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Nitrate and iron nutrition effects on some nitrate assimilation enzymes and metabolites in *Spirulina platensis*

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Abstract: Effects of different sodium nitrate and iron concentrations on the production of some metabolites and pigments and the activities of some nitrate assimilation enzymes [nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS), and glutamate synthase (GOGAT)] were investigated in the growth medium of *Spirulina platensis* (Gamont) Geitler. For this purpose, *S. platensis* was cultivated on Zarrouk's medium containing different sodium nitrate concentrations (10–180 mM). A 100 mM sodium nitrate concentration stimulated growth of this organism as well as the production of pigments and other metabolites and enzyme activities. In the presence of 100 mM sodium nitrate, different concentrations of iron (10–100 µM) were tried in the growth medium of *S. platensis*. The highest enzyme activities were determined in the presence of 100 mM sodium nitrate and 50 µM iron. The highest NR, NiR, GS, and GOGAT activities obtained were 126.92 ± 9.2 U mL^{-1}, 841.16 ± 61.4 U mL^{-1}, 0.1301 ± 0.02 U mL^{-1}, and 46.18 ± 1.8 U mL^{-1}, respectively. These high enzymatic activities may stimulate high amino acid production. Higher enzyme activity may result in higher nutritional value in *S. platensis*, which has wide usage in biotechnology, industry, and biochemistry.

Key words: *Spirulina platensis*, iron, nitrate, nitrate reductase, nitrite reductase, glutamine synthetase, glutamate synthase

1. Introduction

Cyanobacteria are photosynthetic bacteria that use sunlight as an energy source, water as an electron donor, and carbon dioxide as a carbon source to produce food. They use oxygen to support life and usually live in fresh or marine water. They produce organic material and thus they are known as primary producers (Ozturk Urek and Tarhan, 2011; Tian et al., 2014). The cyanobacterium *Spirulina platensis* is a filamentous, nitrate-utilizing, nonnitrogen-fixing, photosynthetic organism that is rich in pigments such as chlorophyll a, carotenoids, and phycobiliproteins. These properties make it very important in nutritional, industrial, and environmental biotechnology (Tarko et al., 2012). It is a healthy food and also has defensive properties against viral diseases and anemia. It is used as a colorant because of its rich pigment content (Henrikson, 2010; Singh et al., 2014). Antioxidant properties and the high vitamin, protein, carbohydrate, and mineral values of *S. platensis* inhibit tumor growth and malnutrition. *S. platensis* is rich in protein content and contains 60%–70% protein by dry weight (Jha et al., 2007), including 9 essential amino acids that are considered high-quality protein (Belay, 2008). Nitrogen is an essential element due to nitrogen assimilation or incorporation of the most important functional and structural macromolecules in organisms, such as amino acids. Nitrogen is the most important element due to its approximately 10% contribution to cyanobacterium cells (Perez-Garcia et al., 2011), with 15% of this rate composed of incorporated proteins and nucleic acids (Inokuchi et al., 2002).

Minerals are the other vital members involved in the growth and enzymatic activities of *S. platensis* (Tarko et al., 2012). The growth and metabolic activity of cyanobacteria may be limited by a variety of these minerals. They affect the growth and metabolic activity of *Spirulina* (Ozturk Urek and Tarhan, 2011). One of the most important elements for the growth of *Spirulina* is iron (Fe). It is a limiting factor for pigment production (chlorophyll a). Investigations into the relationship between chlorophyll a biosynthesis and iron show that iron deficiency causes chlorosis. It decreases chlorophyll a and heme production and represses porphyrin biosynthesis (Belkhodja et al., 1998; Briat et al., 2007; Reinbothe et al., 2010). Additionally, Fe is a significantly important element for nitrogen assimilatory enzymes. The structures of the enzymes consist of Fe as cofactor. Cubic clusters of Fe and

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S constitute a very common type of cofactor in nitrogen assimilatory proteins. Nitrate reductase (NR) binds 1 [4Fe-4S] cluster and 1 molybdopterin cofactor with cysteines (Glass et al., 2009). Nitrite reductase (NiR) is an iron-rich protein containing 1 [4Fe-4S] cluster and 1 heme. The center of the heme group also contains 1 Fe atom (Milligan and Harrison, 2000). Glutamate synthase (GOGAT) is one of the nitrogen assimilatory enzymes containing a [4Fe-4S] cluster in its structure (Glass et al., 2009).

Cyanobacteria can assimilate different kinds of nitrogen sources, mainly nitrate, urea, ammonia, amino acids, peptone, yeast extract, and purines (Perez-Garcia et al., 2011). Nitrate is the available form of nitrogen in nature and is most widely used by cyanobacteria (Herrero et al., 1986). Nitrate assimilation requires large amounts of carbon, protons, and energy. Nitrate assimilation in cyanobacteria consists of 3 basic steps (Perez-Garcia et al., 2011): 1) nitrate is transported into the cytoplasm by its specific transporter, 2) nitrate ions are reduced to ammonium ions by catalyzing 2 enzymes as NR (EC 1.7.7.2) and NiR (EC 1.7.7.1), and 3) ammonium ions incorporate into carbon compounds as the α-amino group of L-glutamate in the glutamine synthetase (GS; EC 6.3.1.2)/GOGAT (EC 1.4.1.14) pathway (Devriese et al., 2001). The first enzyme of nitrate assimilation is NR, which catalyzes the nitrate reduction. Reduced pyridine nucleotides are used as electron donors for the reaction (Shah, 2008). NiR reduces nitrite to ammonium. Ferredoxin is used as an electron donor in the reaction and transfers 6 electrons (Perez-Garcia et al., 2011). GS is an enzyme that catalyzes the assimilation of ammonium into glutamine. Sequentially, GOGAT catalyzes transamidation of glutamine-amido nitrogen to 2-oxoglutarate to form 2 moles of glutamate (Nalbantoğlu, 2000; Sood et al., 2002). Assimilation of nitrogen into macromolecules such as amino acids and proteins requires carbon components in the carbon cycle. Carbon and nitrogen metabolisms interact with each other in cyanobacteria (Inokuchi et al., 2002; Perez-Garcia et al., 2011). They use carbon supplied from assimilated organic carbon by heterotrophic growth or respiration of fixed CO_2 by autotrophic growth. They also share the energy produced in the mitochondrial electron transport chain and TCA cycle. Cyanobacteria need carbon compounds in the form of keto acids (oxaloacetate and 2-oxoglutarate) and ATP and NADH for energy to produce the amino acids in the nitrate assimilation processes. In both autotrophic and heterotrophic growing cells, ATP, NADH, and keto acids are obtained from the TCA cycle (Fernandez and Galvan, 2007).

Nitrate assimilation is a very important metabolic pathway. The process causes biosynthesis of glutamine and glutamate amino acids. These amino acids have biochemical and industrial importance. For example, glutamate and glutamine are used as additives in the food industry. Recently, enzyme activities in nitrogen assimilation and their characteristics were investigated in many plants and some cyanobacteria (Sood et al., 2002; Silveira et al., 2003; Sellers et al., 2004). Nitrate assimilation in the *S. platensis* (Gamont) Geitler organism is still a matter of curiosity. The aim of this study was to investigate the effects of different nitrate concentrations on some nitrate assimilation enzymes such as NR, NiR, GS, and GOGAT in *S. platensis*. In addition, the relationships among different nitrate concentrations (10–180 mM) and some metabolites such as chlorophyll a, total carotenoids, proline, pyruvate, total carbohydrates, and phycobiliprotein [phycocyanin (CPC), allophycocyanin (APC), and phycoerythrin (PE)] contents were investigated in *S. platensis* according to incubation period.

2. Materials and methods

2.1. Microorganism and culture conditions

The cyanobacterium *S. platensis* (Gamont) Geitler 1952 was used in this study. The organism was provided by the Faculty of Aquaculture of Çanakkale Onsekiz Mart University. To prepare and maintain the inoculums, Zarrouk's medium was used (Zarrouk, 1966). The utilized carbonate-bicarbonate buffer gives a pH of 9.0 ± 0.2. *S. platensis* was cultivated in batch cultures containing 1500 mL of medium at 30 °C. For the growth conditions of *S. platensis*, 10, 30 (control conditions), 60, 100, and 180 mM sodium nitrate concentrations were used. After determination of the best sodium nitrate concentration for growth of *S. platensis*, 10 (iron-limited condition), 50 (iron-sufficient condition), and 100 μM (excess-iron-containing medium) iron concentrations were tried in the presence of the optimum sodium nitrate concentration in *S. platensis* growth medium. The reason for trying different iron concentrations in the growth medium was to achieve optimum growth and production of metabolites, pigment, and enzymatic activity in *S. platensis*. The culture was inoculated to an initial optical density (600 nm) of approximately 0.2. The cultures were mixed and bubbled using filtered air continuously. Illumination at 2500 lx light intensity was provided by white fluorescent lamps. The light intensity was measured by a digital light meter (Luxtron LX-101). All the reagents used were of analytical grade.

2.2. Analytical methods

The cells were harvested periodically by centrifugation and were washed with distilled water. The precipitated cells were weighed, and 50 mM phosphate buffer (pH 7) was added at a rate of 12.5 mL/1 g cells. The cells were homogenized at 8000 rpm for 1 min and 9500 rpm for 1 min with 30-s intervals. After centrifugation, the obtained supernatant was used for determination of protein levels

and phycobiliproteins, total carbohydrates, and pyruvate contents. The protein level was assayed using bovine serum albumin as the standard by the method of Bradford (1976). Chlorophyll a and total carotenoid contents were measured as described by Lichtenthaler and Wellburn (1983). CPC, APC, and PE contents were determined by the method of Tarko et al. (2012). Proline content was assayed by the method of Bates et al. (1973). Pyruvate content was assayed by 2,4-dinitrophenyllhydrazine reagent (Friedeman and Haugen, 1943). Total carbohydrate content was obtained with phenol-sulfuric acid by the method of Dubois (1956). Nitrate uptake rate was assayed by the method of Bartzatt and Donigan (2004).

2.3. Enzyme assay procedures

The cells were harvested periodically depending on incubation days by centrifugation and were washed with distilled water. Supernatant was used for nitrate uptake assays. The precipitated cells were weighed and frozen at –20 °C for enzyme assays.

NR activity was determined by the method of Jha et al. (2007). One unit of NR activity was defined as the quantity of enzyme needed for producing 1 μmol of nitrite per minute at 25 °C. Enzymatic activity of NiR was determined by the method of Losada and Paneque (1971). One unit of NiR was defined as the quantity of enzyme needed to reduce 1 μmol of nitrite per minute at 30 °C. GS activity was determined by the method of Berteli et al. (1995). One unit of GS was defined as the amount of enzyme needed to produce 1 μmol of GGH-complex per minute at 30 °C. NADH-GOGAT activity was determined by the method of Chen and Cullimore (1988). NADH oxidation was measured at 340 nm and the enzyme activity was defined as nmol mL^{-1}.

2.4. Statistical analysis

For statistical significance analyses, the Tukey test was used. The values were determined after 3 separate experiments.

3. Results

Changes in nitrate uptake, OD_{600}, dry biomass, pH, protein levels, CPC, APC, PE, chlorophyll a, total carotenoids, proline, pyruvate, total carbohydrates, and some nitrate assimilation enzyme activities in *S. platensis* were investigated in Zarrouk's medium with different sodium nitrate concentrations (10–180 mM) during the incubation period. Zarrouk's medium is used for *S. platensis* studies, and it contains 30 mM sodium nitrate and 35.9 μM iron concentrations. Similarly, the medium is used for industrial production of *Spirulina* (Belay, 2008). Standard Zarrouk's medium was used as the control in our experiments.

3.1. Alterations in nitrate uptake and dry biomass levels depending on different sodium nitrate concentrations in *S. platensis*

In the growth medium of *S. platensis*, the nitrate uptake rate accelerated with increasing nitrate concentrations. As shown in Figure 1a, on the 10th day of the incubation period concentrations of residual nitrate in the growth media containing 10, 30, 60, 100, and 180 mM sodium nitrate were obtained as 39.2 ± 1.4%, 77.43 ± 3.2%, 66.73 ± 3.1%, 48.88 ± 1.7%, and 59.99 ± 2.9%, respectively. According to the results, the highest nitrate uptake was observed in the medium containing 30 mM sodium nitrate.

In growth media containing different sodium nitrate concentrations, the growth rate of *S. platensis* was determined by dry biomass levels depending on incubation time (Figure 1b). The highest growth in organisms was observed on the 14th day in the medium containing 100 mM sodium nitrate. The highest dry biomass was determined on the 14th day (95.06 ± 3.4 mg mL^{-1} in this medium). After the 14th day of the incubation period results did not show important changes. Similarly, the highest OD_{600} and protein levels were determined on the 15th day in the same medium. There were no

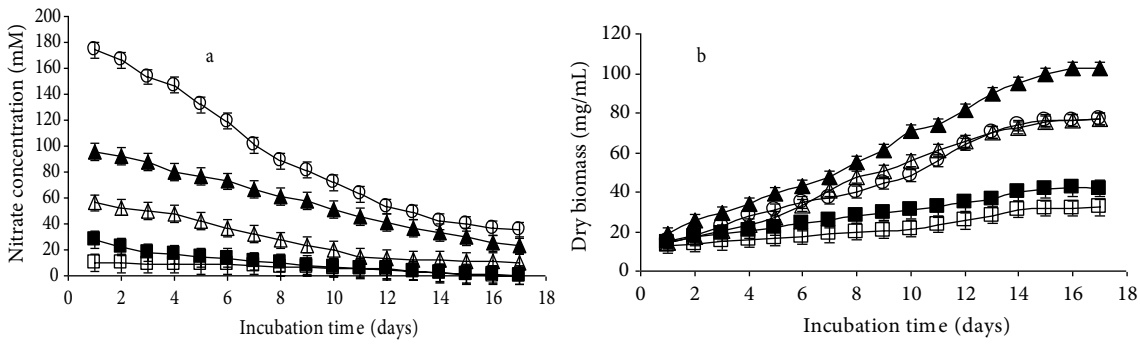

Figure 1. Variations of **a)** nitrate concentrations and **b)** dry biomass levels in *S. platensis* depending on the incubation period in media containing different nitrate concentrations [10 mM (□); 30 mM (■); 60 mM (Δ); 100 mM (▲); 180 mM (o)]. The values are the mean ± SD for 3 separate experiments.

important pH differences among the tested sodium nitrate concentrations during the incubation period.

3.2. Alterations in pigment production with different sodium nitrate concentrations in *S. platensis*

Chlorophyll a, total carotenoids, and CPC contents of *S. platensis* with different sodium nitrate concentrations are depicted in Figure 2. The highest chlorophyll a and total carotenoids contents were determined in the medium containing 100 mM sodium nitrate (106.3 ± 5.2 μg g^{-1} and 33.002 ± 1.3 μg g^{-1}, respectively) on the 14th day. However, the highest CPC content of *S. platensis* was 1.406 ± 0.05 mg g^{-1} in the same medium on the 12th day of the incubation period. Similarly, the highest APC and PE contents were 0.47 ± 0.01 mg g^{-1} and 222 ± 8.7 μg g^{-1}, respectively, in the same medium on the same day; 10 mM sodium nitrate had insufficient nitrogen for pigment production. In this medium the pigment contents of *S. platensis* were several folds lower compared with 100 mM sodium nitrate.

3.3. Alterations in proline, pyruvate, and total carbohydrate contents with different sodium nitrate concentrations in *S. platensis*

Proline, pyruvate, and total carbohydrate contents of *S. platensis* with different sodium nitrate concentrations with respect to incubation time are shown in Figure 3. The highest proline and pyruvate contents were obtained on the 12th day of incubation (36.5 ± 1.3 μmol g^{-1} and 146 ± 7.0 μg g^{-1}, respectively) in the medium containing 100 mM sodium nitrate. The highest total carbohydrate content was 2.824 ± 0.1 mg g^{-1} in medium containing 100 mM sodium nitrate on the 15th day of incubation.

3.4. Alterations in nitrate assimilation enzyme activities depending on sodium nitrate concentration in *S. platensis*

The first enzyme of the nitrate assimilation pathway is NR. Its activity was measured by incubation period in growth media of *S. platensis* containing different sodium nitrate concentrations. The maximum NR and NiR activities were obtained on the 12th and 10th days of the incubation period, respectively. The highest NR and NiR activities were obtained in the medium containing 100 mM sodium nitrate (102.36 ± 3.65 U mL^{-1} and 600.83 ± 19.5 U mL^{-1}, respectively) (Figure 4). The highest NiR activity was approximately 1.24 times higher than that of the control ($P < 0.05$).

GS is responsible for initial assimilation of ammonium, and 100 mM sodium nitrate stimulated GS activity when compared with 10, 30, and 60 mM sodium nitrate. In contrast, 180 mM sodium nitrate had an inhibitory effect on GS activity. GOGAT is the last enzyme of the GS/GOGAT pathway. GS and GOGAT activities were measured during the incubation period with different sodium nitrate concentrations (Figure 5). Maximum GS and GOGAT activities were determined on the 12th day

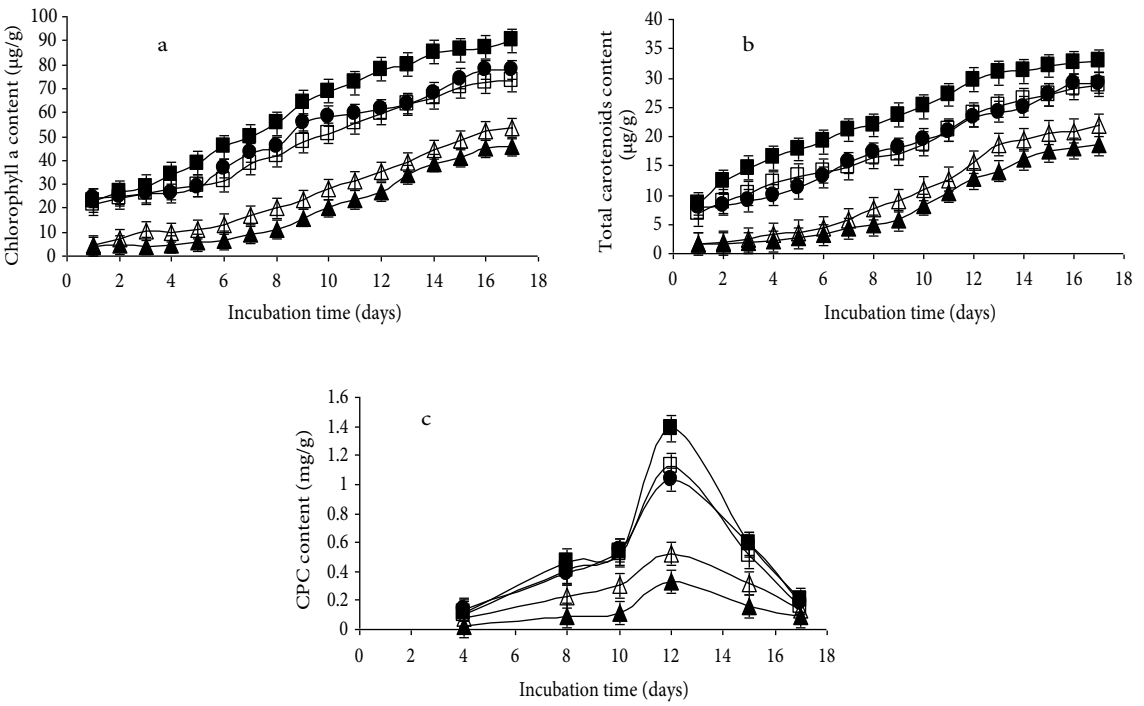

Figure 2. Variations of **a)** chlorophyll a, **b)** total carotenoids, and **c)** CPC contents in *S. platensis* depending on the incubation period in media containing different nitrate concentrations [10 mM (□); 30 mM (■); 60 mM (Δ); 100 mM (▲); 180 mM (o)]. The values are the mean ± SD for 3 separate experiments.

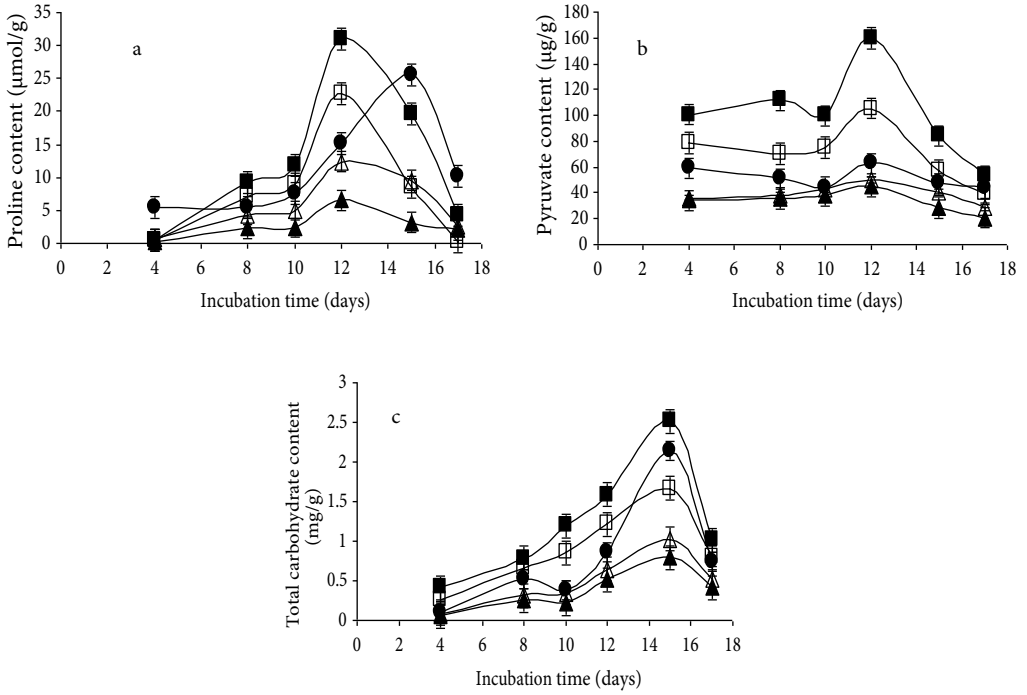

Figure 3. Variations of **a)** proline, **b)** pyruvate, and **c)** total carbohydrate contents in *S. platensis* depending on the incubation period in media containing different nitrate concentrations [10 mM (□); 30 mM (■); 60 mM (Δ); 100 mM (▲); 180 mM (o)]. The values are the mean ± SD for 3 separate experiments.

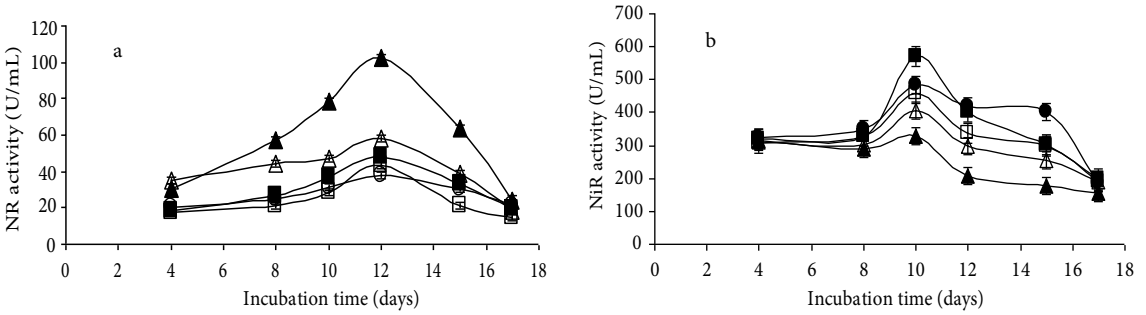

Figure 4. Variations of **a)** NR and **b)** NiR activities in *S. platensis* depending on the incubation period in media containing different nitrate concentrations [10 mM (□); 30 mM (■); 60 mM (Δ); 100 mM (▲); 180 mM (o)]. The values are the mean ± SD for 3 separate experiments.

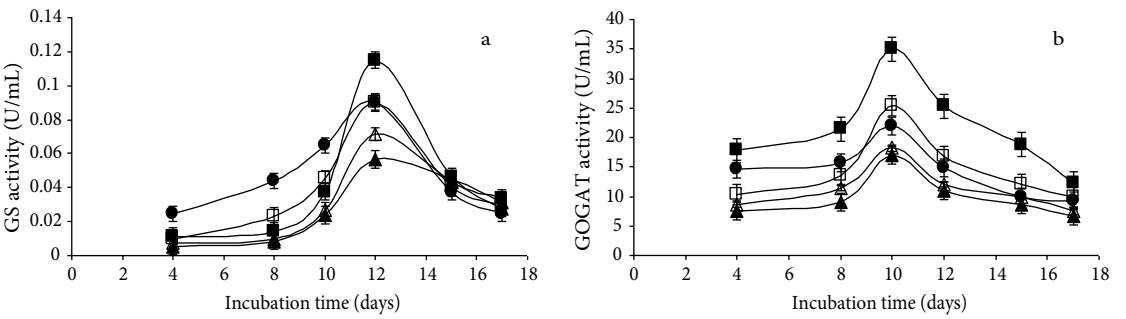

Figure 5. Variations of **a)** GS and **b)** GOGAT activities in *S. platensis* depending on the incubation period in media containing different nitrate concentrations [10 mM (□); 30 mM (■); 60 mM (Δ); 100 mM (▲); 180 mM (o)]. The values are the mean ± SD for 3 separate experiments.

and 10th days of the incubation period, respectively. The highest GS and GOGAT activities were observed in the medium containing 100 mM sodium nitrate (0.1084 ± 0.01 U mL^{-1} and 34.726 ± 1.3 U mL^{-1}, respectively).

3.5. Alterations in nitrate uptake and dry biomass levels with different iron concentrations in *S. platensis*

The best growth, pigment production, and nitrogen assimilation enzyme activities were reached in the medium containing 100 mM sodium nitrate. The effects of various concentrations (10–100 μM) of iron on nitrate uptake and dry biomass levels in the presence of 100 mM sodium nitrate are shown in Figure 6. Nitrate consumption was lower in iron-limited medium than in the other media. On the 17th day of the incubation period, external nitrate concentration was 44.37 ± 3.2 mM in iron-limited medium, whereas it was 22.98 ± 1.6 mM in the control medium. Nitrate was not totally consumed in iron-limited medium. The highest dry biomass of *S. platensis* (106.2 ± 7.1 mg mL^{-1}) was determined in iron-sufficient medium on the 14th day of the incubation period in the presence of 100 mM sodium nitrate as the nitrogen source. Similarly, the highest OD_{600} and protein levels were determined in the same medium on the 14th day.

3.6. Alterations in pigment production with different iron concentrations in *S. platensis*

Chlorophyll a, total carotenoids, and CPC contents of *S. platensis* grown in iron-limited, iron-sufficient, and excess-iron-containing media are shown in Figure 7.

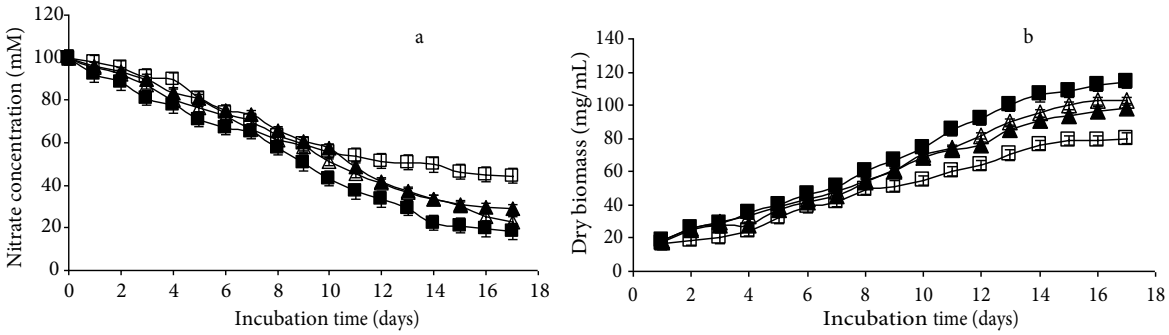

Figure 6. Variations of **a)** nitrate concentrations and **b)** dry biomass levels in *S. platensis* depending on the incubation period in media containing different iron concentrations [10 μM (□); 50 μM (■); 100 μM (▲); control (35.9 μM) (Δ)] using 100 mM sodium nitrate as the nitrogen source. The values are the mean ± SD for 3 separate experiments.

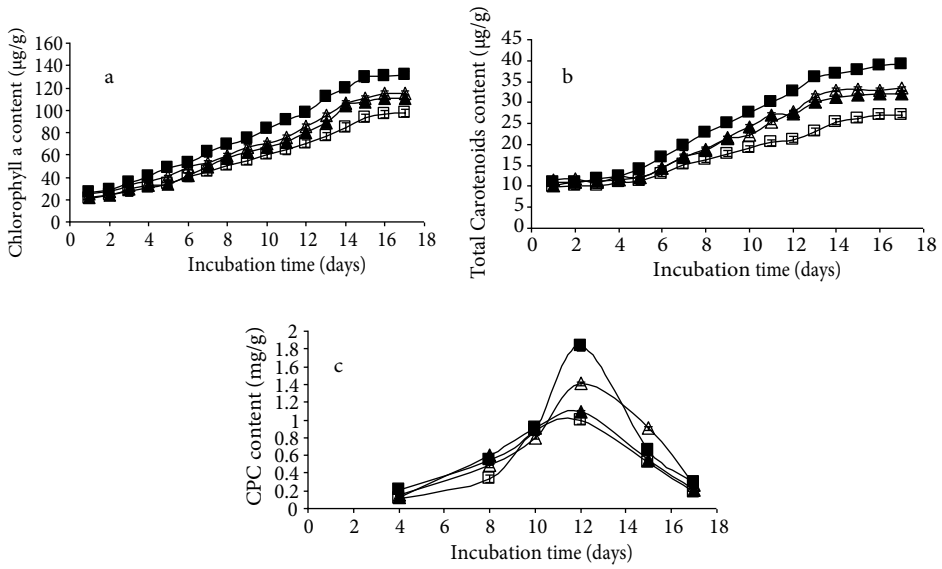

Figure 7. Variations of **a)** chlorophyll a, **b)** total carotenoids, and **c)** CPC contents in *S. platensis* depending on the incubation period in media containing different iron concentrations [10 μM (□); 50 μM (■); 100 μM (▲); control (35.9 μM) (Δ)] using 100 mM sodium nitrate as the nitrogen source. The values are the mean ± SD for 3 separate experiments.

With 100 mM sodium nitrate as the nitrogen source, the highest chlorophyll a and total carotenoids contents were 119.6 ± 8.5 μg g^{-1} and 36.87 ± 2.5 μg g^{-1}, respectively, in iron-sufficient medium on the 14th day. The highest CPC content was 1.827 ± 0.1 mg g^{-1} in the same medium on the 12th day of the incubation period. Similarly, the highest APC and PE contents were determined in the same medium on the same day.

3.7. Alterations in the proline, pyruvate, and total carbohydrate contents with different iron concentrations in *S. platensis*

In the presence of 100 mM sodium nitrate as the nitrogen source, proline, pyruvate, and total carbohydrate contents of *S. platensis* with different iron concentrations with respect to incubation time are shown in Figure 8. The maximum proline and pyruvate contents were obtained on the 12th day of incubation in all tested growth media. The highest proline and pyruvate contents were 44.02 ± 3.6 μmol g^{-1} and 180.13 ± 16 μg g^{-1}, respectively, in iron-sufficient medium. Similarly, the highest total carbohydrate content was obtained in the same medium on the 15th day of the incubation period.

3.8. Alterations in nitrogen assimilation enzyme activities with different iron concentrations in *S. platensis*

In the presence of 100 mM sodium nitrate as the nitrogen source, NR and NiR activities of *S. platensis* with different iron concentrations are depicted in Figure 9. The maximum NR and NiR activities were obtained on the 12th and 10th

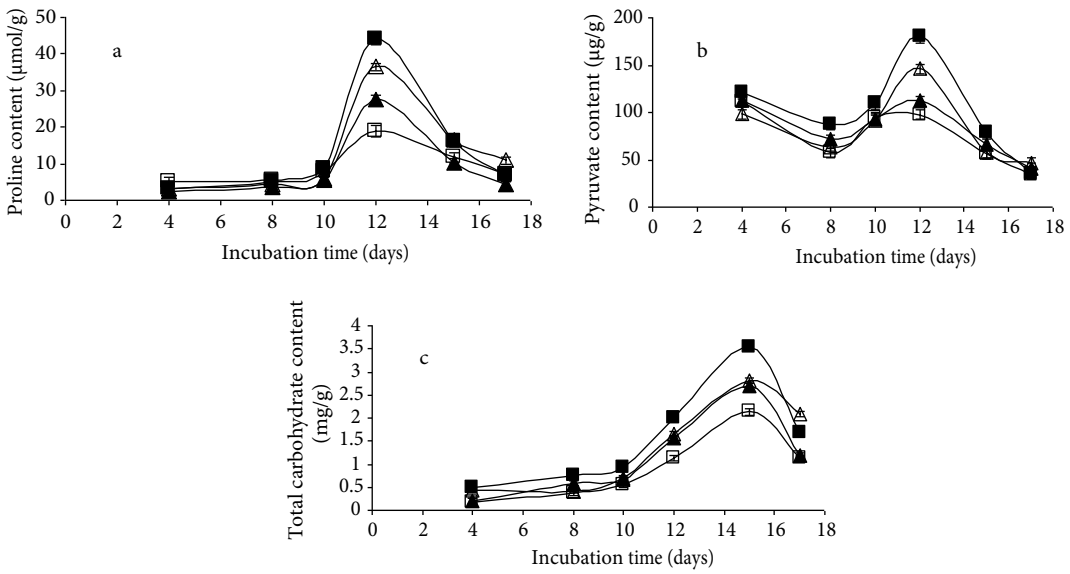

Figure 8. Variations of **a)** proline, **b)** pyruvate, and **c)** total carbohydrate contents in *S. platensis* depending on the incubation period in media containing different iron concentrations [10 μM (□); 50 μM (■); 100 μM (▲); control (35.9 μM) (Δ)] using 100 mM sodium nitrate as the nitrogen source. The values are the mean ± SD for 3 separate experiments.

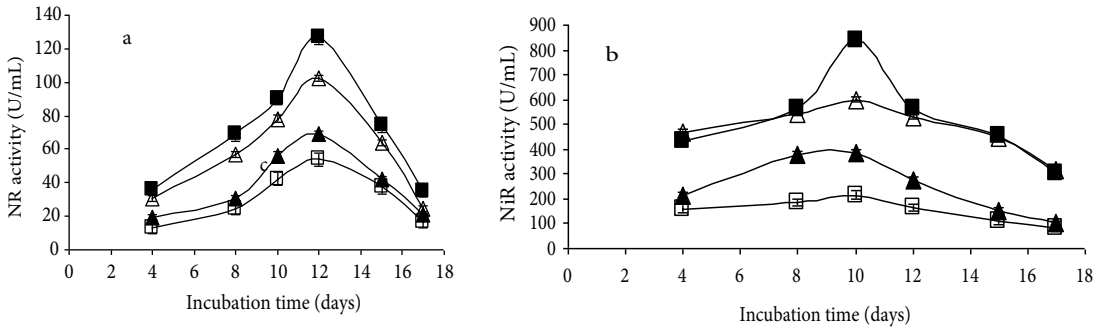

Figure 9. Variations of **a)** NR and **b)** NiR activities in *S. platensis* depending on the incubation period in media containing different iron concentrations [10 μM (□); 50 μM (■); 100 μM (▲); control (35.9 μM) (Δ)] using 100 mM sodium nitrate as the nitrogen source. The values are the mean ± SD for 3 separate experiments.

days of the incubation period, respectively, in all tested media. The highest NR and NiR activities were reached in iron-sufficient medium (126.92 ± 9.2 U mL^{-1} and 841.16 ± 61.4 U mL^{-1}, respectively). In this study, all enzymes were in positive correlation with each other. A positive correlation was seen between NR and NiR activities ($r = 0.997$, $P < 0.01$) with respect to the varying iron concentrations.

When compared to the control with 100 mM sodium nitrate as the nitrogen source, 50 µM iron stimulated GS and GOGAT activities; 10 and 100 µM iron had an inhibitory effect on these enzyme activities. The maximum GS and GOGAT activities were obtained on the 12th and 10th days of the incubation period. The highest GS and GOGAT activities were estimated at 0.1301 ± 0.02 U mL^{-1} and 46.18 ± 1.8 U mL^{-1}, respectively, in iron-sufficient medium (Figure 10). After the 10th day, activity started to decrease in all growth media. A positive correlation was determined between GS and GOGAT activities ($r = 0.963$, $P < 0.05$). Positive correlations were determined between GS and NR ($r = 0.985$, $P < 0.05$) as well as GS and NiR ($r = 0.985$, $P < 0.05$). In addition, positive correlations were obtained between GOGAT and NR ($r = 0.982$, $P < 0.05$) and between GOGAT and NiR ($r = 0.992$, $P < 0.01$).

4. Discussion

Cyanobacteria are generally cultivated in growth media containing many essential minerals and ions. Zarrouk's medium is optimum for the growth of *S. platensis* and has rich mineral and ion content, including Ca, Mg, Mn, Zn, Cu, Mo, nitrate, bicarbonate, and sulfate (Öztürk Ürek and Tarhan, 2012). Nitrate is a vital element and is incorporated in the most important structural components, such as proteins. In addition, nitrate concentration affects the nitrogen assimilation pathway and enzymatic activities (Perez-Garcia et al., 2011). As a limiting factor in growth and metabolite production, iron is one of the most important elements. In addition, it is a significantly vital element for nitrogen metabolism and contributes nitrogen assimilation enzymes such as NR, NiR, and GOGAT (Glass et al., 2009). Nitrate utilization is the first step in nitrogen assimilation and growth of *S. platensis*. Nitrate has a stimulating effect on the biomass of *S. platensis*. In our investigations with Zarrouk's medium, the dry biomass results in medium containing 30 mM sodium nitrate was in accordance with the results of Tarko et al. (2012). According to the results, dry biomass values in the media containing excess sodium nitrate concentrations (60, 100, and 180 mM) were almost 2.0–2.5 times higher ($P < 0.05$). Generally, the effects of 10–60 mM nitrate concentrations were investigated with nitrate assimilation enzymes of *S. platensis* (Devriese et al., 2001; Jha et al., 2007; Logeswaran et al., 2011). We investigated the effects of 10–180 mM sodium nitrate concentrations on nitrate assimilation enzymes and some metabolites in *S. platensis* (Gamont) Geitler. In the presence of 100 mM sodium nitrate as the nitrogen source, nitrate consumption was inhibited in iron-limited medium. The best growth was achieved in iron-sufficient medium. The investigations of Milligan and Harrison (2000) showed that iron deficiency limited the growth of phytoplankton. Similar to their results, in this study, the slowest growth rate was reached in iron-limited medium. It can be concluded that optimum iron concentration may stimulate growth of *S. platensis*. In this study, the best growth, levels of pigments and metabolites, and enzyme activities were determined in iron-sufficient medium using 100 mM sodium nitrate as the nitrogen source.

According to the results, pigment contents of *S. platensis* in the presence of 10 mM sodium nitrate were lower than in the control medium; a 10 mM sodium nitrate concentration may not provide enough nitrogen for algal pigment production. Insufficient production of pigments can be associated with the structure of pigments. The porphyrin rings of chlorophyll a structures contain nitrogen atoms such as ringed groups of PE and APC. Therefore, pigment production requires sufficient nitrogen in growth media. On the 12th day of the incubation period,

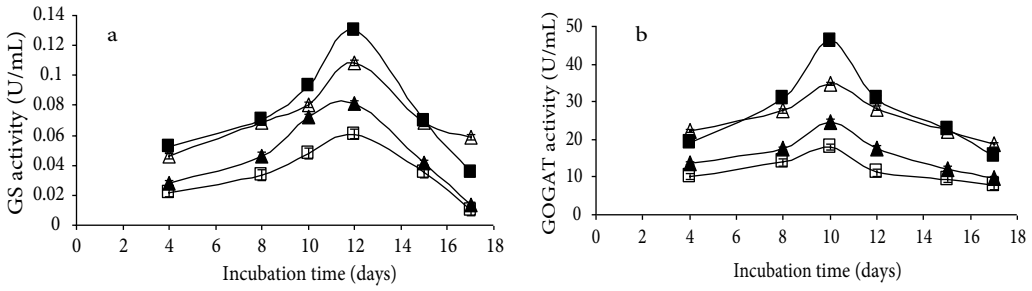

Figure 10. Variations of **a)** GS and **b)** GOGAT activities in *S. platensis* depending on the incubation period in media containing different iron concentrations [10 µM (□); 50 µM (■); 100 µM (▲); control (35.9 µM) (Δ)] using 100 mM sodium nitrate as the nitrogen source. The values are the mean ± SD for 3 separate experiments.

CPC content in the presence of 100 mM sodium nitrate was 1.37 times higher than in control medium ($P < 0.05$). In addition, 180 mM sodium nitrate caused a reduction in the contents of CPC, chlorophyll a, and total carotenoids in all growth media when compared with the control. In the presence of 100 mM sodium nitrate as the nitrogen source, the effects of different concentrations of iron on the pigment production of *S. platensis* were investigated. According to the studies of Milligan and Harrison (2000), iron is a limiting factor in production of pigments such as chlorophyll a, total carotenoids, and phycobiliproteins. Iron limitation inhibits photosystem activity. Thus, in iron-limited conditions, pigment production decreases. Our studies supported these findings. The highest chlorophyll a and total carotenoid contents were determined in iron-sufficient medium, and they were almost 1.12-fold higher than in the control ($P < 0.05$). However, the lowest total carotenoid and chlorophyll a contents were obtained in iron-limited conditions. CPC is a cellular pigment and contains nitrogen atoms in its structure. Thus, in iron-limited conditions, limited biomass production affects CPC production, and its levels decrease (Milligan and Harrison, 2000). Our investigations were in accordance with these results. The lowest phycobiliprotein contents were determined in iron-limited conditions when compared with the control.

Proline contains imino groups. Therefore, nitrogen concentration of the growth medium is highly important for its biosynthesis. It derives from L-glutamate with a group of reactions (Nelson et al., 2008). This shows that nitrogen concentration and assimilation processes are very important for proline production. According to the results, the highest proline content was detected in the medium containing 100 mM sodium nitrate. This can be correlated with the optimum biomass production in this medium. The highest biomass content of the organism might contain the greatest amount of structural components such as amino/imino acids. In the presence of different concentrations of iron, the highest proline production was determined in iron-sufficient medium using 100 mM sodium nitrate as the nitrogen source.

Total carbohydrate and pyruvate contents affect nitrate assimilation. Carbon and nitrogen metabolisms are strongly linked with each other. The energy provided from the TCA cycle is used for the enzymatic reactions as electron transport. Additionally, pyruvate supports the TCA cycle and thus affects 2-oxoglutarate concentration. The 2-oxoglutarate transforms glutamate with a transamination reaction, and this affects the GS/GOGAT pathway. It can be concluded that 2-oxoglutarate and pyruvate play a role as allosteric regulators for GS and GOGAT activities. In this study, 100 mM sodium nitrate provided sufficient nitrogen for metabolite production, whereas 10 mM sodium nitrate was ineffective. In the presence of different concentrations of iron, the highest pyruvate content of the organism was determined in iron-sufficient medium, and it was almost 1.23 times higher when compared with the control ($P < 0.05$). Similarly, 50 μM iron stimulated total carbohydrate production, and the highest total carbohydrate content was estimated in iron-sufficient medium. It was almost 1.25-fold higher than that of the control ($P < 0.05$).

After nitrate uptake into the cytoplasm, NR and NiR work to reduce it to ammonium (Perez-Garcia et al., 2011). Investigations of cyanobacteria indicate that NR and NiR work together in the catalyzing transformation of nitrate to ammonium (Fernandez and Galvan, 2007). In the first reaction, nitrate is converted nitrite by NR, and, sequentially, NiR catalyzes nitrite reduction to ammonium (Perez-Garcia et al., 2011). Nitrate concentration affects enzymatic activities in nitrate assimilation in cyanobacteria (Sood et al., 2002; Ali et al., 2007; Jha et al., 2007; Logeswaran et al., 2011). According to the results, the highest NR activity was determined on the 12th day of the incubation period in the medium containing 100 mM sodium nitrate. It was 2.14 times higher when compared to the control ($P < 0.05$). It may be that 100 mM sodium nitrate was the optimum concentration for NR activity among the tested sodium nitrate concentrations. However, 180 mM sodium nitrate inhibited NR activity, and it showed an approximate decrease of 2.71 times compared with activity in 100 mM sodium nitrate ($P < 0.05$). The highest NiR activity was obtained in the medium containing 100 mM sodium nitrate and was 1.24 times higher when compared to the control ($P < 0.05$). Logeswaran et al. (2011) used Zarrouk's medium containing 30 mM sodium nitrate as a control and determined variations in nitrate assimilation enzyme activities with different growth conditions in *S. platensis* strain PCC 7345. Control results of NiR and NR activities supported our specific activity results in the same medium. In this study, NR and NiR activities were several folds higher in the medium containing 100 mM sodium nitrate. After the reduction of nitrate, assimilation of ammonium is catalyzed by the GS/GOGAT pathway. GS catalyzes glutamine production, and GOGAT catalyzes glutamate production (Lu et al., 2005; Vanoni and Curti, 2005). According to the results, GS activity rose with the increasing nitrate concentrations up to 180 mM. The highest GS and GOGAT activities were reached in the medium containing 100 mM sodium nitrate. They were 1.2 and 1.6 times higher, respectively, when compared with the control ($P < 0.05$), and 180 mM sodium nitrate inhibited GS and GOGAT activities. Growth medium containing 100 mM sodium nitrate is optimum for nitrate assimilation of *S. platensis* (Gamont) Geitler. Zarrouk's medium containing 30 mM nitrate is not sufficient for

the highest nitrate assimilation enzyme activities, some metabolites, and growth parameters.

In the presence of 100 mM sodium nitrate as a nitrogen source, the effects of different concentrations of iron were investigated in the NR, NiR, GS, and GOGAT activities of *S. platensis*. The structures of NR and NiR contain Fe-S clusters. NR has a [4Fe-4S] cluster whereas NiR has 1 [4Fe-4S] cluster in addition to 1 iron atom from the heme group (Glass et al., 2009). Therefore, the iron requirement of NiR is more than the NR requirement. Thus, iron supplementation and starvation affect NiR significantly. Our results were in accordance with this information, and iron was more effective on NiR than NR. In this study the lowest NR and NiR activities were determined in iron-limited conditions. The highest NR and NiR activities were obtained in iron-sufficient medium, and they were almost 1.24 and 1.4 times higher, respectively, compared with control ($P < 0.05$). In addition, iron nutrition was also affected by the GS/GOGAT pathway. The highest GS activity was determined in iron-sufficient medium. However, GS activity was dramatically decreased in iron-limited medium. The structure of GOGAT also contains a [3Fe-4S] cluster (Glass et al., 2009). The highest GOGAT activity was determined in iron-sufficient medium, and it was almost 1.33-fold higher than in the control ($P < 0.05$). However, the lowest GOGAT activity was obtained in iron-limited medium and it was approximately 1.93-fold lower compared with the control ($P < 0.05$).

In conclusion, the highest NR, NiR, GS, and GOGAT activities were determined in medium containing 50 µM iron in the presence of 100 mM sodium nitrate. This research was aimed at achieving the highest enzyme activities using different concentrations of sodium nitrate and iron. The results showed that enzyme activities and growth, metabolite, and pigment levels were several folds higher using 100 mM sodium nitrate in the presence of 50 µM iron. We achieved the best nitrate assimilation enzyme activities under these conditions. Higher enzyme activities may result in greater amounts of amino acids, such as glutamine and glutamate, which have biotechnological, industrial, and biochemical importance. In addition, a 50 µM iron concentration and 100 mM sodium nitrate stimulated algal growth and contents of some metabolites such as protein, proline, pyruvate, total carbohydrate, and antioxidant-featured pigments (phycobiliproteins, total carotenoids, and chlorophyll a). The high contents of these metabolites and compounds in *S. platensis* can make it a rich food source.

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