] have shown that addition of acetate and Fe^{2+} enhanced the carotenoids formation in *H. pluvialis*, emphasizing a possible synergic effect between both organic and mineral compounds. However, there is no information on the effect of Fe^{2+} addition with acetate or malonate in growth medium on carotenoid accumulation in *D. salina*.

This study presents results obtained in batch conditions with acetate, malonate and iron additions at various concentrations. Effects on growth and β-carotene synthesis were especially considered and compared to the classical protocol of nitrogen starvation which was retained as a reference.

2. Materials and methods

2.1. Organism and culture conditions

D. salina (CCAP 19/18) was collected from the culture collection of IFREMER-Nantes (France). Micro-algae were first cultivated in an airlift photobioreactor operated in continuous mode with standard growth conditions [28]. A constant cell concentration of 8×10^5 cell/ml was thus obtained containing 3.2 pg/cell of β-carotene. The growth medium contains 1.5 M NaCl, 12 mM KNO_3, 0.4 mM KH_2PO_4, 5 mM $MgSO_4$, 185 μM H_3BO_3, 0.2 mM $CaCl_2$, 2 μM $FeCl_3$, 7 μM $MnCl_2$, 1 μM $ZnCl_2$, 1 μM $CoCl_2$, 1 μM $CuCl_2$, 1 μM $(NH_4)_6Mo_7O_{24}$, 5 μM Na_2EDTA. The pH of the medium was adjusted to 7.5 by an automatic injection of CO_2 and the temperature was maintained at 25 °C by air blowing.

Various protocols were then evaluated using 400 ml of algal culture from the airlift photobioreactor placed in Roux bottles in batch conditions (600 ml). Same temperature was applied as in the growth phase, with a continuous illumination (400 $\mu E/m^2$ s) by using six fluorescent tubes (OSRAM L13W/12-950) and a constant agitation by a magnetic stirrer (200 rpm).

2.2. Assays

For nitrate starvation, algal culture was centrifuged at 2000 rpm during 5 min, washed, resuspended in modified medium, and placed in Roux bottle. To study the effect of nitrate concentration on carotenoid production and growth, different initial nitrate concentrations (0, 1.2, 6 and 12 mM KNO_3) in the growth medium have been applied.

For subsequent experiments, a KNO_3 concentration of 12 mM was chosen to test the effect of malonate, acetate and iron concentration. In these experiments, volumes of sodium malonate ($C_3H_2Na_2O_4$) solution (2.25 M, pH 7) or sodium acetate (CH_3COONa) solution (2.25 M, pH 7) and ferrous sulphate ($FeSO_4$) solution (22.5 mM, pH 1.5) were directly added to algal culture to obtain the desired initial concentrations in the growth medium investigated.

For all these expriments we tried to unify the values of the biomass concentration and the β-carotene content at around 8×10^5 cells/ml and 3 mg/l, respectively.

2.3. Analyses

The cell number was determined by a direct counting, using a light microscope with a Malassez cell. This was used for growth analysis and to determine β-carotene content of cells.

Total pigment analysis and β-carotene isomers were measured using HPLC (Fig. 1). Ten-millilitre aliquot of cell suspension was centrifuged at 6000 rpm for 5 min. The pellet containing the cells was extracted with 5 ml of acetone and centrifuged again at 6000 rpm during 10 min. Acetone was separated under vacuum evaporation. The residue was mixed with solvent composed of 81% methanol (MeOH), 15% methyl *t*-butyl ether (MTBE) and 4% water, and analysed on a HPLC equipped with a tunable absorbance detector. A reversed-phase 250 mm × 4.6 mm C_{30} column (YMC) was used.

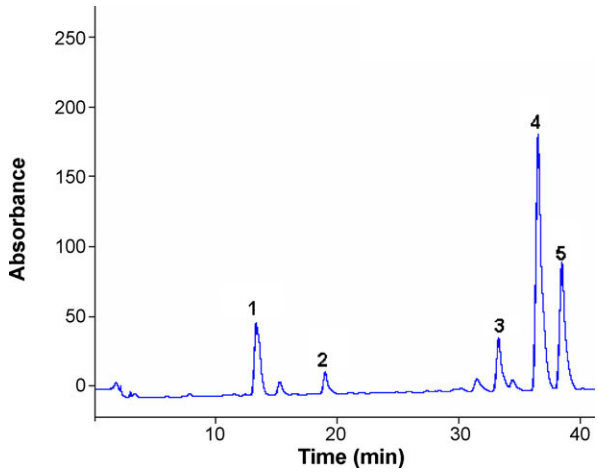

Fig. 1. HPLC separation of isomers of β-carotene extracted from carotenoids-rich cells of *Dunaliella salina* (detection at 450 nm).

Peak no.	Compound	Retention time (min)
1	Chlorophyll b	13.30
2	Chlorophyll a	18.98
3	α-carotene	33.25
4	all-trans-β-carotene	36.53
5	9-cis-β-carotene	38.45

The mobile phase consisted on: solvent A (81% MeOH, 15% MTBE and 4% H_2O) and solvent B (6% MeOH, 90% MTBE and 4% H_2O). The elution gradient was run as following: 0 min 100%A to 100%B in 90 min.

The flow rate was 1 ml min^{-1}. The detection wavelength for integration was 450 nm.

Standards of β-carotene, chlorophyll a and chlorophyll b were purchased from Sigma-Aldrich and used for calibration.

3. Results and discussion

3.1. Effect of nitrate concentration

Among the nitrate concentrations tested (Fig. 2), the highest β-carotene cellular content (35 pg/cell) was found in the nitrate-free culture. These values drastically dropped to 24 and 12 pg/cell, respectively, at the initial nitrate concentration of 1.2 and 6 mM, respectively. The control (12 mM) shows the lowest increase in β-carotene cellular content.

An increase in β-carotene cellular content, which is obtained under stress conditions, is not necessarily associated with an increase in total β-carotene concentration in the culture, due to the loss of cells when stress conditions are applied (Fig. 2b). Ben-Amotz [29] studied the properties of *Dunaliella bardawil* grown outdoors in a two-stage system and reported that volumetric productivity of β-carotene in the production stage (containing stressed cells, but in less number) was lower than during the growth stage (containing more cells but with a lower intracellular β-carotene content). Biomass concentration is thus important in terms of total productivity (Fig. 2c). The highest biomass (10^6 cell/ml) has been obtained for the maximum of nitrate concentration. This is not surprising, as nitrate plays a very important role in the cell division rate. It has been reported that under nitrogen-starved conditions, the specific activity of nitrate reductase, an enzyme responsible for assimilation of nitrate in culture medium, is very low, that greatly affects the cellular metabolism (such as the cell division) of the algae [30], resulting in extremely slow growth rates, with a possible decrease in biomass for too low concentrations of nitrate in the medium.

Analysis of pigment contents emphasizes the pigment modification when cells are placed under nitrogen deprivation. The β-carotene intracellular content reaches a maximum value

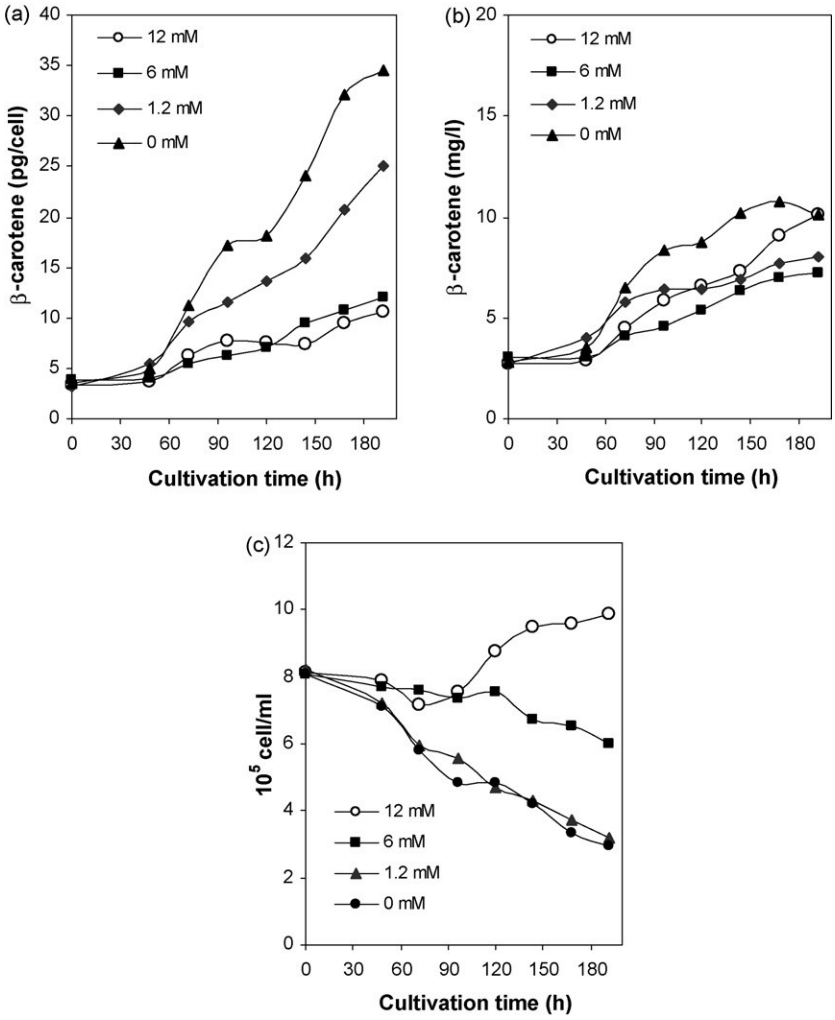

Fig. 2. (a) Evolution of β-carotene cellular content, (b) volumetric β-carotene production and (c) cellular concentration of *D. salina* as a function of cultivation time under different nitrate concentrations.

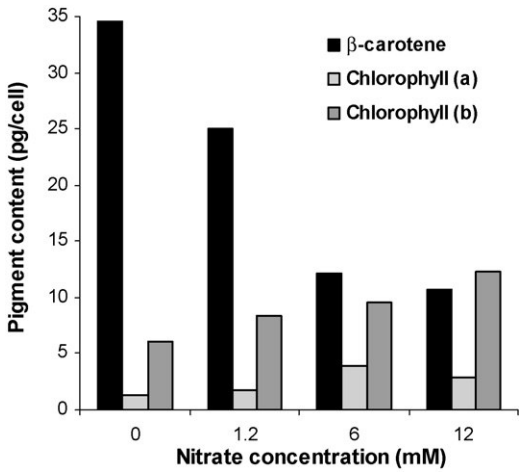

Fig. 3. *Dunaliella salina* pigment content after 8 days of batch culture as a function of various initial nitrate concentrations.

(35 pg/cell), while chlorophylls (a and b) decrease significantly (Fig. 3). Finally, a complete reorganisation of the pigment content of the cell is achieved, with a β-carotene-to-chlorophyll ratio above 4 (Fig. 4), chlorophyll cellular content being around 8 pg/cell under total nitrogen deprivation.

Despite a reduction in culture density under total nitrate deprivation, an important part of the culture survives during several days. This indicates that *D. salina* certainly presents a nitrogen intracellular store which has been reported to support cell survival [31]. Boussida and Vonshak [32] have reported that nitrate is essential for carotenoid accumulation in *H. pluvialis*, and also Orosa et al. [25] have suggested that nitrate plays a very important role in the accumulation of secondary carotenoids. Nitrate deprivation will thus have negative effects on both growth, but also on β-carotene synthesis in long-term cultivation. Despite effectiveness of this protocol in batch conditions for *D. salina*, nitrogen depletion is certainly not suitable for a continuous production. To reduce the negative influence of nitrate starvation on resulting growth, intermediate nitrate concentration can be applied, but with a significant decrease in β-carotene cellular content. In the following, an alternative protocol is investigated, based on an enrichment of the growth medium with iron and acetate or malonate.

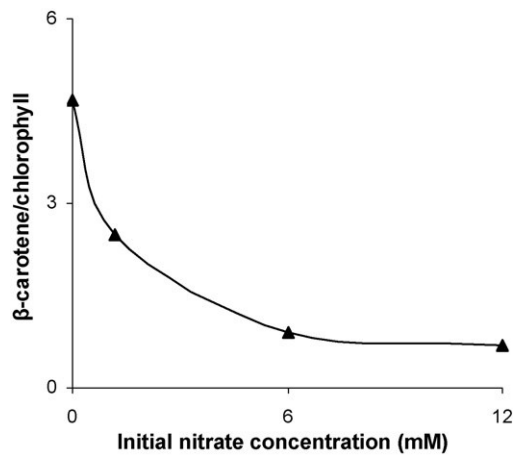

Fig. 4. β-carotene/chlorophyll ratio as a function of different nitrate concentrations after 8 days.

3.2. *Effect of iron and acetate concentration*

3.2.1. *Iron effect*

The only addition of Fe^{2+} in the basic medium at 450 μM affects greatly the growth, resulting either in a reduction of growth rate without significant β-carotene production, or causing the loss of culture after only 72 h (data not shown). Only Fe^{2+} addition with acetate gives satisfactorily results. It was then retained to investigate influence of Fe^{2+} concentration, but in the presence of a constant initial concentration of acetate. An initial acetate concentration of 45 mM was used, as established by Kobayashi et al. [23]. Kinetics of growth and β-carotene production were also measured for growth without the Fe^{2+} and acetate addition (reference conditions). The β-carotene formation was found to be drastically enhanced in the case of acetate and Fe^{2+} addition (Fig. 5a), and the cellular β-carotene produced after 192 h was found (31 pg/cell) three times higher than in the reference conditions. This important accumulation of β-carotene is certainly explained by the oxidative stress [23,33,34]. As a matter of fact, carotenoids have two important roles in photosynthetic organisms. They act as accessory light-harvesting pigment, trapping light energy and transferring it on to chlorophylls. Also, and more importantly in this case, carotenoids protect the photosynthetic apparatus from environment stress, and especially from oxidative stress caused by active oxygen species, as for example, superoxide anion ($O_2^{-\bullet}$) generated by photooxidation and hydroxyl radical ($HO^\bullet$) generated by Fenton reaction [35]. As shown by Orosa et al. [36] and Shaish et al. [37], oxidative stress, caused by intensive illumination, is an effective way for inducting accumulation of carotenoids, respectively, in *H. pluvialis* and in *D. bardawil*.

Response of microorganisms to oxidative stress is, however, not simple. Robinowitch et al. [38] have shown that the algal metabolism adapts itself to the oxidative stress not only by the accumulation of β-carotene but also by increasing the activities of catalase and SOD (superoxide-dismutase). This was observed with *D. bardawil* [37]. The underlying mechanism is represented below:

$$2O_2^{-\bullet} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \quad (1)$$

$$2H_2O_2 \xrightarrow{Catalase} 2H_2O + O_2 \quad (2)$$

High level photo-oxidation under high irradiance can lead to the generation in vivo of active oxygen molecules, such as $O_2^{-\bullet}$ which is converted by SOD into hydrogen peroxide (H_2O_2) (Eq. (1)). The H_2O_2 is next converted by catalase into water and oxygen (Eq. (2)). Shaish et al. [37] showed that the addition of azide, an inhibitor of catalase, greatly enhanced the β-carotene synthesis by *D. bardawil* that is a response against the accumulation of reactive oxygen species. Thus, intracellular β-carotene may function together with SOD and catalase (Eqs. (1 and 2)) to

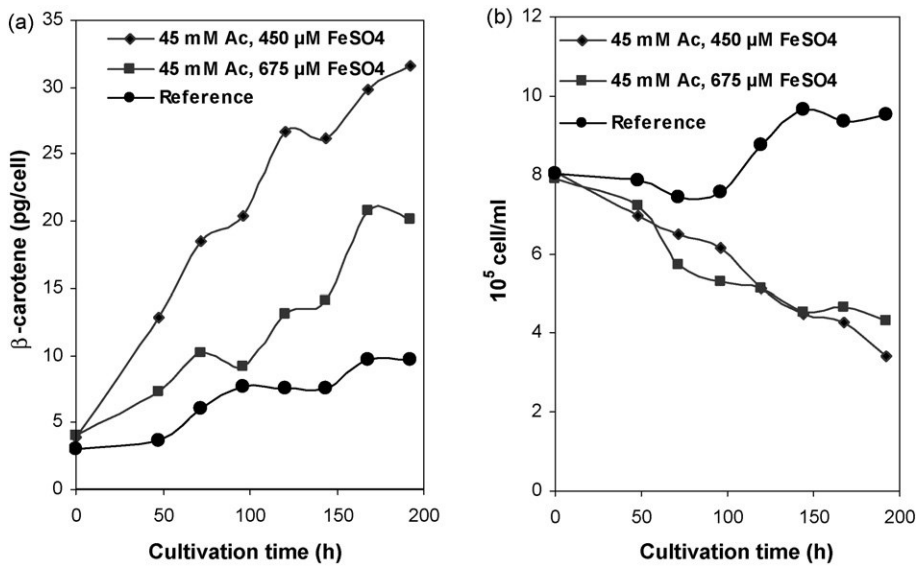

Fig. 5. (a) Evolution of β-carotene intracellular content and (b) cellular concentration of *D. salina* as a function of cultivation time under different ferrous concentrations. 12 mM of nitrate concentrations in the growth medium has been applied.

preserve viability towards the continuous production of hydroxide radicals in cells.

Fe^{2+} can interfere in this mechanism, by generating $HO^{\bullet}$ via iron-catalyzed Fenton reaction [23]:

$$Fe^{2+} + H_2O_2 \rightarrow OH^- + HO^{\bullet} + Fe^{3+} \quad (3)$$

Because this active oxygen molecule cannot be converted by SOD or catalase, this may explains the increase of β-carotene biosynthesis when Fe^{2+} is added. However, the excess Fe^{2+} addition induces stress conditions via active oxygen molecules generation, resulting in a negative effect on cell growth. This is observed in Fig. 5b where loss of biomass is observed in the cases of 450 and 675 µM Fe^{2+} additions. For 675 µM Fe^{2+}, a decrease of β-carotene content is also obtained (Fig. 5a). Salguero et al. [39] suggests a relation with chloroplastic oxygen species dissipation processes. In the case of excess addition of ferrous ions, carotenoids are unsaturated, as a protection mechanism against the overoxidative damage. Such phenomenon could explain results at 675 µM. Moreover, Bhosale [40] and Johnson [41] considered the physiological role of carotenoids in red yeasts as antioxidant in situation of high oxidative stress.

For subsequent experiments, a $FeSO_4$ concentration of 450 µM was chosen to test the effect of acetate and malonate concentration.

3.2.2. Acetate effect

The only addition of acetate in the medium at 45 mM (initial concentration) modifies the growth rate. As observed previously by Browitzka et al. [1], acetate as carbon source appears to be influent by enhancing growth. But again, metabolic response is not so simple if carotenoids accumulation is considered. Kakizono et al. [42] and Ip et al. [30] showed that excess acetate addition generate relative shortage of nitrogen, inducing carotene accumulation triggered by a high carbon/nitrogen (C/N) ratio, respectively, in *H. pluvialis* and *Chlorella Zofingiensis*. This is explained by a decrease of nitrogen consumption by cells although there is nitrogen in the culture medium [43,44]. But in our experiments, only a slight synthesis of β-carotene was observed with the addition of acetate (Fig. 6a).

Maximum cellular β-carotene content was obtained in the presence of Fe^{2+} and at the higher acetate concentration tested, in culture with 67.5 mM of acetate and 450 µM of $FeSO_4$. Up to two times more carotenoids per cell were accumulated than in culture with 45 mM of acetate and 450 µM of $FeSO_4$ (Fig. 6a), and than with the nitrogen deprivation (35 pg/cell).

The effect of acetate concentration on the β-carotene-to-chlorophyll ratios of *D. salina* is illustrated in Fig. 6d. Maximum β-carotene-to-chlorophyll ratio is observed at higher concentration of acetate (67.5 mM). This ratio could be a good indicator of the physiological state of the culture [25]. In favourable growth conditions, this value was about 0.5 (Fig. 6d). When oxidative stress is applied, a value above 12 was obtained. Though a direct comparison is difficult because of the difference in protocols and certainly in Dunaliella strain, the higher ratio obtained (192 h) is ten times higher than the values obtained by Ben-Amotz et al. [13], where *D. salina* was cultivated under continuous illumination and 4 mM of NaCl concentration. This emphasizes the potential of specific stress conditions investigated in this study. However, despite a great increase of β-carotene synthesis compared to nitrate deprivation, it can also be observed a decrease in cell density (Fig. 6b). Because acetate has generally a positive effect on growth, as observed in first 130 h of the culture with only acetate addition, such result remains to be further investigated. The relationship between acetate and Fe^{2+} addition is indeed certainly complex, both having a large influence on the overall metabolism, including growth and carotenoid synthesis. But with the great increase in β-carotene production stated in these preliminary results (Fig. 6c), it seems optimistic to find

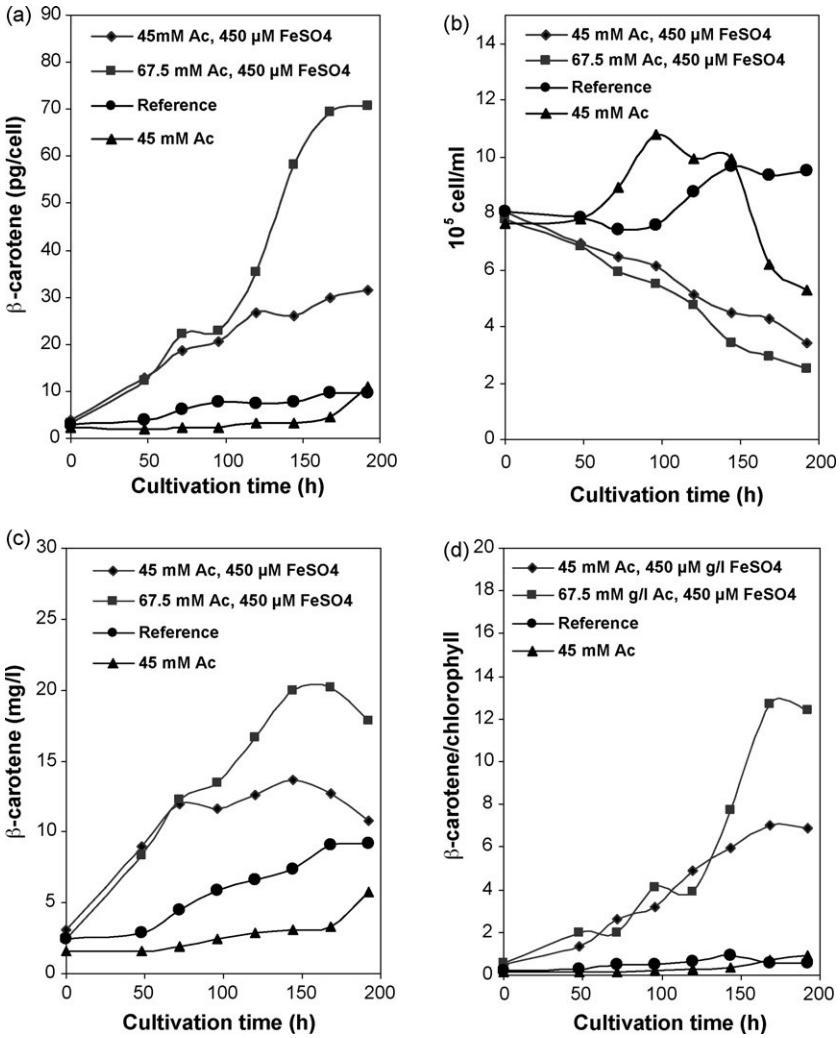

Fig. 6. (a) Evolution of β-carotene cellular content, (b) cellular concentration of *D.* salina, (c) volumetric β-carotene production and (d) carotene/chlorophyll ratio as a function of cultivation time under different acetate concentrations (Ac). 12 mM of nitrate concentrations in the growth medium has been applied.

a compromise where stress conditions based on Fe^{2+} addition will induce carotenoid accumulation, while keeping growth in a reasonable range.

3.3. *Effect of malonate and iron concentration*

Same tendencies have been observed when replacing acetate by malonate, but with a difference in kinetics, the effect being faster in case of malonate condition. Again, though lower concentrations are achieved than with acetate, the cellular β-carotene content (33 pg/cell) estimated in the culture after 120 h of addition of malonate may be considered important when compared to that of nitrogen deprivation (Fig. 7a). It must be noticed that a similar effect was observed in *P. rhodozyma*, where the addition of a carotenoid pathway precursor (mevalonic acid) was proved to have a strong effect on the accumulation of carotenoids [22]. However, an increase in β-carotene content of cells is not necessarily coupled with an increase in volumetric production of β-carotene (Fig. 7c). Others parameters such as the cell growth rate are also important (Fig. 7b).

In the case of the sole addition of malonate, an increase in cellular β-carotene concentration is observed (13.4 pg/cell after only 120 h). Those results are encouraging to find conditions involving β-carotene accumulation while keeping cell division. Compared to acetate resulting in our experiments in cellular concentration reduction, using malonate seems to be more promising. However, when Fe^{2+} is added, the cellular concentration also decreases (Fig. 7b). This confirms that organic carbon sources and Fe^{2+} ions have a complex influence on *D. salina* response that remains to be further investigated.

Table 1 summarizes the results of the present study. Additions of acetate (or malonate) and iron increase significantly the β-

Table 1
β-Carotene content of *Dunaliella salina* at different stress conditions

	β-carotene (pg/cell)
Nitrate starvation	35
67.5 mM of acetate and 450 µM of $FeSO_4$	70
67.5 mM of malonate and 450 µM of $FeSO_4$	33

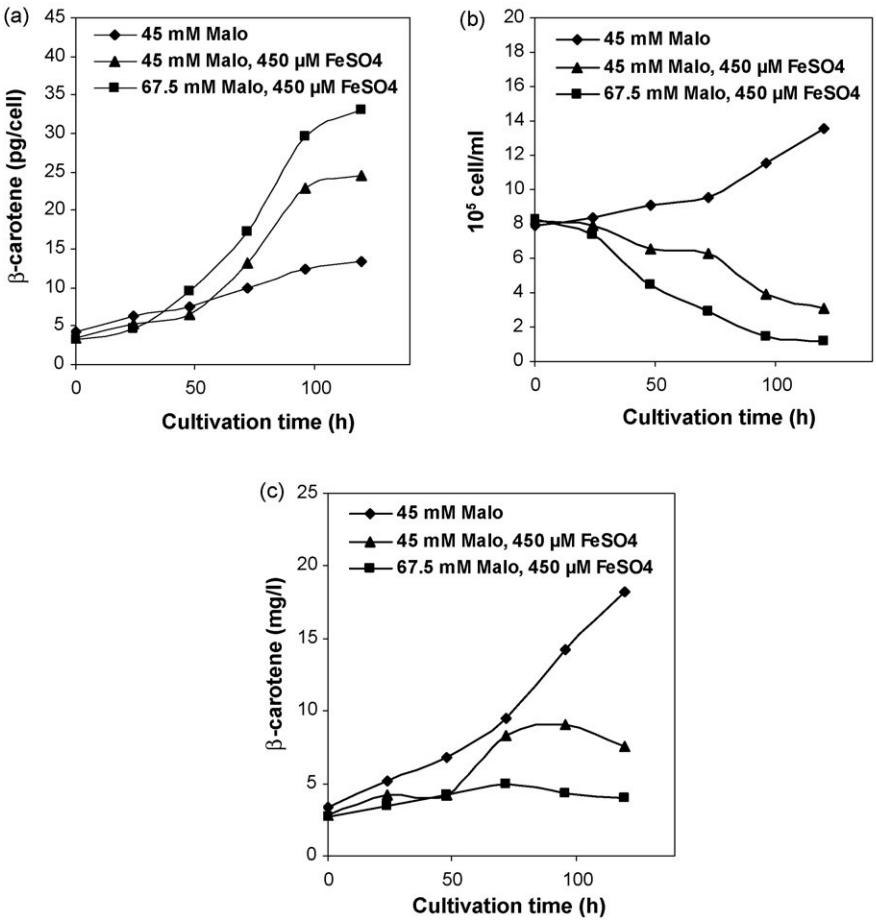

Fig. 7. (a) Evolution of β-carotene cellular content, (b) cellular concentration of *D. salina* and (c) volumetric β-carotene production as a function of cultivation time under different malonate concentrations (Malo). 12 mM of nitrate concentrations in the growth medium has been applied.

carotene content in *D. salina* when comparing to the effect of nitrogen starvation.

4. Conclusion

Adding organic carbon source in complement of Fe^{2+} appears as an interesting solution to cumulate a high intracellular β-carotene quantity. However, the physiological oxidative stress is not simple and the relationship between organic carbon source and Fe^{2+} addition is complex, both having a wide influence on the overall metabolism, possibly not restricted to growth and carotenoid synthesis. Replacing acetate by malonate, *D. salina* response was slightly modified, with both growth and β-carotene accumulation with malonate only. But, when Fe^{2+} was simultaneously added, the growth rate was not maintained and a lower concentration of cellular β-carotene content than with acetate was obtained.

The protocol based on an enrichment of the basic medium with acetate (or malonate) and Fe^{2+} appears as a promising alternative for continuous production of β-carotene by *D. salina* in intensified photobioreactor. However, it remains necessary to determine the optimum concentration of Fe^{2+} and the organic carbon source to find a good compromise between the β-carotene synthesis caused by stress condition and the growth rate that needs to be sufficient in the objective of a continuous process.

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