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RESEARCH ARTICLE

Season variability of iron effects on periodic culture of microalgae *Dunaliella viridis* Teod.

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Abstract Effects of different iron concentrations (final concentrations of iron in Artari's medium: 3.7, 37.0, 74.0, and 185.0 $mmol \cdot L^{-1}$) on growth rate and contents of protein, triacylglycerides, and β-carotene in *Dunaliella viridis* cells at cultivation in different months were investigated. It was shown that the dose-dependent effects of iron were notable for season variability. In the 1st experimental series (October, 2007), iron at researched concentrations did not affect growth rate of culture and protein, triacylglyceride, and β-carotene contents in cells. In experimental series conducted respectively in November 2007, December 2007, and February 2008, the dose-dependent stimulation of microalgae growth was observed. For each of these experimental series, there were particular dose dependences of protein, triacylglyceride, and β-carotene contents in microalgae cells at cultivation on media with iron at different concentrations. Meanwhile, for all of the four experimental series conducted in different months, variability of growth rate and analyzed parameters of microalgae *Dunaliella viridis* as control (cultivation without iron) was shown. It is suggested that these functional differences of control cultures of microalgae in different months caused variability in the dose-dependent effects of iron in a *Dunaliella viridis* culture. The possibility of iron usage for increasing microalgae biomass and for enriching it by β-carotene in *Dunaliella viridis* culture with initial low productivity and low β-carotene content is considered.

Keywords *Dunaliella viridis* Teod, iron, protein, β-carotene, triacylglyceride (TG)

1 Introduction

Iron is a cofactor of enzymes catalyzing redox reactions in animals, plants, fungi, and bacteria (Fontaine et al., 2002). Iron is involved in the reaction centers of photosynthetic electron transport in photosynthesized organisms (Paz et al., 2007), in reaction centers of Fe-hydrogenases that mediate a light driven hydrogen evolution after an anaerobic adaptation (Happe and Kaminski, 2002). In algae cells, iron steps in carotene synthesis regulation (Pascal and Dorne, 1994; Choi et al., 2002; Rick, 2006), in desaturation of fatty acids (Ivanov et al., 2007), in formation of damaging oxygen radicals (Paz et al., 2007) and apoptosis (Bidle and Bender, 2008).

The halotolerant alga *Dunaliella salina* has 150-kDa transferring-like protein involved in iron uptake. (Fisher et al., 1998; Paz et al., 2007). Internalized iron is stored in special vacuolar structures (Nagasaka et al., 2003; Paz et al., 2007; Royt et al., 2007). In these structures, iron is associated with bioorganic chelators (Royt et al., 2007). These iron-storing vacuolar structures are shown to play an important role in the formation of tolerance of alga cells to aluminum (Nagasaka et al., 2002).

These data suggest that iron deficiency can cause considerable disturbances of microalgae metabolism and decrease of their productivity by mass cultivation technology. However, the biological effects of iron excess (concentrations exceeding the physiological necessary levels) in microalgae cultures that are biotechnological object are not studied. Judging from the literature, one may assume that iron excess in a culture medium of microalgae can cause alterations in lipid and carotenoid metabolism and intracellular iron accumulation in the form of chelate organic compounds. In prospect, microalgae biomass can be used as a biologically active addition that normalizes iron homeostasis in organisms of humans and animals.

In the current study, the effects of iron at different concentrations in a culture medium on growth rate and the contents of iron, protein, lipids, and β-carotene in *Dunaliella viridis* Teod. cells, which were used in different technologies of mass cultivation, were investigated.

In recent studies of *Dunaliella viridis* Teod, periodic culture seasonal variability in growth rate and biomass composition at cultivation under standard conditions was

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shown (Menzyanova, 2002). One may assume that seasonal features of the functional state of a *Dunaliella viridis* culture (season functional epigenotype) can determine the specificity of the biological effects of iron in a microalgae culture. In other words, the effects of different concentrations of iron on growth rate of culture and biomass composition can depend on the initial functional state of microalgae culture and can differ essentially during a calendar year.

To reveal possible seasonal variability of iron effects, different experimental series were conducted in October 2007 (the 1st series), November 2007 (the 2nd series), January 2008 (the 3rd series), and March 2008 (the 4th series).

2 Materials and methods

The experiments were conducted with algologically pure culture of *Dunaliella viridis* Teod. The microalgae was cultivated on standard Artari's medium at round-the-clock illumination, T= 23°C–24°C. Iron ($FeCl_3 \cdot 5H_2O$) was added to the experimental culture at the moment of passage to final concentrations of 3.7, 37.0, 74.0, and 185.0 mmol·L^{-1}. On the 21st day of cultivation, cells were collected by centrifugation (3000 g/min). In cell biomass contents of proteins, lipids and β-carotene were determined (Bozhkov and Menzyanova, 1995). For iron content determination, biomass samples after extraction of pigments and fats were digested with the mix of acids (H_2SO_4:HCl:HNO_3, 1:1:1). Concentration of iron in the remaining digest solution was determined by using atomic absorptive spectrophotometer ("Saturn", Ukraine, Donetsk).

Experiments were conducted in October 2007 (the 1st series), November 2007 (the 2nd series), January 2008 (the 3rd series), and March 2008 (the 4th series), respectively.

3 Results

3.1 Effects of different iron concentrations on microalgae culture growth

Dose dependence of the growth rate of microalgae culture on iron concentration in the medium was not the same in different experimental series (Fig. 1). In the 1st experimental series, addition of 3.7, 37.0, 74.0, or 185.0 mmol·L^{-1} of iron into the cultural medium did not change the microalgae growth rate; on the 21st day of cultivation, cell concentration was the same as in the control.

In the 2nd experimental series, the dose-dependent stimulation of microalgae culture growth was found. The addition of 37.0, 74.0 and 185.0 mmol·L^{-1} of iron was accompanied by an increase in cell number in a 21-day-old culture by 1.3, 1.6, and 1.4 times, respectively, as compared with the control. The stimulating effects of different iron concentrations (37.0, 74.0, and 185.0 mmol·L^{-1}) were the same. It was observed that the curve of dose dependence reached a plateau between 37.0 mmol·L^{-1} and 185.0 mmol·L^{-1}.

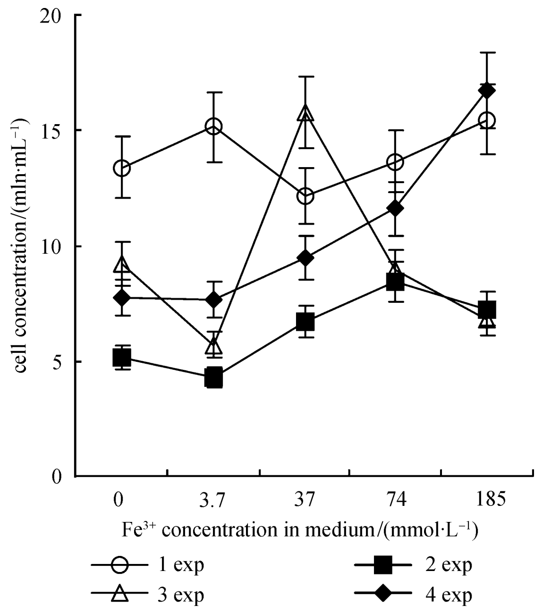

Fig. 1 *Dunaliella viridis* cell concentration (mln·mL^{-1}) on the 21st day of growth on standard Artari's medium and on media with iron at different concentrations. The means of four independent experiments and their standard errors are shown. exp: experimental series.

In the 3rd experimental series, considerable growth-stimulating effect was noted for 37.0 mmol·L^{-1} of iron: the cell number in 21-day-old culture in this variant was 1.7 times more compared with control. A variant with 74.0 mmol·L^{-1} of iron did not change the growth rate, and 185.0 mmol·L^{-1} of iron inhibited the growth of the culture (1.4 times less compared with the control).

In the 4th experimental series, there was the other dose dependence. Introduction of 74.0 and 185.0 mmol·L^{-1} iron was accompanied by a 1.5-fold and a 2.2-fold increase, respectively, in the number of algae cells in the 21-day-old culture compared with the control.

Thus iron effects on microalgae growth rate showed seasonal variability.

The most obvious stimulation on microalgae culture growth was found in the 2nd, 3rd, and 4th experimental series for different iron concentrations (37.0, 74.0, and 185.0 mmol·L^{-1}). One may assume that the variability of iron effects can be caused by no similar growth rate of the control culture in four experimental series. In the 1st experimental series, the growth rate of control culture was the highest and the introduction of iron to the medium neither stimulated nor inhibited the growth of experimental variants. In the 2nd, 3rd, and 4th experimental series, the growth rate of the control culture was lower than that in the 1st experimental series: the cell number on the 21st day of growth was 2.6, 1.5, and 1.7 times less, respectively, than that in the 1st experimental series (Fig. 1). In cultures with a low growth rate, the dose-dependent growth-stimulating effects of different iron concentrations were found.

In other words, in cultures with initially low productivity, iron introduction increased biomass yield considerably. In cultures with initially high productivity, iron did not affect biomass yield. This allows using iron to increase biomass yield of *Dunaliella viridis* in periods of seasonal decrease in microalgae growth rate.

One may assume that iron effect on growth rate of *Dunaliella viridis* was determined by the initial functional state of culture in different seasons of one year. For microalgae *Dunaliella viridis*, the functional state of the culture to a great extent depends on cell population composition: the ratio of vegetative haploid gametophytes, diploid zygotes, and polyploid cysts (Masyuk, 1973). Variability of iron effects can be related to features of "response" reactions of different cell subpopulations of *Dunaliella viridis*.

The features of functional state of microalgae cell population can cause stimulation of intracellular iron accumulation, which in turn can affect the process of proliferation of microalgae cells and determine culture growth rate.

One may assume that iron effects on the growth rate of microalgae are related to the activity of intracellular iron binding. In this study, we determined the iron content in microalgae cells after introduction of different amounts of iron in culture medium and analyzed the correlation between growth rate and iron content in cells.

3.2 Iron content in microalgae cells on the 21st day of cultivation on media with iron at different concentrations

Cultivation of *Dunaliella viridis* on a medium with an initial iron concentration of 3.7 mmol·L^{-1} did not cause an increase in the iron content in microalgae cells compared to the control (Fig. 2). At the same time, dose-dependent accumulation of iron in microalgae cells on media with 37.0, 74.0, and 185.0 mmol·L^{-1} of iron was found. It is worth mentioning that in different experimental series, dose dependence had some particularities, and activity of intracellular iron binding (after introduction of iron with the same concentration) varied considerably (Fig. 2).

In the 2nd and 3rd experimental series on cultivation on medium with an initial iron concentration of 37.0 mmol·L^{-1} microalgae, cells differed considerably in iron content (by 16.6 times).

Meanwhile, in the 2nd experimental series, elevation of iron concentration in the medium (37.0–74.0 mmol·L^{-1}) did not cause changes in iron content in microalgae cells. Further elevation of iron concentration in the medium to

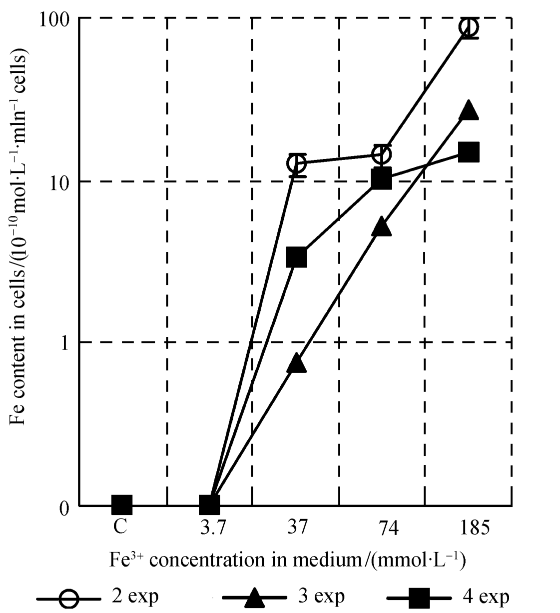

Fig. 2 Iron content (10^{-10} mol·L^{-1}·mln^{-1} cells) in *Dunaliella viridis* cells on the 21st day of growth on standard Artari's medium (C) and on media with iron at different concentrations. exp: experimental series.

185.0 mmol·L^{-1} caused secondary increase in iron content in cells (by 6.1 times compared to 74.0 mmol·L^{-1}).

In the 3rd experimental series, a twofold elevation of initial iron concentration in the medium (37.0 mmol·L^{-1} and 74.0 mmol·L^{-1}) caused a sevenfold increase in iron content in cells. Further, a 2.5-fold elevation of iron concentration in the medium (74.0 mmol·L^{-1} and 185.0 mmol·L^{-1}) caused an increase in the iron content in cells by 5.2 times.

Thus, at the same iron concentrations in a culture medium, intracellular iron binding in microalgae cells varied considerably (Fig. 2). One of the reasons (or consequence) of such variability of intracellular iron content could be different growth rates of the culture. Analysis of the correlation between intracellular iron content and growth rate of culture (cell concentration on the 21st day of growth) revealed dissimilarity of relationships between these parameters (Fig. 3).

Cultivation on the medium with iron at initial concentrations of 37.0 mmol·L^{-1} and 185.0 mmol·L^{-1} found an inverse relationship between growth rate and iron content in cells on the 21st day of cultivation (Fig. 3)—decrease of culture growth rate was accompanied by an increase in iron content in cells. Whereas cultivation on the medium with iron at an initial concentration of 74 mmol·L^{-1} found no relationship between these parameters (Fig. 3B).

The obtained results suggested a "dose-dependent" pattern of intracellular iron metabolism, its influence on different metabolic pathways, which finally led to variability of such integral parameter as growth rate of

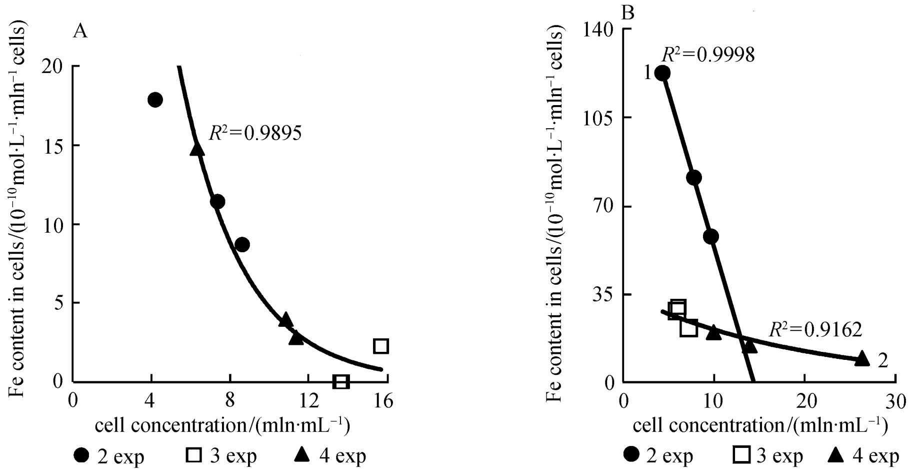

Fig. 3 Relation of iron content (10^{-10} mol·L^{-1}·mln^{-1} cells) in *Dunaliella viridis* cells to growth rate of culture (cell concentration on the 21st day of growth) on media with iron at different concentrations. exp: experimental series. A: with an initial concentration of 37 mmol·L^{-1}; B: with an initial concentration of 185 mmol·L^{-1}. On Fig. A trend line with exponential approximation. On Fig. B trend with linear (1) and exponential (2) approximation. R^2: measure of reliability of approximation.

microalgae culture. On the other hand, variability of iron effects on the level of growth rate of culture may depend on morphofunctional characteristics of microalgae culture (ratio of cell subpopulations) in different experimental series.

In different experimental series, we also determined the contents of protein, β-carotene, and lipids in microalgae cells in the control and analyzed dose dependence of these parameters at cultivation on media with iron at different concentrations.

3.3 Protein content in microalgae cells at cultivation on media with iron at different concentrations

In the 4th experimental series, the protein content in microalgae cells in the control differed significantly (Fig. 4). The protein content in cells of the 2nd and 4th experimental series was almost 13-fold different. These data suggested essential differences in protein synthesis activity in microalgae cells in different experimental series. It is possible that these differences caused the formation of differing dose dependences in protein content in cells at the cultivation of microalgae on media with iron at different concentrations. Meanwhile, in the 1st and 3rd experimental series, introduction of 3.7 mmol·L^{-1} and 185 mmol·L^{-1} of iron in culture medium , respectively, did not change the protein content in microalgae cells.

In the 2nd experimental series (the lowest protein content in the control), the introduction of iron at different concentrations in culture medium was accompanied by an increase in protein content in cells. Considerable increase in protein content (1.9-fold compared with the control) was found after an introduction of 3.7 mmol·L^{-1} iron. After introduction of 74 mmol·L^{-1} of iron, protein content in microalgae cells was maximal (3.8-fold compared with control). Further elevation of iron concentration in the medium to 185 mmol·L^{-1} did not cause changes in protein content (Fig. 4).

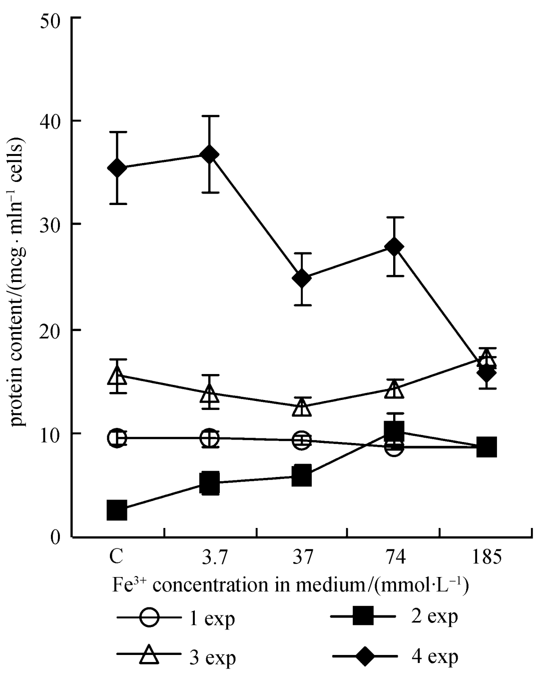

Fig. 4 Protein content (mcg·mln^{-1} cells) of *Dunaliella viridis* cells at growth on standard Artari's medium (C) and on media with iron at different concentrations in the 21-day-old culture. The means of four independent experiments and their standard errors are shown. exp: experimental series.

In the 4th experimental series, protein content in the control was the highest, and introduction of iron at different concentrations caused dose-dependent decreases in protein content in microalgae cells. The curve of dose dependence showed a "stair like" shape (Fig. 4).

Introduction of 3.7 mmol·L^{-1} iron did not change the protein content in cells compared with the control. Introduction of 37.0 and 74.0 mmol·L^{-1} iron decreased the protein content by 1.4 and 1.3 times compared with the control, respectively. Elevation of initial iron concentration to 185 mmol·L^{-1} was accompanied by a decrease in protein content by 2.2 times compared with the control.

The results showed that there were three variants of iron effects at the research concentrations (from 3.7 to 185.0 mmol·L^{-1}) on protein content in microalgae cells. These variants were determined by the activity of protein storage in cells during growth (21 days). In microalgae cultures without notable storage of protein in cells, the introduction of iron caused a dose-dependent increase in protein content in cells (the 2nd series). In microalgae cultures that stored considerable protein content, the introduction of iron caused a dose-dependent decrease in protein content in cells (the 4th series). In cultures with "intermediate" activity of protein storage during growth (the 1st and the 3rd series), the introduction of iron at different concentrations did not change the protein content in cells.

The revealed effects could be related both to the existence of Fe-dependent elements in protein synthesis system and to the changes in protein synthesis activity during intracellular iron accumulation. Indeed, after introduction of 185.0 mmol·L^{-1} of iron to culture medium, it showed an inverse relationship between protein content and iron content (Fig. 5). Therefore, accumulation of iron to 75×10^{-10} mol·L^{-1}·mln^{-1} cells was accompanied by a decrease in protein content by almost five times. Further accumulation of iron in cells did not change protein content significantly. After an addition of 37.0 mmol·L^{-1} and 74.0 mmol·L^{-1} of iron, the protein content and iron content in microalgae cells did not correlate.

A dissimilar relationship between growth-stimulating effects of iron, activity of intracellular iron accumulation, and protein content was found. Therefore, in the 4th experimental series, Fe-dependent growth stimulation and intracellular iron accumulation were accompanied by a decrease in protein content in cells. In the 2nd series, the highest Fe-dependent growth stimulation (74.0 mmol·L^{-1}) was accompanied by an increase in protein content in cells. In the 3rd series, a significant growth-stimulating effect of iron (37.0 mmol·L^{-1}) did not change the protein content in cells.

One may assume that such variability of iron effects on protein synthesis is related to seasonal functional differences (season functional epigenotypes) of microalgae culture in different experimental series.

At the following research stage, the effects of different iron concentrations on secondary metabolism (triacylglyceride (TG) and β-carotene) of microalgae were analyzed.

3.4 TG content in microalgae cells at cultivation on medium with iron at different concentrations

The TG content in microalgae cells on the 21st day of cultivation differed considerably in controls of four experimental series. The TG content differed by threefolds at the 1st and 2nd experimental series (Fig. 6). Differences in the activity of intracellular storage of TG suggested that microalgae cultures in four experimental series had different levels of lipid metabolism. Consequently. dose-dependent iron effects on lipid metabolism of microalgae varied significantly.

Thus, in microalgae culture with high levels of TG storage during growth (the 1st series), 185.0 mmol·L^{-1} of iron decreased TG content by 1.3 times. Lower iron concentration did not change the TG content (Fig. 6).

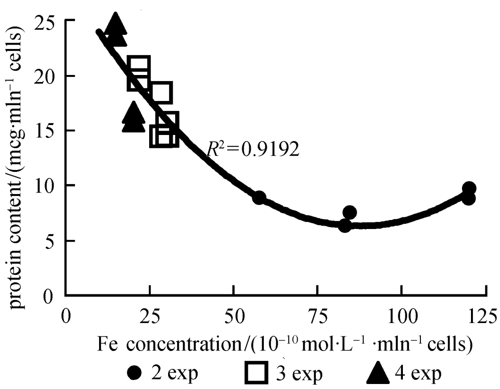

Fig. 5 Relationship of protein (mcg·mln^{-1} cells) in *Dunaliella viridis* cells to the concentration of iron in cells (10^{-10} mol·mln^{-1} cells) at cultivation in the medium with 185 mmol·L^{-1} iron in the 21-day-old culture. R^2: measure of reliability of approximation. exp: experimental series.

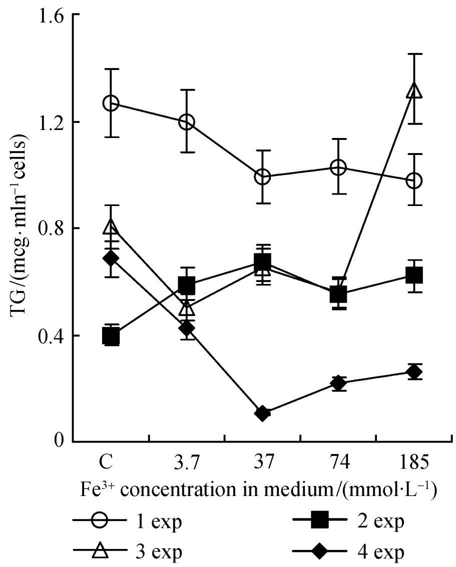

Fig. 6 Triacylglyceride (TG) content (mcg·mln^{-1} cells) in *Dunaliella viridis* cells on the 21st day of cultivation on standard Artari's medium (C) and on media with iron at different concentrations. The means of four independent experiments and their standard errors are shown. exp: experimental series.

In the microalgae culture with low activity of TG storage (the 2nd series) during growth, iron addition (at all concentrations) increased the TG content by 1.4–1.7 times. The TG content was almost the same after the addition of iron at 3.7, 37.0, 74.0, and 185.0 mmol·L^{-1}.

In the 3rd and 4th series, dose curves of TG content had "U-liked" shape: low iron concentration decreased TG content in cells, and higher ones caused secondary increase in TG content in cells.

Thus, in the 3rd series. 3.7 mmol·L^{-1} of iron decreased the TG content by 1.6 times, whereas 185.0 mmol·L^{-1} of iron increased its content by 1.6 times (compared to the control). In the 4th series, the "hypolipidemic iron effect" was more apparent: 37.0 mmol·L^{-1} of iron decreased TG content in microalgae cells by 6.3 times compared to the control. Further elevation of iron concentration caused repeated increase in the TG content. It is worth mentioning that the relation between the activity of TG storage and iron content in cells was found only for 74.0 mmol·L^{-1} of iron: iron accumulation accompanied by an increase in the TG content in cells (Fig. 7).

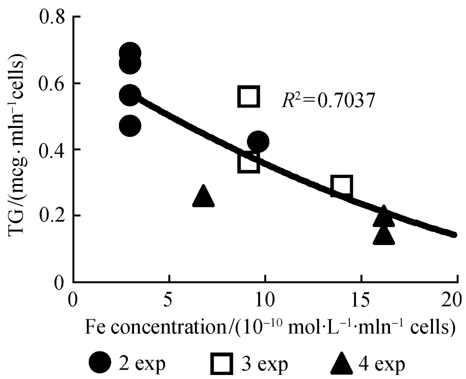

Fig. 7 Relation of the triacylglyceride (TG) content (mcg·mln^{-1} cells) in *Dunaliella viridis* cells to the iron concentration (10^{-10} mmol·L^{-1}·mln^{-1} cells) in cells at cultivation on medium with 74 mmol·L^{-1} iron. R^2: measure of reliability of approximation. exp: experimental series.

Activity of β-carotene storage in microalgae cells during growth in control variants in the 4th experimental series differed essentially. In the 2nd series, the β-carotene content in cells on the 21st day of cultivation was 26 times lower than that in the 1st series. The differences between carotene contents in the 1st, 3rd, and 4th series were not significant (Fig. 8).

The effect of different iron concentrations on β-carotene content depended on the initial activity of β-carotene storage in microalgae cells (Fig. 8). In cultures with high activity of β-carotene storage iron, addition of iron did not affect β-carotene content in microalgae cells (the 1st series).

Apparent dose-dependent activation of β-carotene storage after iron addition was shown in the 2nd series.

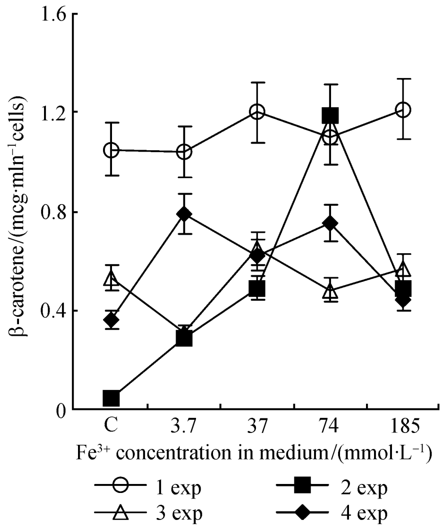

Fig. 8 β-carotene content (mcg·mln^{-1} cells) in *Dunaliella viridis* cells on the 21st day of growth on standard Artari's medium (C) and on media with iron at different concentrations. The means of four independent experiments and their standard errors are shown. exp: experimental series.

Iron at 74.0 mmol·L^{-1} increased the β-carotene content by 29 times compared to the control. Addition of 185.0 mmol·L^{-1} of iron caused secondary decrease in β-carotene content in microalgae cells.

In the 3rd experimental series, Fe-dependent activation of β-carotene storage was not found. Furthermore, 3.7 mmol·L^{-1} of iron decreased the β-carotene content in microlgae cells.

In the 4th series, the introduction of 3.7, 37.0, and 74.0 mmol·L^{-1} iron caused the increase in β-carotene in cells to almost the same extent (1.7–2.0 times) compared to the control. Iron at 185.0 mmol·L^{-1} caused a secondary decrease in β-carotene content (Fig. 8).

Thus, iron is proposed to be an inductor of β-carotene storage in microalgae cultures with initial low level of its storage. In cultures of microalgae with high levels of β-carotene storage, iron effects were less pronounced or absent (Fig. 8).

The pattern of correlation between the activity of β-carotene storage and accumulation of iron in cells depended on iron concentration in the medium of cultivation. Therefore, after iron introduction at 37.0 mmol·L^{-1}, the activity of β-carotene storage and iron content in cells did not correlate (data not shown). At 74.0 mmol·L^{-1} of iron concentration, the direct relationship between the β-carotene and iron contents in cells was found.

At 185.0 mmol·L^{-1} of iron concentration, these parameters were related inversely (Fig. 9).

Such variability of relations suggested the dose particularity of iron effects on microalgae metabolism. Iron effects on β-carotene content in cells may be caused

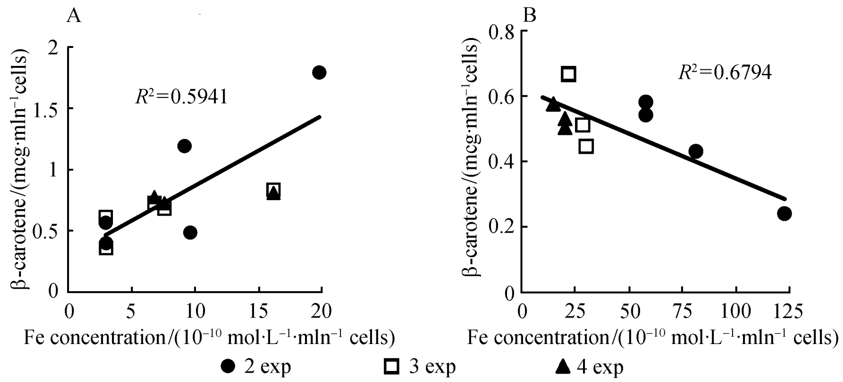

Fig. 9 Relation of β-carotene (mcg·mln^{-1} cells) in *Dunaliella viridis* cells to iron content (10^{-10} mol·L^{-1}·mln^{-1} cells) in cells at cultivation on media with iron at different concentrations. A: with initial concentration of 74 mmol·L^{-1}; B: with initial concentration of 185 mmol·L^{-1}. R^2: measure of reliability of approximation. exp: experimental series.

by regulatory effects of iron on carotenogenesis and formation of metabolically inert pool of β-carotene. On the other hand, iron effects could be mediated by Fe-dependent alterations in microalgae cell population (increase in cell numbers with high level of β-carotene storage).

Metabolic cycles of carotenoids and TG in cells of microalgae *Dunaliella* are known to be related closely (Rabbani et al., 1998). In our experiments, a direct relationship between β-carotene and TG contents was found in control microalgae culture (on standard Artari's medium). However, after introduction of iron at different concentrations in the culture medium, there was no relation between these parameters (Fig. 10). Disappearance of this relation (TG and β-carotene) is considered a result of formation of Fe-dependent functional epigenotypes of microalgae with particular systems of TG and carotenoids metabolism regulation.

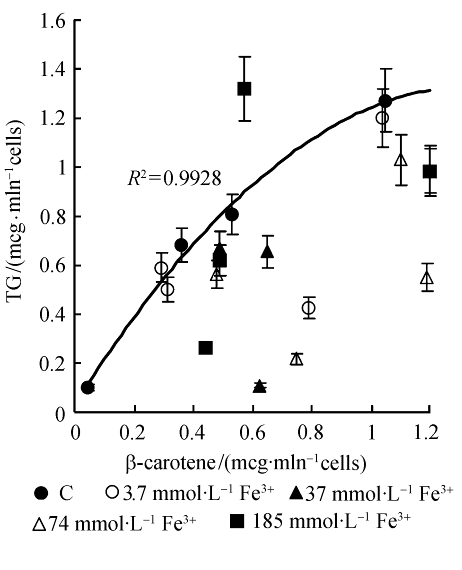

Fig. 10 Relation of triacylglyceride (TG) content to β-carotene content in the cells of the 21-day-old *Dunaliella viridis* culture. C: control culture (standard Artari's medium). R^2: measure of reliability of approximation.

Thus, under conditions of periodic cultivation on standard Artari's medium in different seasons, the variability of growth rate, protein content, TG, and β-carotene in *Dunaliella viridis* cells was revealed. The reasons for such seasonal variability under standard conditions of periodic cultivation have been discussed in detail (Bozhkov et al., 2008). Season features of functional activity of microalgae cause variability in iron effects on growth rate and activity of primary and secondary metabolism.

Particular ratios of growth rate, protein content, and contents of TG and β-carotene in microalgae cells after iron introduction in culture medium in different experimental variants may be considered as a result of formation of specific Fe-dependent epigenotypes. It is worth mentioning that a variety of Fe-dependent functional epigenotypes of microalgae depended not only on different iron concentrations in culture medium. Variability of Fe-dependent epigenotypes was found also on media with the same iron concentration and was related to season culture features. The petal diagrams illustrate well "dose" and "intradose" variability of Fe-dependent functional epigenotypes of microalgae (Fig. 11).

4 Conclusions

(1) Iron effects on growth rate, contents of protein, TG, and β-carotene in *Dunaliella viridis* cells depended on the seasonal features of the culture in different experimental series. (2) In cultures with high growth rate, iron introduction did not affect growth rate, contents of protein, TG, and β-carotene in cells. (3) Under conditions of seasonal decrease in microalgae growth rate, iron introduction in culture medium caused dose-dependent stimulation of growth and increase in β-carotene content. (4) The rate of intracellular iron accumulation was notable for season variability.

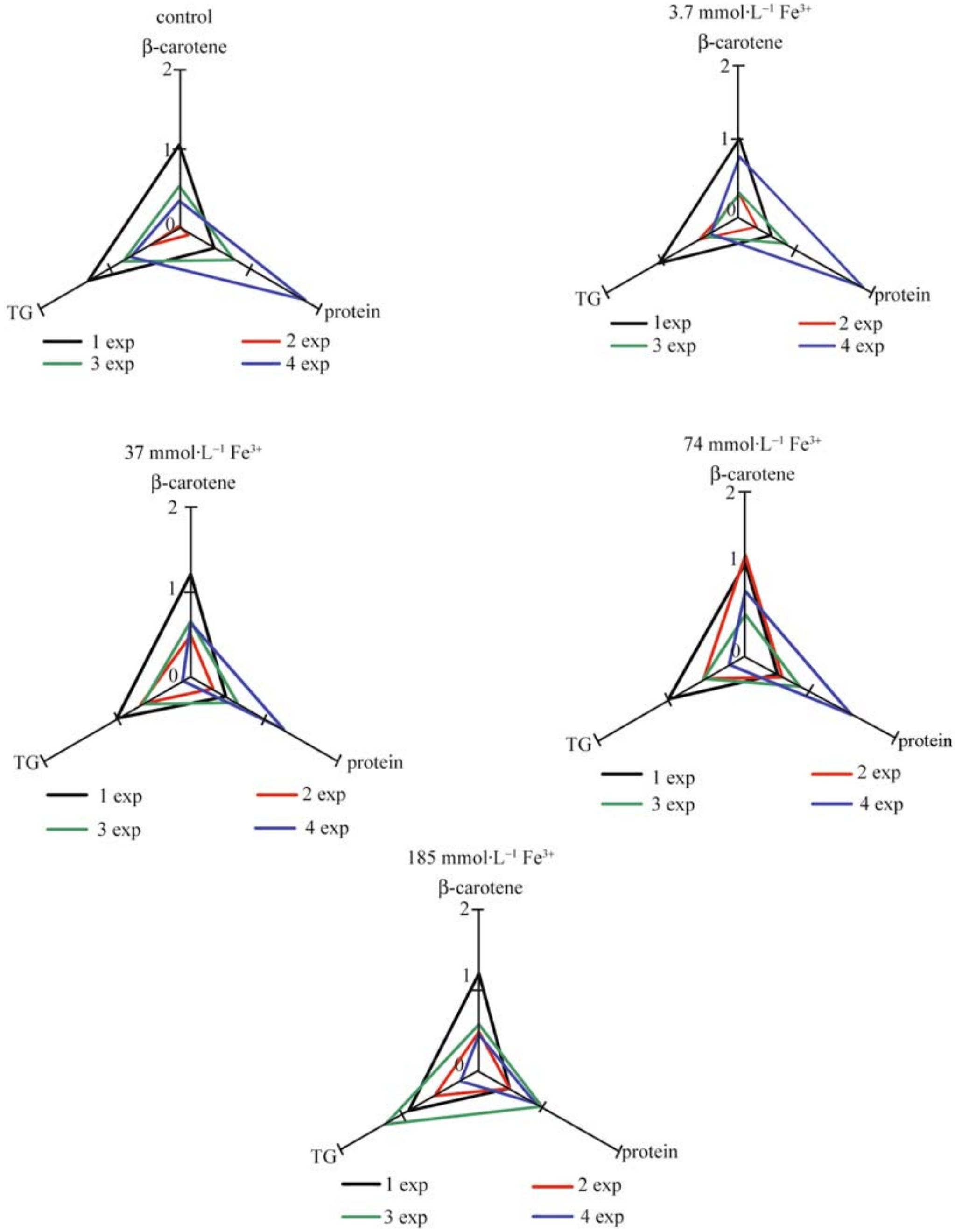

Fig. 11 Variability of functional epigenotypes of *Dunaliella viridis* at cultivation on standard Artari's medium (control) and after the addition of iron at different concentrations. Functional epigenotype was determined as a relation of triacylglyceride (TG), β-carotene, and protein content in microalga cells. On petal diagrams, results of 4 independent experiments are presented. exp: experimental series.

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