DOKUZ EYLÜL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

EFFECT OF DIFFERENT NITROGEN SOURCES ON SOME MICROALGAE NITROGEN ASSIMILATING ENZYMES

by
Merve ESEN

June, 2014
İZMİR

EFFECT OF DIFFERENT NITROGEN SOURCES ON SOME MICROALGAE NITROGEN ASSIMILATING ENZYMES

A Thesis Submitted to the
Graduate School of Natural and Applied Sciences of Dokuz Eylül University
In Partial Fulfilment of the Requirements for the Degree of Master of Science
in Chemistry Program

by
Merve ESEN

June, 2014
İZMİR

M.Sc THESIS EXAMINATION RESULT FORM

We have read the thesis entitled **"EFFECT OF DIFFERENT NITROGEN SOURCES ON SOME MICROALGAE NITROGEN ASSIMILATING ENZYMES"** completed by **MERVE ESEN** under supervision of **PROF. DR. RAZİYE ÖZTÜRK ÜREK** and we certify that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

Prof. Dr. Raziye ÖZTÜRK ÜREK

Supervisor

Prof. Dr. Leman Tarhan

(Jury Member)

Prof Dr. Aysegül Pala

(Jury Member)

Prof.Dr. Ayşe OKUR
Director
Graduate School of Natural and Applied Sciences

ACKNOWLEDGMENTS

I would like to express my gratitude to my thesis advisor, Prof. Dr. Raziye ÖZTÜRK ÜREK for her supervision, her guidance, support, patience, humanity and abilities to pursue a chemistry career as an independent researcher throughout this thesis.

My heartfelt thanks to Prof. Dr. Leman TARHAN for her generous support, encouragement and constructive suggestions. Additionally, I am very thankful to my laboratory friends for helping involved in my experience.

I would like to express my sincere gratefulness to my mother Yüksel ESEN, my father Mehmet ESEN, and my sister Kübra Nur ESEN for their patience and support throughout my thesis.

Finally, I owe my fiance, Mehmet Ertuğrul TAVUKÇU a debt of gratitude, for his patience, support and understanding during my thesis.

The thesis is supported by Dokuz Eylül University Scientific Research Project Coordination Unit, 2013.KB.FEN.026.

Merve ESEN

EFFECT OF DIFFERENT NITROGEN SOURCES ON SOME MICROALGAE NITROGEN ASSIMILATING ENZYMES

ABSTRACT

Effects of different concentrations of sodium nitrate (ten-one hundred eighty milimolar) and ammonium nitrate (five-sixty milimolar) on the growth, nitrate/ammonium uptake rate, production of some pigments and metabolites, and some nitrogen assimilation enzymes such as nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS) and glutamate synthase (GOGAT) in *Spirulina platensis* (Gamont) Geitler were investigated.

In the presence of sodium nitrate as nitrogen source, the highest growth of the organism, levels of pigment and metabolite production and enzyme activities were reached in the medium containing one hundred milimolar sodium nitrate. The highest NR, NiR, GS and GOGAT activities were two point two; one point two; one point two and one point six-fold higher, respectively, than control. However, one hundred eighty milimolar sodium nitrate showed toxic effect on these parameters and enzyme activities were several folds lower when compared with control.

When preferred ammonium nitrate as nitrogen source, ten milimolar ammonium nitrate stimulated growth, production of pigments and metabolites, and enzyme activities whereas thirty and sixty milimolar ammonium nitrate caused inhibition on these parameters. The highest NR, NiR, GS and GOGAT activities were one point two; one point two; one point three and one point six-fold higher, respectively, than control. The reasons of ammonium nitrate usage as nitrogen source were being economic and giving chance us to examine different pathways of ammonium and nitrate in nitrogen assimilation.

If ten milimolar ammonium nitrate and one hundred milimolar sodium nitrate were compared the highest yield of growth, metabolite production and enzyme activities were reached in the presence of one hundred milimolar sodium nitrate. In

conclusion, the best growth, metabolite and pigment production and enzyme activities were reached in the media containing one hundred milimolar sodium nitrate. Higher enzymatic activities may stimulate high amount of amino acid production such as glutamine and glutamate.

Keywords: Ammonium nitrate, glutamate synthase, glutamine synthetase, nitrate reductase, nitrite reductase, sodium nitrate, *Spirulina platensis*.

FARKLI AZOT KAYNAKLARININ BAZI MİKROALGAL AZOT ASİMİLASYON ENZİMLERİ ÜZERİNE ETKİSİ

ÖZ

Spirulina platensis (Gamont) Geitler'de, sodyum nitrat (on-yüz seksen milimolar) ve amonyum nitratın (beş-altmış milimolar) farklı derişimlerinin, büyüme, nitrat/amonyum alım hızları, pigment ve metabolitlerin üretimi ve nitrat redüktaz (NR), nitrit redüktaz (NiR), glutamin sentetaz (GS) ve glutamat sentaz (GOGAT) gibi bazı azot asimilasyonu enzimleri üzerindeki etkileri araştırıldı.

Azot kaynağı olarak sodyum nitratın varlığında, en fazla büyüme, pigment ve metabolit düzeylerinin üretimi ve enzim aktivitelerine yüz milimolar sodyum nitrat içeren büyüme ortamında ulaşılmıştır. En yüksek NR, NiR, GS ve GOGAT aktiviteleri kontrol ile kıyaslayınca, sırasıyla, iki onda iki; bir onda iki; bir onda iki ve bir onda altı kat fazla idi. Ancak, yüz seksen milimolar sodyum nitrat, bu parametreler üzerinde toksik etki göstermiştir ve enzim aktiviteleri kontrol ile kıyaslanınca oldukça düşüktür.

Azot kaynağı olarak amonyum nitrat tercih edildiğinde, on milimolar amonyum nitrat büyüme, pigment ve metabolitlerin üretimi ve enzim aktivitelerini stimüle ederken otuz ve altmış milimolar amonyum nitrat bu parametreler üzerinde inhibisyona neden olmuştur. En yüksek NR, NiR, GS ve GOGAT aktiviteleri kontrol ile kıyaslayınca, sırasıyla, bir onda iki; bir onda iki; bir onda üç ve bir onda altı kat fazla idi. Azot kaynağı olarak amonyum nitrat kullanılmasının sebepleri, ekonomik olması ve azot asimilasyonunda amonyum ve nitratın farklı yolaklara sahip olmasının incelenmesi imkânı vermesidir.

On milimolar amonyum nitrat ve yüz milimolar sodyum nitrat kıyaslanırsa, en verimli büyüme, pigment ve metabolit üretimi ve enzim aktiviteleri, yüz milimolar sodyum nitrat varlığında tespit edilmiştir. Sonuç olarak, en iyi büyüme, pigment ve metabolit üretimi ve enzim aktiviteleri, yüz milimolar sodyum nitrat içeren büyüme

ortamında tespit edilmiştir. Yüksek enzim aktiviteleri, fazla miktarda glutamin ve glutamat gibi amino asitlerin üretimine yol açabilir.

Anahtar kelimeler: Amonyum nitrat, glutamat sentaz, glutamin sentetaz, nitrat redüktaz, nitrit redüktaz, sodyum nitrat, *Spirulina platensis*.

CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

CHAPTER ONE
INTRODUCTION

1.1 Cyanobacteria

Cyanobacteria are photosynthetic and prokaryotic bacteria that use light and CO_2 with pigments, mainly chlorophyll a, and then produce glucose and O_2 with them. They usually live in wetlands and terrestrial areas; commonly in rivers, freshwater like lakes, ponds and springs. They are primary producers that play an essential role in nitrogen, carbon and oxygen metabolisms of aquatic life (Vincent, 2009).

Cyanobacteria are mainly called as blue-green algae because they have some similar properties with microalgae such as appearance and having essential pigments. One of the similarities among the cyanobacteria and microalgae is photosynthesizing. As in microalgae; cyanobacteria produce oxygen with using water as the electron donor. Chlorophyll and carotenoids are naturally occurring pigments present, mainly, in photosynthetic organisms, including microalgae and cyanobacteria (Öztürk Ürek, & Tarhan, 2012). In cyanobacteria, chlorophyll is a green molecule similarly in plants. Chlorophyll structure has pyrrole rings and they are known as "porphyrin pigments" by scientists. Especially; chlorophyll a is the major pigment in cyanobacteria (Figure 1.1). Chlorophyll a is one of the specific forms of chlorophyll and it is used in oxygenic photosynthesis. It absorbs energy from wavelengths of violet-blue and orange-red light, mostly. It also reflects green/yellow light, and contributes to the observed green color of most plants (Raven, Evert, & Eichhorn, 2005). One of the assignments of chlorophyll a is transferring resonance energy in the antenna complex, ending in the reaction center where specific chlorophylls P700 and P680 are located. The molecular structure of chlorophyll a consists of a N-ring with a magnesium center, side chains, and a hydrocarbon tail. Chlorophyll a contains a central magnesium ion covered in a 4-nitrogen-ion ring known as “chlorin ring” (Figure 1.1a). The chlorin ring is a heterocyclic compound. It derives from a pyrrole ring that encases a metal. The magnesium within the center uniquely defines the structure of the chlorophyll molecule. Chlorophyll a molecule contains methyl group

(CH_3) as side chain that attaches to the porphyrin ring (Figure 1.1b). A long hydrocarbon tail attaches to the porphyrin ring of the chlorophyll a (Figure 1.1c) (Woodward et al., 1990; Raven, Evert, & Eichhorn, 2005).

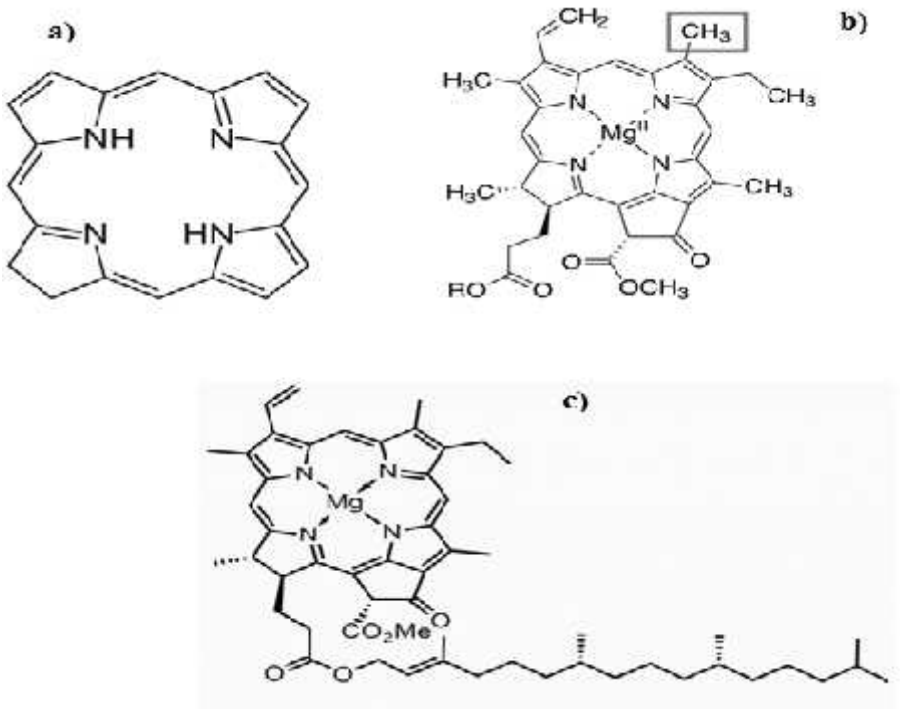

Figure 1.1 Chlorophyll a molecules. a) Chlorin ring without the central Mg b) Central of the chlorophyll a c) Structure of chlorophyll a molecule.

Carotenoids are the members of isoprenoids family and they have forty carbon atoms. They are the most important photosynthetic pigments with protection of chlorophyll and thylakoid membrane from photo oxidative damage. The most visible carotenoids present in biomass of cyanobacteria are β-carotene, cryptoxanthin, zeaxanthin, myxoxanthopyll, oscillaxanthin and echinenone (Mendiola et al., 2007). β-carotene is a kind of the hydrocarbon carotenoids. The structure of β-carotene consists of chromophore characteristic due to the presence of eleven conjugated double bonds (Figure 1.2a). It is synthesized mostly by algae, plants, and some photosynthetic microorganisms. β-carotene has the prime role with bearing pro-vitamin A activity (Dey, & Rathod, 2013). It has also intermediary position in the biosynthetic pathway of various carotenoids such as zeaxanthin and phytoene. Carotenoid molecules have an important role in photosynthesis with taking part in

transferring the excitation energy to the reaction centre. The auxiliary antennas of carotenoids are pigment-protein complexes and their primary activity is surrounding the reaction centre for transferring the excitation energy of pigments appearing in them. Another major function of carotenoids is to protect of photosynthetic mechanisms against photo oxidation of unsaturated fatty acids in chlorophyll (Kerfeld, 2004). Cyanobacteria cells also consist of carotenoids bounded by specific proteins which occur in the biomass of cyanobacteria growing in natural environments and cultivated in laboratories. These complexes are soluble in water and bind neither chlorophyll nor other pigments (Tarko, Duda-Chodak, & Kobus, 2012).

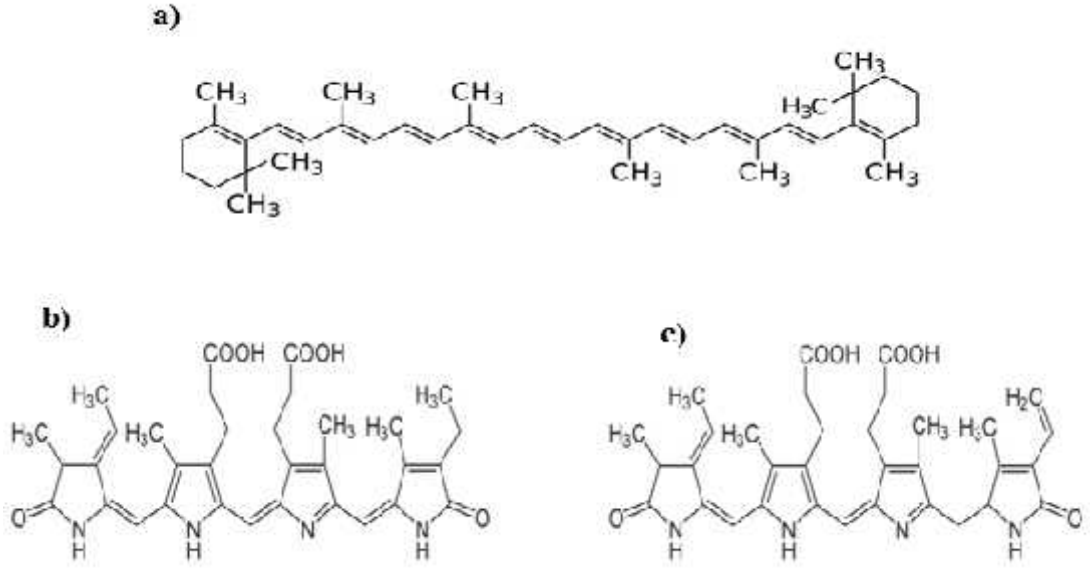

Figure 1.2 The chemical structure of bioactive compounds present in *Arthrospira* cells. (a) β-carotene, (b) phycocyanobilin and (c) phycoerythrobilin (Tarko, Duda-Chodak, & Kobus, 2012).

The other crucial pigments found in cyanobacteria are phycobiliproteins. They have been investigated due to their involvement as vital accessory pigments in photosynthesis (Hirata, Tanaka, Ooike, Tsunomura, & Sakaguchi, 2000; Singh, Kate, Banerjee, 2005). In general, they are found in prokaryotic cyanobacteria. They are major light-harvesting complexes and harvest efficiently the sunlight, then transfer the energy to chlorophyll a in the thylakoid membranes. The phycobiliproteins are linked to the thylakoid membranes and locate near the reaction center chlorophyll a

of the photosystem II in cyanobacteria (Sun, Wang, & Qiao, 2006). Phycobiliproteins are water-soluble pigments, so they cannot exist within the membrane like carotenoids can. They aggregate to form clusters that adhere to phycobilisomes. The phycobiliproteins are categorized into three groups based on the presence of different chromophores among them: (1) Phycocyanin (PC; absorption light from 590 to 630 nm); (2) Allophycocyanin (APC; absorption light from 620 to 665 nm) and (2) Phycoerythrin (PE; absorption light from 480 to 570 nm) (Tarko, Duda-Chodak, & Kobus, 2012). PC is one of the most important members of phycobiliprotein family with being pigment-protein complex from the light-harvesting, along with APC and PE (Glazer, 1989). It has a deep blue color with owing to the open tetrapyrrole chromophore group which is tightly linked to the apoprotein. Phycocyanobilin is the chromophore of PC and APC (Figure 1.2b) and phycoerythrobilin is the chromophore group of PE (Figure 1.2c). Phycocyanobilin has a similar structure as bilirubin that is a powerful remover for reactive oxygen species *in vivo*. Thus it shows that phycocyanobilins have antioxidant properties and protect the living cell from oxidative stress. A cyanobacterium, *Spirulina platensis*, also has the PE which gives red pigmentation (Hirata, Tanaka, Ooike, Tsunomura, & Sakaguchi, 2000). PE is an auxiliary pigment to the main chlorophyll pigments responsible for photosynthesis. The light energy is captured by PE then it is passed to the reaction centre (Ficner, & Huber, 1993). Generally, phycobiliproteins are made up of two monomers as α and β. They are two different polypeptide chains, α and β, and tightly attached chromophores. Purified biliprotein structures are mainly in forms of trimers $(\alpha\beta)_3$ (PC and APC) or hexamers $(\alpha\beta)_6$ (PE). The structures of trimer PC or APC biliproteins are disk-shaped aggregates, about 11 nm in diameter and about 3 nm in thickness, with a center cavity about 3.5 nm in diameter whereas hexamer PE proteins, which are organized by two trimers stacking, are 6 nm in thickness (Sun, Wang, & Qiao, 2006). Cyanobacteria are rich PC sources and used as a natural colorant for food additive in industry. PE amount in *S. platensis* is little when compared with APC and CPC (Hirata, Tanaka, Ooike, Tsunomura, & Sakaguchi, 2000).

Cyanobacteria lack membrane-bound organelles because of their prokaryotic properties. However they have some specialized functioning structures such as circular DNA, nucleoids, phycobilisome containing thylakoid membranes. Cyanobacteria can live over a wide temperature range with the highest limit of 75°C. However optimum growth of the organism is at warm temperatures. They prefer alkaline growth conditions mostly pH 9. They are also UV tolerant microorganisms and have various enzymatic defenses against reactive oxygen species (Vincent, 2009).

1.2 *Spirulina platensis*

Spirulina also called *Arthrospira* is a prokaryotic, photosynthetic, filamentous, spiral-shaped, multicellular, nitrate-utilizing and non-nitrogen fixing cyanobacteria with tremendous importance in environmental, industrial, and nutritional biotechnology (Figure 1.3) (Jha, Ali, & Raghuram, 2007).

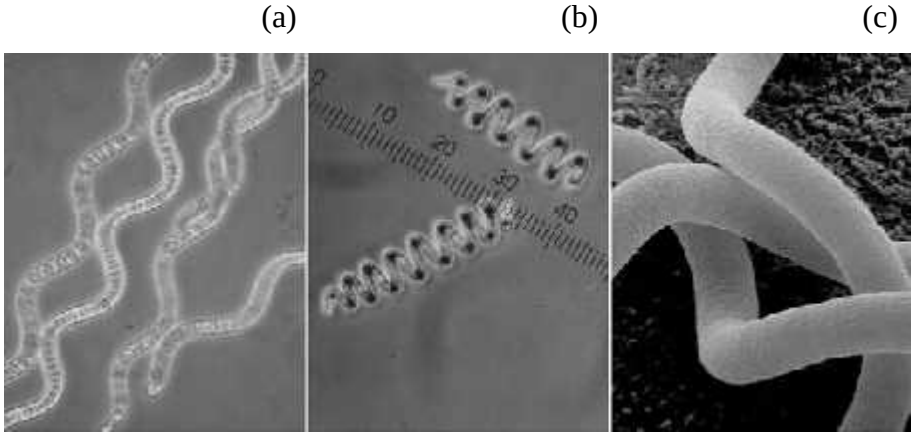

Figure 1.3 Structure of *Spirulina*. (a) Long filamentation of structure, (b) spiral helical shape and (c) electron microscope view of *Spirulina*.

The species of *Spirulina* genus are mixotrophic microalgae thus they can live both autotrophically and heterotrophically. The optimum temperature range for the growth is 30-45°C. They can also survive in temperature range as 60-70°C however no growth is determined at temperatures below 18°C (Tarko, Duda-Chodak, & Kobus, 2012). *Spirulina* species are alkaliphilic organisms and the optimum pH for their growth is 8.5-10.5. Such high alkalinity of the environment is available for *Spirulina*

species for making microbiological purity in the medium during their commercial cultivation and also protects themselves from microbial contaminations. The chemical composition of the growth medium is another essential factor for *Spirulina*. It was found that high concentrations of sodium and carbonate ions are vital to make environment high alkaline. In addition, the light intensity is a major factor for optimum growth that takes place at the intensity of 120-200 μmol photons/m^2s and 10-15% of the total solar radiation intensity in the wavelength range of 400-700 nm. Excess intensive illumination usage with carbon dioxide deficiency makes photoinhibition in the growth medium probably caused by H_20_2 accumulation (Tarko, Duda-Chodak, & Kobus, 2012).

One of most important species of *Spirulina* is *Spirulina platensis* that is a rich source of micro elements such as Se, Mg, Cr, Cu, Ca, Na Fe, P, I, Mn, K, and Zn. *S. platensis* is a very rich source of vitamins; mainly B-group, in particular A, D, E vitamins. Additionally, unsaturated fatty acid content of *S. platensis*, including (particularly beneficial to human health) Ω-3 and Ω-6 is major components for cell structure (Tarko, Duda-Chodak, & Kobus, 2012). Dry matter of *S. platensis* consists of lipids and carbohydrates as 4-9% and 8-16%, respectively, (Becker, 2007). The biomass of *S. platensis* contains approximately 60-70% protein. This is known as high-quality protein due to containing as many as 9 essential amino acids: Lysine, histidine, leucine, isoleucine, methionine, tryptophan, phenylalanine, threonine, and valine (Gershwin, & Belay, 2008).

1.2.1 Health Benefits of Spirulina

Spirulina has several benefits for human health with its cellular composition and phytonutrients richness. There are some examples for effect of *Spirulina* on human health: 1) reducing risk of cancer, 2) improving the immune system, 3) having anti-aging properties, 4) having anti-viral activity, 5) decreasing cholesterol levels, 6) accelerating wound healing, 7) avoiding malnutrition, 8) reducing radiation sickness, 9) building healthy lactobacillus (Mathew et al., 1995; Gemma, Mesches, Sepesi,

Choo, Holmes, & Bickford, 2002; Hirahashi, Matsumoto, Hazeki, Saeki, Ui, & Seya, 2002).

Spirulina is a beneficial food for cholesterol reduction. Feeding with *Spirulina* was showed decreasing serum cholesterol levels approximately 4.5% in human in a month. The reduction of cholesterol can be associated with partly owing to the high content γ-linolenic acid (Henrikson, 2010).

The β-carotene is one of the most important pigments with reducing free radicals which transform the cells and cause variable diseases such as cancer. *Spirulina* is ten times more β-carotene sources than any other plants. Investigations at the Harvard University School of Dental Medicine showed that consumption of β-carotene extracts, obtained from *Spirulina* caused reduction in mouth cancer (Schwartz, & Shklar, 1987). Studies with hamsters showed that applying the β-carotene extract to oral cancer tumors reduced the tumor number and size; also sometimes tumors disappeared. Additionally, phycocyanin is one of the most abundant pigments found in *Spirulina*. It has high benefits for health such as anti-cancer effects and immune system boosting with its antioxidant properties (Suda, Schwartz, & Shklar, 1986).

Spirulina structure consists of vitamin A which is vital in preventing eye diseases. In addition, iron and vitamin B_{12} found in *Spirulina* can be use for anemia treatments. *Spirulina* also has a positive effect on Parkinson‘s disease, sclerosis, malnutrition, cardiac disease and wounds cure (Henrikson, 2010).

Calcium-Spirulan (Ca-SP) is an extract of sulfated polysaccharides and it consists of galactose, fructose, xylose, rhamnose, ribose, glucose, mannose, galacturonic acid, glucuronic acid. Calcium sulfate obtained from *Spirulina* showed activity against HIV, Human Cytomegalovirus, Mumps Virus, Measles Virus, Influenza A Virus and Herpes Simplex Virus. The studies in this field are for searching these extracts that inhibit the AIDS virus replication and allows these patients to improve their health (Ayehunie, Belay, Baba, & Ruprecht, 1998).

1.2.2 Spirulina in Industry

Spirulina is a major natural colorant in industry. It is a valuable food source as using Japanese chewing gums due to its colorant featured pigment contents such as phycocyanin, chlorophyll a and β-carotene. *Spirulina* is used as aquarium fish food in Taiwan and Japan; also in United States. It is used to enhance color, speed the growth and sexual maturation of exotic birds and canaries. Adding *Spirulina* to the silages of horses and cattles showed that the fertility in females and the quantity of sperms in males are increased. *Labeo rohita* (rohu), an Indian carp, was being fed with *Spirulina* and after that greater growth was obtained. In addition, *Spirulina* enhances the mononuclear phagocyte system function of chickens thus this increases their disease resistance (Henrikson, 2010; Zahroojiana, Moraveja, & Shivazada, 2011).

Consumption with *Spirulina* is becoming widespread all around the world. Many countries such as France, Chile, Germany, Cuba, Switzerland, Sweden, Spain, Portugal, Holland, Belgium, United Kingdom, Denmark, Australia, and New Zealand use *Spirulina* in various areas in industry. Marketing food complements, which include *Spirulina* as the major component can be an example of this. In France *Spirulina* was used as a vegetable pate and in Sweden the bread with low calorie enriched with *Spirulina* was produced (Henrikson, 2010).

1.2.3 Spirulina as Food

Spirulina is known as a good food source with its rich nutritional values. It is rich in natural pigments such as phycobiliproteins, chlorophyll and carotenoids. Additionally, it contains high amount of carbohydrate, protein and essential fatty acids; also it contains numerous minerals. Thus these properties make *Spirulina* amazing food source for human and animals. *Spirulina* is produced as powder, tablets and capsules to making easy for consuming (Henrikson, 2010).

Studies have demonstrated that feeding with *Spirulina* reduces body weight by 1.4±0.4 kg after a month. *Spirulina* is consumed for low carbohydrate diet and exercise to eat low calorie containing meals and avoid fattening snacks. *Spirulina* is called as “green superfood” and due to these properties and it can help satisfy body’s appetite. It doesn’t contain drug or chemical that causes damages to human health. It is simply concentrated and easily digested natural nutrition source. Especially important to dieters, it is rich in iron. Because low calorie diets of women generally lacked of iron (Henrikson, 2010).

Spirulina is mainly consumed by sportsman and athletes due to its high protein and mineral contents. In addition; *Spirulina* contains γ-linoleic acid which stimulates prostaglandins, master hormones that regulate cells of the body, including circulation, skin, heart and musculature. It helps mending and creating muscle mass. In heavy training, loss of iron is big problem and causes some health problems as anemia. *Spirulina* prevents from anemia because it has ten times more iron than spinach (Henrikson, 2010).

Spirulina is an excellent food not only for human but also for the animals. *Spirulina* provides five benefits for health of aquarium fish: 1) being a source of natural vitamins and minerals, 2) providing mucoproteins for healthy skin, 3) being rich in phycocyanin for better health and prevent obesity, 4) containing essential fatty acids for proper organ development, 5) being a natural source for coloring agents such as carotenoids. Feeding with *Spirulina* can result in beautiful, healthy and longer lived fish. Additionally flamingos are fed with *Spirulina* for improvement their health and colors. Birds such as parrot, lovebird, canary, finch and other breeders use *Spirulina* to accelerate growth, increase coloration, and improve fertility rates and sexual maturity. It enhances attractive yellow skin coloration in chickens and increases the deep yellow color of egg yolks. Investigations in chickens show that adding a small percentage of *Spirulina* in the diet stimulates macrophage production, improves immune system and resistance to disease (Henrikson, 2010).

1.3 Nitrogen assimilation in *S. platensis*

S. platensis is best known with being rich and high quality protein source. Due to these properties of *S. platensis*; nitrogen is the most important bio-element with contributing approximately 10% to cyanobacterium cells (Perez-Garcia, Escalante, de-Bashan, & Bashan, 2011). It is an essential element with incorporating in the most important functional and structural macromolecules, such as aminoacids, in organisms and it is referred to as nitrogen assimilation. Proteins and nucleic acids contain about 15% nitrogen (Inokuchi, Kuma, Miyata, & Okada, 2002).

Nitrogen is a basic element for living organisms because of being a major component of the two important biological macromolecules: Nucleic acids and proteins. Nitrogen exists different oxidation states, from N(V) to N(-III), in the biosphere. Nitrogen cycle includes interconversions of these nitrogen species. In this process bacteria play a predominant role. Nitrogen is removed from the environment with two processes: Nitrification and denitrification. Nitrification is the oxidative conversion of ammonia to nitrate and denitrification is a respiratory process whereby nitrate is successively reduced to nitrite, nitrogen oxides (NO and N_2O), and dinitrogen (N_2). In the nitrogen cycle, inorganic nitrogen is converted to a biologically useful form by nitrate assimilation or dinitrogen fixation and the further process is incorporation of ammonia into carbon skeletons (Fiore, Jarett, Olson, & Lesser, 2010). Nitrate reduction is the first step for the assimilation process and has important agricultural, environmental, and public health implications. Assimilatory nitrate reduction, performed by microorganisms and higher plants, is one of the most basic biological processes, transforming more than 104 megatons of inorganic nitrogen each year (Herrero, Muro-Pastor, & Flores, 2001; Inokuchi, Kuma, Miyata, & Okada, 2002).

Various nitrogen containing compounds can be used as nitrogen sources by different organisms. Inorganic ions such as ammonium or nitrate and organic compounds like amino acids, urea, and some nitrogen-containing bases can be examples for the nitrogen sources used in nitrogen assimilation (Herrero, Muro-

Pastor, & Flores, 2001). Cyanobacteria are able to assimilate wide diverse of nitrogen sources, generally ammonia, nitrate, and urea, as well as yeast extract, peptone, amino acids, and purines (Perez-Garcia, Escalante, de-Bashan, & Bashan, 2011). Many biochemical and molecular biological studies have advanced our knowledge of nitrogen assimilation, and revealed that many enzymes have role for this biological process (Inokuchi, Kuma, Miyata, & Okada, 2002). Nitrogen assimilation requires large amount of carbon, protons and energy. Cell membranes of cyanobacteria consist of specific transport systems for the uptake of different forms of nitrogen, but generally, most of them enter the cell by diffusion, depending on the pH of the culture medium or their concentration. Nitrate and nitrite are reduced by the enzymes such as nitrate and nitrite reductase, respectively. As a nitrogen source for cyanobacteria, urea is converted to ammonium by urease. In addition; dinitrogen is reduced to ammonium by the nitrogenase complex by some cyanobacteria. Other forms of nitrogen as nitrogen sources like certain amino acids require specific transport systems (Figure 1.4) (Muro-Pastor, & Florencio, 2003).

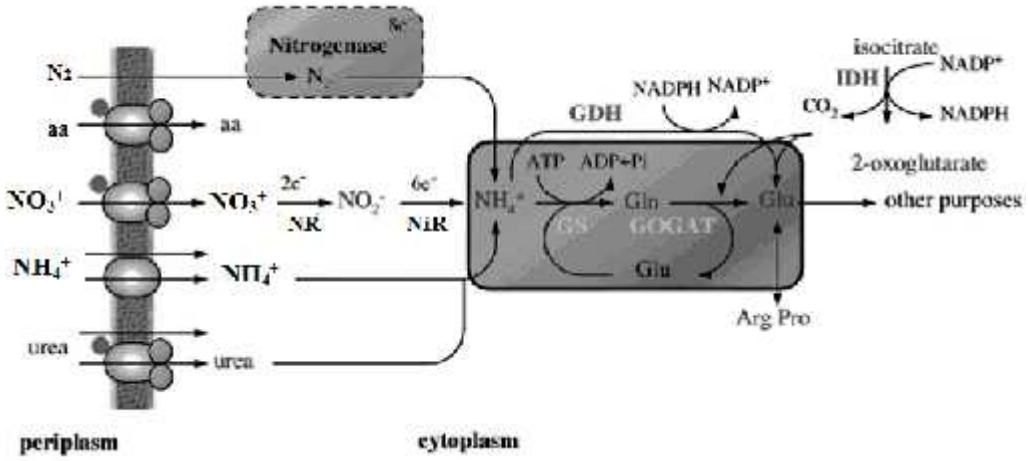

Figure 1.4 Nitrogen uptake and metabolism processes in cyanobacteria (Muro-Pastor & Florencio, 2003).

Studies show that nitrate is most widely used by cyanobacteria and the available form of nitrogen in nature (Herrero, & Guerrero, 1986). Cells of cyanobacteria firstly transport nitrate into the cytoplasm and then reduce it to ammonia in the process

(Perez-Garcia, Escalante, de-Bashan, & Bashan, 2011). Nitrate assimilation in cyanobacteria consists of three basic steps:

a) Nitrate/Ammonium Uptake:

The assimilation of nitrate involves its incorporation into the cell through an active transport system and its intracellular two-step reduction to ammonium sequentially catalyzed by nitrate reductase and nitrite reductase (Figure 1.5). Nitrate is frequently found in the environment at relatively low concentrations (e.g., in the µM range), thus specific nitrate uptake systems are required to concentrate this nutrient inside the cells before nitrate reduction can take place (Flores, Frias, Rubio, & Herrero, 2005). Nitrate is firstly transported into the cytoplasm by the specific transporters (Devriese, Tsakaloudi, Garbayo, León, Vílchez, & Vigara, 2001). An ATP-binding cassette (ABC)-type transporter constituted by the products of the nrtA, nrtB, nrtC, and nrtD genes is involved in nitrate-nitrite uptake by the freshwater cyanobacteria (Herrero, Muro-Pastor, & Flores, 2001). ABC-type uptake permeases are encoded by nrtA, nrtB, nrtC and nrtD genes. ABC-type permeases couple ATP hydrolysis then transport the solutes across cell membranes (Flores, Frias, Rubio, & Herrero, 2005). The nitrate uptake system in plants must be versatile and robust because plants have to transport sufficient nitrate to satisfy total demand for nitrogen. The energy that drives nitrate uptake comes from the proton gradient maintained across the plasma membrane by the H^+-ATPase (Crawford, 1995).

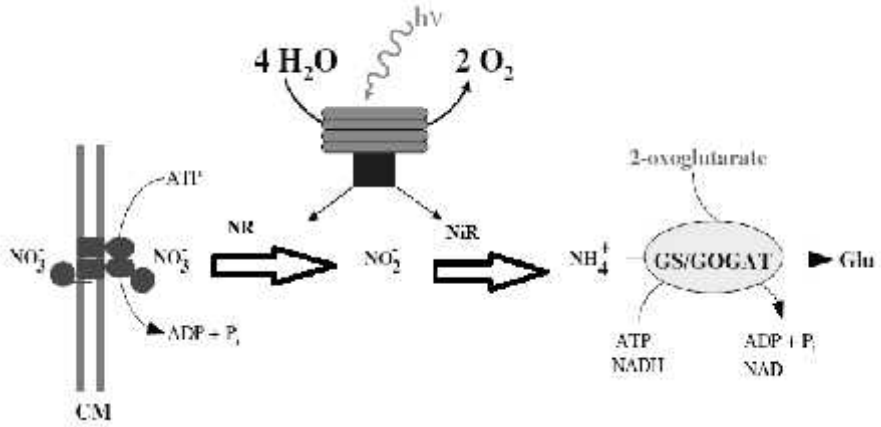

Figure 1.5 Nitrate Transportation and Assimilation (Flores, Frias, Rubio, & Herrero, 2005).

Ammonium is a kind of nitrogen source incorporated into carbon skeletons in photosynthetic organisms by ammonium assimilation pathway. In general, the nitrogen forms found in nature are oxidized forms such as dinitrogen, nitrate, and nitrite. However, ammonium is the most reduced inorganic form of nitrogen available for nitrogen assimilation process. All of these forms of nitrogen sources require their reduction to ammonium with the process which needs an energy expense. That is the reason why ammonium is preferred as a nitrogen source by the most of the photosynthetic organisms (Muro-Pastor, & Florencio, 2003). In addition, ammonium is the most energetically efficient source, since less energy is required for its uptake when compared with nitrate uptake (Perez-Garcia, Escalante, de-Bashan, & Bashan, 2011). Although ammonia appears to easily permeate biological membranes, ammonium transporters have been characterized in numerous organisms. In bacteria, fungi, and plants, high-affinity uptake of ammonium is mediated by transporters belonging to the ammonium transporter/methylamine permease/rhesus superfamily (Lanquar et al., 2009). They are monocomponent permeases which seem to be necessary for uptake of ammonium when it is present at low concentrations (i.e., below 1 mM) in the extracellular medium or when the organisms grow in a rather acidic medium (which is not the case for cyanobacteria) (Herrero, Muro-Pastor, & Flores, 2001).

b) Nitrate Reduction to Ammonium:

The assimilatory reduction of nitrate to ammonium is a significant pathway of the nitrogen cycle for environment (Flores, Frias, Rubio, & Herrero, 2005). After the uptake of nitrate into the cell, the next step in the nitrogen assimilation pathway is reduction of nitrate to nitrite (Crawford, 1995). Enzymes of nitrate reductase (NR, EC 1.7.1.1) and nitrite reductase (NiR, EC 1.7.7.1) catalyze reduction of nitrate to nitrite. First enzyme of nitrate assimilation is NR (EC 1.7.1.1) which catalyzes the nitrate reduction to nitrite forms. Reduced pyridine nucleotides are used as electron donors for the reaction. NR catalyzes the following reactions (Inokuchi, Kuma, Miyata, & Okada, 2002):

- $NO_3 + NADH + H^+ \rightarrow NO_2^- + NAD^+ + H_2O$ (1.1)

NiR (EC 1.7.7.1) catalyzes the resulting nitrite; reduction from nitrite to ammonium uses ferredoxin as the electron donor in a reaction that involves the transfer of six electrons (Inokuchi, Kuma, Miyata, & Okada, 2002):

- $NO_2^- + 6\ ferredoxin_{(red)} + 8\ H^+ \rightarrow NH_4^+ + 6\ ferredoxin_{(oxi)} + 2\ H_2O$ (1.2)

c) Ammonium Assimilation:

The ammonium resulting from nitrate reduction is incorporated into carbon skeletons via glutamate dehydrogenase (GDH) or the glutamine synthetase (GS)/glutamate synthase (GOGAT) cycle, the latter being the predominant ammonium assimilation pathway in phototrophic organisms (Flores, Frias, Rubio, & Herrero, 2005). Ammonium is catalyzed by GS (EC 6.3.1.2), which produces glutamine, and GOGAT (EC 1.4.1.14), which produces two molecules of glutamate from glutamine plus one molecule of 2-oxoglutarate (2-OG) (Lu, Yuan, Zhang, Ou, Zhou, & Lin, 2005; Vanoni, & Curti, 2005). GS transfers ammonium to glutamate to form glutamine:

- Glutamate+ ATP → γ-glutamyl phosphate + ADP (1.3)
- γ-glutamyl phosphate + NH_4^+ → Glutamine + Pi + H^+ (1.4)

Totally; Glutamate + NH_4^+ + ATP → Glutamine + ADP + Pi + H^+ (1.5)

GOGAT catalyzes glutamate formation reaction with the conversion of glutamine and 2-OG to two molecules glutamate and uses NADH for electron donor (Inokuchi, Kuma, Miyata, & Okada, 2002):

- Glutamine + 2-OG + NADH + H^+ → 2 Glutamate + NAD^+ (1.6)

1.3.1 Nitrogen Assimilation Enzymes in S. platensis

a) Nitrate Reductase (NR)

Nicotinamide adenine dinucleotide (NADH)-dependent assimilatory NR has heterodimer structure with a 45-kDa flavin adenine dinucleutide (FAD)-containing diaphorase which carries a [4Fe–4S] cluster (Figure 1.6). This enzyme uses NADH an organic cofactor that accepts electrons from ferredoxin, as its electron donor (Moreno-Vivian, Cabello, Martinez-Luque, Blasco, & Castillo, 1999). Each NADH-NR subunit contains three prosthetic groups: FAD, cytochrome b557, and molybdopterin. During the reaction, electrons donated by NADH reduce FAD which transfers the electrons to cytochrome b557. After that, electrons tunnel to the active site of Mo in the molybdopterin, where nitrate is reduced to nitrite (Glass, Wolfe-Simon, & Anbar, 2009).

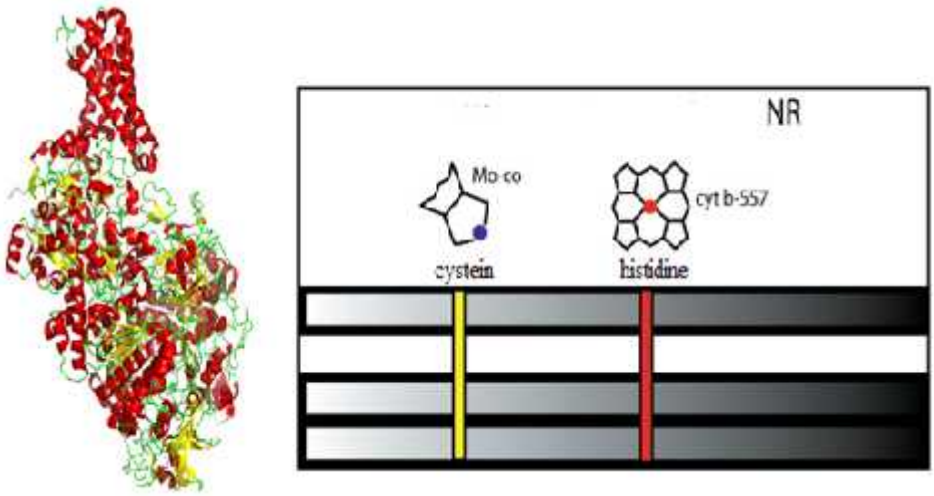

Figure 1.6 Structural diagram of NR and schematic of NR protein alignments (Glass, Wolfe-Simon, & Anbar, 2009).

b) Nitrite Reductase (NiR)

After the reduction of nitrate, a six-electron reduction of nitrite to ammonium is essential for N incorporation (Glass, Wolfe-Simon, & Anbar, 2009). NiR catalyzes the reaction which reduces nitrite to ammonium (Perez-Garcia, Escalante, de-Bashan,

& Bashan, 2011). NiR is a monomer (60-70 kDa) and iron-rich protein containing one [4Fe-4S] cluster at the active side and one siroheme as the prosthetic group (Figure 1.7). The [4Fe-4S] cluster transfers electrons from Fd to Fe in siroheme, where six electrons reduce nitrite to ammonium (Inokuchi, Kuma, Miyata, & Okada, 2002).

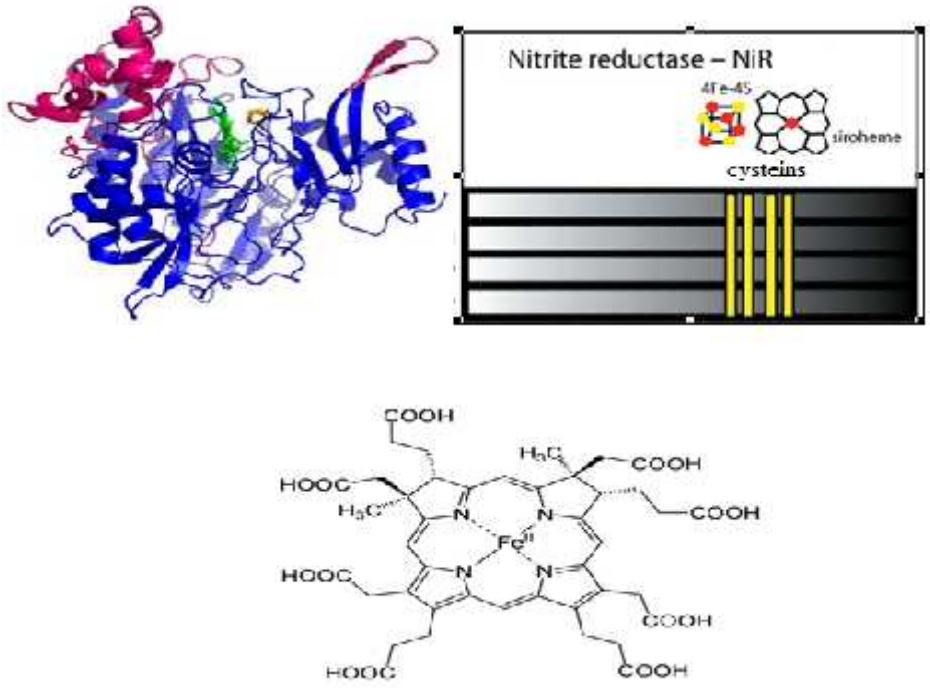

Figure 1.7 Nitrite reductase. a) Structure of ferredoxin dependent NiR and schematic of NiR protein alignments. b) The prostetic group of enzyme: Siroheme (Murphy, Siegel, Tove, & Kavin, 1974; Glass, Wolfe-Simon, & Anbar, 2009).

c) Glutamine Synthetase (GS)

GS is a large, multi-subunit protein complex and presents in thylakoid membranes. For the activity, it requires divalent cations, such as Mg^{2+} or Mn^{2+} (Inokuchi, Kuma, Miyata, & Okada, 2002; Glass, Wolfe Simon, & Anbar, 2009) (Figure 1.8). The enzyme is formed of 12 identical subunits and 12 each active sites. Each active site occurs substrate binding site (ammonium ion, nucleotide, and amino acid) containing structures is called ‘bifunnel’. Glutamate is linked at the bottom of the active site and ATP is linked to the top of the bifunnel. Divalent cations such as Mg^{2+} or Mn^{2+} locate in the middle of the biofunnel with two cation binding active sites. One of them plays

role in phosphoryl transfer of ATP to glutamate and the second stabilizes active GS and helps with the binding of glutamate. Among the GS rings; hydrogen bonding and hydrophobic interactions helps holding and stability of GS structure (Eisenberg, Gill, Pfluegl, & Rotstein, 2000).

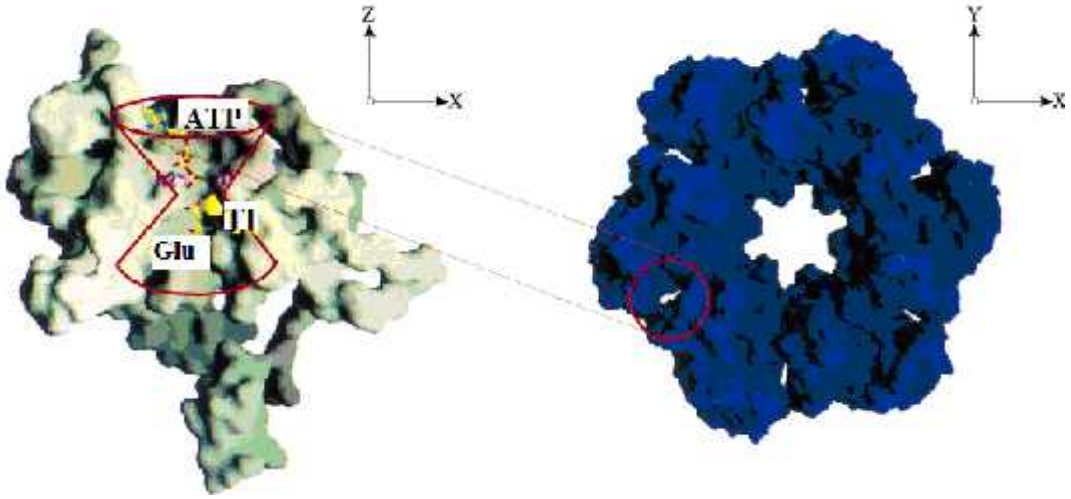

Figure 1.8 Structure of glutamine synthetase with 12 subunites

d) Glutamate Synthase (GOGAT)

GOGAT is a complex iron-sulfur flavoprotein which catalyzes the reductive transfer of L-glutamine amide group to the C-2 carbon of 2-OG, yielding two molecules of L-glutamate (Figure 1.9).

The predominant form of pyridine nucleotide dependent GOGAT uses NADH as the source of electron donor. The protein of enzyme comprises an α-subunit of 162 kDa and a β-subunit of 52.3 kDa. The structure of the α-subunit of the enzyme contains two domains, one carrying out the glutamine amidotransferase reaction and one flavin mononucleotide (FMN) and [3Fe-4S] dependent transport of electrons to 2-iminoglutarate in a similar manner (Vanoni, & Curti, 1999).

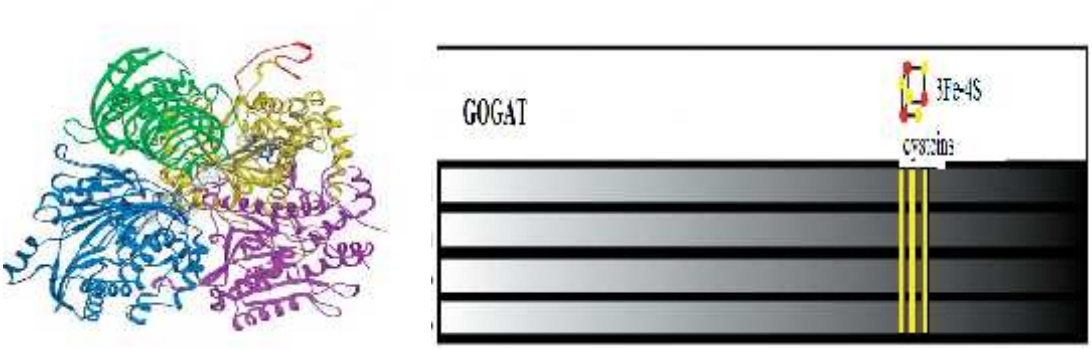

Figure 1.9 Structure of GOGAT and schematic of GOGAT protein alignments (Glass, Wolfe Simon, & Anbar, 2009).

For this process, in cyanobacteria, electrons travel from electron donor through a [3Fe-4S] cluster in GOGAT to the organic molecule FMN at the active site of glutamate formation (Ravasio et al., 2002).

1.4 Relationship with Between Nitrogen and Carbon Metabolisms

The assimilation of nitrogen into macromolecules such as amino acids and proteins requires carbon containing components in carbon cycle. Carbon and nitrogen metabolisms interact with each other in cyanobacteria (Figure 1.10) (Perez-Garcia, Escalante, de-Bashan, & Bashan, 2011). They use carbon that 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-OG) and ATP for energy and NADH to produce the amino acids glutamate, glutamine, and aspartate to assimilate of ammonium to form amino acids. In both autotrophic and heterotrophic growing cells, ATP, NADH and keto-acids are obtained from TCA cycle (Fernandez, & Galvan, 2007).

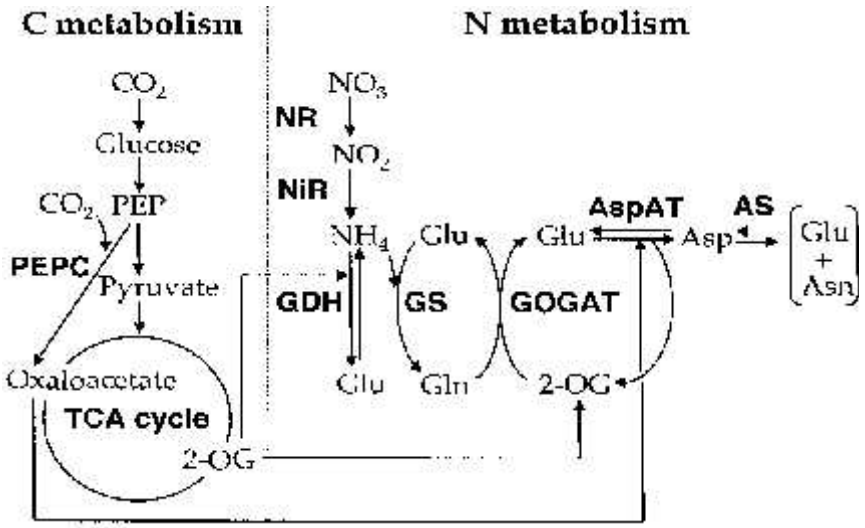

Figure 1.10 Carbon and nitrogen flow in a cell.

The aim of this study was to investigate the effects of different concentrations of various nitrogen sources (sodium nitrate: 10-180 mM; ammonium nitrate: 5-60 mM) on some nitrate assimilation enzymes such as NR, NiR, GS and GOGAT in *S. platensis*. In addition, the relationship among different concentrations of various nitrogen sources and some metabolites such as chlorophyll a, total carotenoids, proline, pyruvate, total carbohydrate and phycobiliprotein (CPC, APC and PE) contents was investigated in *S. platensis* depending on incubation period. According to the results, the best concentration and nitrogen source will be obtained for *S. platensis* growth, pigment and metabolite production and some nitrogen assimilation enzymes.

CHAPTER TWO
MATERIAL AND METHODS

In this chapter; materials and experimental processes about thesis are explained.

2.1 Microorganism and Culture Conditions

The cyanobacterium *S. platensis* (Gamont) Geitler 1952 was used in this study. The organism was provided from Çanakkale 18 Mart University, Turkey, Faculty of Aquaculture.

2.1.1 Growth Medium Composition and Growth Conditions

To prepare and maintain the inoculums, Zarrouk's medium (Tables 2.1 and 2.2) was used (Zarrouk, 1966). The utilized carbonate-bicarbonate buffer used for adjusting pH of 9.0±0.2 and growth medium was sterilized in the autoclave for 20 min. at 121°C. *S. platensis* was cultivated in batch cultures containing 750 ml of medium. Culture was inoculated to an initial optical density (600 nm) of ca. 0.2. The cultures were mixed and bubbled using filtered air continuously. Illumination at 2500 lux 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.

Table 2.1 The Zarrouk's medium composition (Tarko, Duda-Chodak, & Kobus, 2012).

Compound	**Amount (g/L)**
$NaHCO_3$	18 g
$NaNO_3$	2.5 g
K_2SO_4	1.0 g
NaCl	1.0 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
Na_2EDTA	0.08 g
$CaCl_2$	0.04 g
$FeSO_4.7H_2O$	0.01 g
Trace Element solution	1 ml

Table 2.2 Trace element solution composition of the Zarrouk's medium (Tarko, Duda-Chodak, & Kobus, 2012).

Trace Element Solution Compounds	**Amount (g/L)**
H_3BO_3	2.86
$MnCl_2.4H_2O$	1.8
$ZnSO_4.7H_2O$	0.22
$CuSO_4$	0.08
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.02
Vitamin B_{12}	$5x10^{-6}$

- The growth medium components are shown on Tables 2.1 and 2.2;

- Effects of different sodium nitrate concentrations (10, 30, 60, 100 and 180 mM) and different ammonium nitrate concentrations (5, 10, 30, and 60 mM) as nitrogen source were investigated on the growth, production of some metabolites such as chlorophyll a, total carotenoids, proline, pyruvate, total carbohydrate and phycobiliprotein (CPC, APC and PE) and activities of some

nitrate assimilation enzymes such as NR, NiR, GS and GOGAT, were investigated in *S. platensis*.

- In the studies with using sodium nitrate as nitrogen source; 10 mM sodium nitrate provided insufficient nitrogen whereas 60 and 100 mM sodium nitrate provided sufficient nitrogen for the growth and metabolism of *S. platensis.* However 180 mM sodium nitrate had excess nitrogen and it had inhibitory effect on the growth and metabolism of this organism.

- In the studies with using ammonium nitrate as nitrogen source; 5 mM ammonium nitrate provided insufficient nitrogen whereas 10 mM ammonium nitrate was the optimum condition for the growth and metabolism of *S. platensis*. However 30 and 60 mM ammonium nitrate had excess nitrogen and it had inhibitory effect on the growth and metabolism of this organism.

2.2 Methods

For this thesis; all methods were assayed spectrophotometrically. Additionally, biomass and pH values were measured.

2.2.1 Preparing Crude Extract

The cells were harvested periodically depending on incubation days by centrifugation (12000 x g, 10 min, 4°C), were washed with distilled water. The precipitated cells were weighted and phosphate buffer (pH 7.0) added by a rate 12.5 mL for 1 g cells. The cells were homogenized by 8000 rpm 1 min. and 9500 rpm 1 min. with 30 seconds intervals. Cell debris was removed by centrifugation at 12000 x g, 4°C for 10 min. After the centrifugation; the obtained supernatant was used for the determination of protein levels and phycobiliprotein, total carbohydrate and pyruvic acid contents.

2.2.2 Analysis of Samples

2.2.2.1 Measurement of Optical Density (OD)

For measuring optical density of cells; 1 mL cell suspension was put into the quartz cell and absorbance was measured at 600 nm. Increasing of optical density was observed during the incubation period for 17 days.

2.2.2.2 Determination of Dry Biomass

For determination of dry biomass; 1 mL cell suspension was put on watch glass that was previously tarred and waited in the drying-oven for 2 hours at 105°C. Sample was cooled in the desiccator and weighted. Dry biomass amount was calculated from the difference among the sample and tare of watch glass.

2.2.2.3 Determination of pH

pH levels of *S. platensis* were measured by using pH-metre during the incubation period.

2.2.2.4 Determination of Protein Concentrations

Protein concentration of crude extract was determined by the method of Bradford (1976). Preparing of the solutions that were used in Bradford method:

Dye Stock Reagent: 100 mg Commassie Brillant Blue G-250 was solved in 50 mL 95% ethanol solution. 12.5 mL H_3PO_4 85% was added and total volume was adjusted 1000 mL with distilled water. Solution was kept in a dark bottle at 4°C.

Absorbances of the samples were measured at 595 nm. 900 µL of dye reagent was added to 100 µL of sample and after waiting for 2 min. absorbance was measured against the blank. The blank consisted 100 µL distilled water instead of sample.

Calibration curve was drawn with using bovine serum albumin as standard with known concentrations (0-200 μg/L). Protein levels were calculated from the calibration equation as y=0.06x (R^2: 0.9834).

2.2.2.5 Determination of Chlorophyll a and Total Carotenoid Contents

Chlorophyll a and total carotenoids contents were measured as described by Lichtenthaler and Wellburn (1983). 5 mL of algal suspension was centrifuged at 12000 x g, 4°C for 10 min. Pellet was weighted and homogenized in 5 mL of ethanol 95% by 8000 rpm 1 min. and 9500 rpm 1 min. with 30 seconds intervals. Cell debris was removed by centrifugation at 12000 x g, 4°C for 10 min. After the centrifugation; absorbance of the obtained supernatant was measured at 470, 664.2 and 648.6 nm. Calculations were shown as it below:

Chl a: 13.36 x $A_{664.2}$ – 5.19 x $A_{648.6}$
Chl b: 27.43 x $A_{648.6}$ –8.12 x $A_{664.2}$
Chl a + Chl b: 5.24 x $A_{664.2}$ – 22.24 x $A_{648.6}$
Total Carotenoids: (1000 x A_{470} – 2.13 x Chl a – 97.64 x Chl b)/209

- Results were divided cell weight and unit was shown as μg/g.

2.2.2.6 Determination of Phycobiliprotein (CPC, APC, PE) Contents

Phycocyanin, allophycocyanin and phycoerythrin contents were determined by the method of Tarko, Duda-Chodak and Kobus (2012). Absorbances of crude extract at 562, 620 and 652 nm gives phycobiliprotein (CPC, APC and PE) contents. Calculations were shown as it below:

CPC: [(A620 – 0.474 x A652)/5.34]/ g wt
APC: [(A652 – 0.208 x A620)/5.09]/ g wt
PE: [(A652 – 2.41 x CPC – 0.849 x APC)/9.62]/ g wt

- Units of CPC, APC were shown as mg/ g whereas unit of PE was shown as µg/g.

2.2.2.7 Determination of Proline Content

Proline content was assayed according to the method of Bates, Waldren and Teare (1973). In the process acid-ninhydrine reagent was used.

Acid-ninhydrin reagent: Prepared by warming 1.25 g ninhydrin in 30 mL glacial acetic acid and 20 mL 6 M phosphoric acid, with agitation, until dissolved. Kept cool (stored at 4°C) the reagent remains stable 24 hours.

For Crude Extract: Cell suspension was centrifuged at 12000 x g, 4°C for 10 min. The precipitated cells were weighted and 3% aqueous sulphosalicylic acid added by a ratio 10 mL for 1 g cells. The cells were homogenized by 8000 rpm 1 min. and 9500 rpm 1 min. with 30 seconds intervals. Cell debris was removed by centrifugation at 12000 x g, 4°C for 10 min. The obtained extract was used for proline assay process.

1 mL of extract was reacted with 1 ml acid-ninhydrin reagent and 1 mL of acetic acid glacial in a test tube for 1 hour at 100°C and mixture was transferred to ice-bath immediately. The reaction mixture was extracted with 2 mL toluene, mixed vigorously about 15 seconds and left at room temperature for 30 min. until separation of the two phases. The chromophore-containing toluene (1 mL, upper phase) was warmed to room temperature and its optical density was measured at 520 nm using toluene for a blank. The proline concentration was determined from a standard curve using L-Proline with known concentrations (10-80 µg/mL). Amounts (µg/mL) of proline were determined from the standart curve equation as $y=0.0241x$ (R^2: 0.9899) and calculated on a fresh weight basis as follows:

[(µg proline/mL x mL toluene)/115.5 µg/ µmole]/ [(g sample)/5] = µmoles proline/ g of fresh material

2.2.2.8 Determination of Pyruvate Content

Pyruvate content was determined with using 2,4-dinitrophenyllhydrazine as colorant reagent by the method of Friedeman and Haugen (1943).

2, 4-dinitrophenyllhydrazine reagent: 0.1 g of 2,4-dinitrophenyllhydrazine was dissolved in 100 mL of 2N HCl solution. The reagent was stored in dark bottle at 4°C.

1 mL of 2, 4-dinitrophenyllhydrazine reagent was added to 600 µL of crude extract, vortexed and waited for 5 min. 1.2 mL of 2N NaOH solution was added to the mixture and vortexed again. Its optical density was measured at 520 nm. Distilled water was used instead of crude extract in the blank. The amount of pyruvate content was calculated from standart curve with using known concentrations (1-25 µg/mL) of pyruvic acid as standart. Amounts (µg/mL) of pyruvate were determined from the standart curve equation as y=0.0325x (R^2: 0.9977) and calculated on a fresh weight as µg/g.

2.2.2.9 Determination of Total Carbohydrate Content

Total carbohydrate content was determined by phenol-sulphuric acid method of Dubois, Gilles, Hamilton, Rebers and Smith (1956). According to the assay procedure; 1 mL of crude extract, 1 mL of phenol 5% and 5 mL of concentrated H_2SO_4 was mixed in a test tube, vortexed and waited for 20 min. Optical density of the mixture was measured at 470 nm. The amount of total carbohydrate content was calculated from the standard curve with using known concentrations of (10-200 µg/mL) glucose monohydrate as standard. Amounts (µg/mL) of total carbohydrate were determined from the standard curve equation as y=0.0066x (R^2: 0.9981) and calculated on a fresh weight as mg/g.

2.2.2.10 Nitrate Uptake Assay

Nitrate uptake rate was assayed by the method of Bartzatt and Donigan (2004).

For crude extract; cell suspension was centrifuged at 12000 x g, 4°C for 10 min. Obtained supernatant was used for nitrate uptake assay.

Diphenylamine reagent: 0.1 g of diphenylamine was dissolved in 30 mL of 14.4 M H_2SO_4 solution. The reagent was stored in dark bottle at 4°C.

The method was based on measuring nitrate depletion from external medium. To 100 µL of spent medium, 450 µL diphenylamine reagent and 425 µL of 18 M H_2SO_4 were added. The level of nitrate was determined at 600 nm. The amount of nitrate ions was determined using known amounts (0.05-0.5 mM) of sodium nitrate as standard. Concentrations (mM) of nitrate from external medium were determined from the standard curve equation as $y=2.01x$ (R^2: 0.9966).

2.2.2.11 Ammonium Uptake Assay

For crude extract; cell suspension was centrifuged at 12000 x g, 4°C for 10 min. Obtained supernatant was used for ammonium uptake assay.

The reagent solutions used for phenol-hypochlorite method (Weatherburn, 1967) were prepared as follows:

Phenol reagent solution was prepared by mixing equal volume from solution A_1 and B_1. 5 g of phenol was dissolved in 50 mL of distilled water (solution A_1), and 25 mg of sodium nitroprusside was dissolved in 50 mL of distilled water (solution B_1).

Alkaline hypochlorite solution was prepared by mixing equal volume from solution A_2 and B_2. Solution A_2: 5 g of sodium hydroxide was dissolved in 100 mL

of distilled water. Solution B_2 was commercial hypochlorite solution containing 26 g/L of NaOCl.

The first step of the analysis method was to incubate 2.0 mL of supernatant at room temperature for 15 min. Later, 500 µL of phenol solution and 500 µL of alkaline hypochlorite solution were respectively added to incubated sample. The last solution was mixed with vortex and incubated for 5 min. in 60°C-water bath. The absorbance was measured at 630 nm against a blank sample. The blank sample was prepared by using 2.0 mL of distilled water instead of the sample supernatant and the same analysis procedure was followed. Standard curve was prepared in the range of 0-500 µg of ammonium sulfate ($(NH_4)_2SO_4$) per mL of liquid, and the same procedure was performed. Concentrations (mM) of ammonium from the external medium were determined from the standard curve equation as $y=0.1299x$ (R^2: 0.9982).

2.2.2.12 Enzyme Assays

2.2.2.12.1 Nitrate Reductase (NR) Assay. NR activity was determined by measuring NADH dependent production of nitrite.

NR activity was determined by the method of Redinbaugh and Campbell (1985). For crude extract: 1 g of frozen/thawed alga was weighted and suspended in 10 mL of 0.2 M phosphate buffer (pH 7.5) with 5 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM dithiothreitol (DTT) (Granbom et al., 2004). The cells were homogenized by 8000 rpm 1 min., 9500 rpm 1 min., 13500 rpm 1 min., 20500 rpm 1 min. and 24000 0.5 min. with 30 seconds intervals. Clear extract was obtained after centrifugation at 12000 x g, 4°C for 10 min. and used for NR enzyme activity assay. The assay solution consists of 1.80 mL of 25 mM phosphate buffer (pH 7.5) contained 10 mM sodium nitrate and 0.05 mM EDTA, and 0.1 mL of extract. To start the reaction 0.1 mL of 2 mM NADH was used as reducing donor. The blank was lacked NADH. The assay solution was vortexed and incubated for 2 min. and the temperature was 30°C. To colourize the mixture; 1 mL of 58 mM sulfanilamide

in 3M HCl plus 0.77 mM N-(1-Naphthyl) ethylenediamine dihydrochloride (NED) was added and the mixture was stayed for 10 min. at 30°C. A pink colour was developed and nitrite concentration, measured at 540 nm. Concentrations of occurring nitrite ions were determined with using known amounts (3-30.4 µM) of sodium nitrite by the standard curve equation as y=0.0466x (R^2: 0.9975). One unit of NR was shown as the quantity of enzyme needed for producing 1 µmol of nitrite per minute at 30°C.

2.2.2.12.2 Nitrite Reductase (NiR) Assay. The enzymatic activity of nitrite reductase (NiR) was determined with using Losada and Paneque method (1971).

For crude extract; 1 g of frozen/thawed alga was weighted and suspensed in 5 mL of 20 mM phosphate buffer (pH 7.4). The cells were homogenized by 8000 rpm 1 min., 9500 rpm 1 min., 13500 rpm 1 min., 20500 rpm 1 min. and 24000 1 min. with 30 seconds intervals. Clear extract was obtained after centrifugation and used for NiR enzyme activity determination.

For assay process; 100 µL of extract was incubated in the reaction mixture with 0.4 mL of 0.1 M phosphate buffer (pH 7.4), 100 µL of 15 mM sodium nitrite, 200 µL of 5 mM methyl viologen (MV) and 200 µL of 86.15 mM sodium dithionite in 190 mM sodium bicarbonate. Occurring violent color was stopped by stirring on vortex thus reaction was stopped. For colourizing nitrite ions; 2 mL of 15% HCl containing 1% sulfanilamide and 2 mL of 0.02% solution of NED was added the reaction mixture. A pink colour was developed and nitrite concentration, measured at 540 nm. The blank consisted of all the components except for sodium nitrite. The concentrations of reduced nitrite ions were obtained using known amounts (12-100 µM) of nitrite as standard by the standard curve equation as y=0.0145x (R^2: 0.9975). One unit of NiR was shown as the quantity of enzyme needed to reduce 1 µmol of nitrite per minute at 30°C.

2.2.2.12.3 Glutamine Synthetase (GS) Assay. GS activity was determined with using of Berteli, Corrales, Guerrero, Ariza, Pilego and Valpuesta (1995) processes as the hydroxamate biosynthetic method.

For crude extract; 1.0 g of the frozen/thawed alga extracted in 5 mL of 0.1 M HEPES buffer (pH 7.6) with 5 mM DTT, 0.2 mM EDTA and 10 mM $MgCl_2$ at 0-4 °C. The cells were homogenized by 8000 rpm 1 min., 9500 rpm 1 min., 13500 rpm 1 min. and 20500 rpm 1 min. with 30 seconds intervals. Clear extract was obtained after centrifugation at 12000 x g, 4°C for 10 min. and used for GS enzyme activity assay.

Reaction solution contained 600 µL of 0.25 M Tris-HCl buffer (pH 7.0), 200 µL of 0.03 M ATP (pH 7.0), 200 µL of 0.5 M $MgSO_4$, 200 µL of 1M hydroxylamine hydrochloride (pH 7.0) and 500 µL of extract. The solution was incubated at 30°C for 30 min. and γ-glutamylhydroxamate (GGH) as brown complex was formed after the addition of 750 µL $FeCl_3$ solution. $FeCl_3$ solution consisted of 0.37 M $FeCl_3$, 0.2 M trichloroacetic acid (TCA) and 0.67 M HCl.

The amount of the brown GGH-complex was measured at 540 nm. The blank contained all of the components except for ATP. The amount of occurring GGH-complex determined with the standard curve equation using known amounts (50-200 µg) of GGH as $y=0.9128x$ (R^2: 0.9999). One unit of GS was shown as the amount of enzyme needed to produce 1 µmol of GGH-complex per minute at 30°C.

2.2.2.12.4 Glutamate Synthase (GOGAT) Assay. NADH dependent GOGAT activity is determined by the method of Chen and Cullimore (1988).

For crude extract; 1 g of frozen/thawed alga was weighted and suspensed in 5 mL of 20 mM phosphate buffer (pH 7.5) with 0.5 mM EDTA and 0.1 mM DTT at 4°C. The cells were homogenized by 8000 rpm 1 min., 9500 rpm 1 min. and 13500 rpm 1 min. with 30 seconds intervals. Clear extract was obtained after centrifugation and used for enzymatic activity ass

The assay solution contained 500 µL of 40 mM phosphate buffer (pH 7.5), 100 µL of 10 mM 2-OG, 100 µL of 10 mM L-glutamine, 50 µL of 0.14 mM NADH and 250 µL of crude extract. The blank was contained 750 µL of 40 mM phosphate buffer (pH 7.5) instead of NADH, 2-OG and glutamine. After the enzyme addition; reaction was started. NADH oxidation was watched for 2 min. and measured at 340 nm. The enzyme activity was calculated as nmol mL^{-1}.

2.3 Statistical Analysis

For statistical significance analyses, the Tukey test was used. The values were determined after three separate experiments. Comparisons were also made with Pearson correlation.

CHAPTER THREE
RESULTS AND DISCUSSION

Changes in nitrate/ ammonium uptake , OD_{600}, dry biomass, pH and protein levels, also, CPC, APC, PE, chlorophyll a, total carotenoids, proline, pyruvate and total carbohydrate contents and some nitrate assimilation enzyme activities in *S. platensis* were investigated in the Zarrouk's medium with different sodium nitrate (10-180 mM) and ammonium nitrate (5-60 mM) concentrations during the incubation period.

3.1 Effects of Different Sodium Nitrate Concentrations on Some Nitrogen Assimilation Enzymes and Metabolites in *S. platensis*

Standard Zarrouk's medium containing 30 mM sodium nitrate was used as control in our experiments. Similarly, the medium is used for industrial production of *Spirulina* (Gershwin, & Belay, 2008).

3.1.1 Variations at Nitrate Uptake Levels Depending on Sodium Nitrate Concentrations in S. platensis

The cyanobacterium cells contain accounting from 1 to 10% of nitrogen. The cyanobacteria can utilize variable nitrogen sources mainly nitrate and ammonium in assimilation processes. In nature, nitrogen is found in oxidized forms. Nitrate is a kind of oxidized form of nitrogen (Perez-Garcia, Escalante, de-Bashan, & Bashan, 2011). The reason of the preference of sodium nitrate as nitrogen source is being oxidized form in environment. The first step of nitrate assimilation is transporting it into the cytoplasm. It is transported into the cytoplasm by the specific transporters and an ATP molecule is used (Devriese, Tsakaloudi, Garbayo, León, Vílchez, & Vigara, 2001). In addition, nitrate uptake into the cell is a limiting step for the growth and production of the metabolites and pigments with contributing the structure of them. In the growth medium of *S. platensis*, nitrate uptake rate accelerated with increasing nitrate concentration. As shown in Figure 3.1; on the 10^{th} day of incubation period, levels of extracellular nitrate depletion 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; 59.99±2.9%, respectively. According to the results, the highest nitrate uptake was observed in the medium containing 30 mM sodium nitrate. After the 14th day of the incubation period, nitrate consumption from the external medium was decreased. In the media containing 60, 100 and 180 mM sodium nitrate, it was not consumed completely. In the media containing 10 and 30 mM sodium nitrate, it was almost consumed completely and external nitrate concentrations were 0.102±0.01 and 0.406±0.01 mM, respectively, on the 17th day of incubation period.

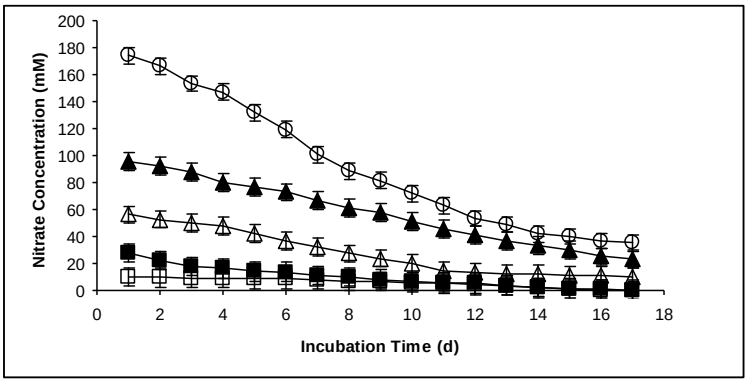

Figure 3.1 Variations of nitrate uptake levels in *S. platensis* depending the incubation period in media containing different sodium nitrate concentrations [10 mM (□); 30 mM (■); 60 mM (Δ); 100 mM (▲); 180 mM (o)]. The values are the mean ± SD for experiments of three separate experiments.

3.1.2 Variations at the Growth, Dry Biomass, Protein and pH Levels Depending on Sodium Nitrate Concentrations in S. platensis

Cyanobacteria are generally cultivated in the growth media containing many essential minerals and ions. Zarrouk's medium is optimum for *S. platensis* growth with rich mineral and ion contents such as Ca, Mg, Mn, Zn, Cu, Mo, nitrate, bicarbonate and sulfate (Öztürk Ürek, & Tarhan, 2012). Nitrate is an essential nutrient for algal growth, so sufficient amounts of nitrate has a stimulant effect on biomass, metabolites and activities of nitrogen assimilation enzymes in *S. platensis*. By this time, generally effect of 10 mM to 60 mM sodium nitrate concentrations were investigated for nitrate assimilation enzymes in *S. platensis* (Devriese, Tsakaloudi, Garbayo, León, Vílchez, & Vigara, 2001; Jha, Ali, & Raghuram, 2007;

Logeswaran, Kohli, & Vani, 2011). It was investigated that the effect of 10-180 mM sodium nitrate concentrations on the growth, activities of some nitrate assimilation enzymes and production of some metabolites in *S. platensis* (Gamont) Geitler organism. The use of some excess sodium nitrate concentrations (60 and 100 mM) caused an increase in the biomass production whereas 180 mM caused inhibition on it in our studies. In addition 10 mM sodium nitrate was contained insufficient nitrate for the growth, production of pigments and metabolites and some nitrogen assimilation enzyme activities in *S .platensis*.

In the growth media containing different sodium nitrate concentrations, growth rate of *S. platensis* determined by OD_{600}, dry biomass, protein and pH levels depending on the incubation time (Figure 3.2). In our investigations, optical density of *S. platensis* showed linear rise until 14^{th} day of incubation time in all the tested nitrate concentrations. After the 14^{th} day of incubation period, there were not important changes in OD_{600} levels. As seen in Figure 3.2a, the highest growth of organism was determined by OD_{600} levels and it was observed on the 14^{th} day in the medium containing 100 mM sodium nitrate. On the other hand, 10 mM sodium nitrate provided insufficient nitrogen for *S. platensis* growth. OD_{600} levels measured in the medium containing 10 mM sodium nitrate were almost lower when compared with the control. However, OD_{600} levels measured in the media containing 60, 100 and 180 mM sodium nitrate were higher than control. Increasing of OD_{600} may be associated with dry biomass and protein levels. The highest dry biomass was determined on the 14^{th} day as 95.06±3.4 mg mL^{-1} in the medium containing 100 mM sodium nitrate (Figure 3.2b). However in the presence of 10 mM sodium nitrate, the maximum dry biomass was approximately 1.3 times lower than control condition ($p<0.05$). On the 14^{th} day of incubation period, the dry biomass of the organism was obtained as 31.1±1.1 in the medium containing 10 mM sodium nitrate. In our investigations, dry biomass results in presence of 30 mM sodium nitrate were in accordance with the results of Tarko, Duda-Chodak and Kobus (2012). According to the results; dry biomass levels in the presence of some excess sodium nitrate concentrations (60 and 100 mM) were almost 2.5-3 times higher than the results of Tarko, Duda-Chodak and Kobus (2012) ($p<0.05$). After the 14^{th} day of incubation

period, dry biomass levels did not show important change in all growth media. Protein levels increased with high sodium nitrate concentrations. The reason of this may base on protein structure. Protein structure involves high percentage of nitrogen. Excess nitrogen concentrations might have stimulant effect on protein production when compared to control. The highest protein content was determined on the 15th day in the medium containing 100 mM sodium nitrate as 315±9.8 ppm (Figure 3.2c). It was almost 1.43 times higher than control ($p<0.05$). Insufficient sodium nitrate concentration (10 mM) inhibited growth and protein levels of the organism. The lowest growth and protein level of this organism was determined in the medium containing 10 mM nitrate. After the 15th day of incubation period, protein content of *S. platensis* did not change in all media. pH values were similar in the media containing 10, 30 and 60 mM nitrate (9-10.5) and higher than the media containing 100 and 180 mM nitrate (9-10.17). There were not important pH differences between the tested nitrate concentrations (Figure 3.2d).

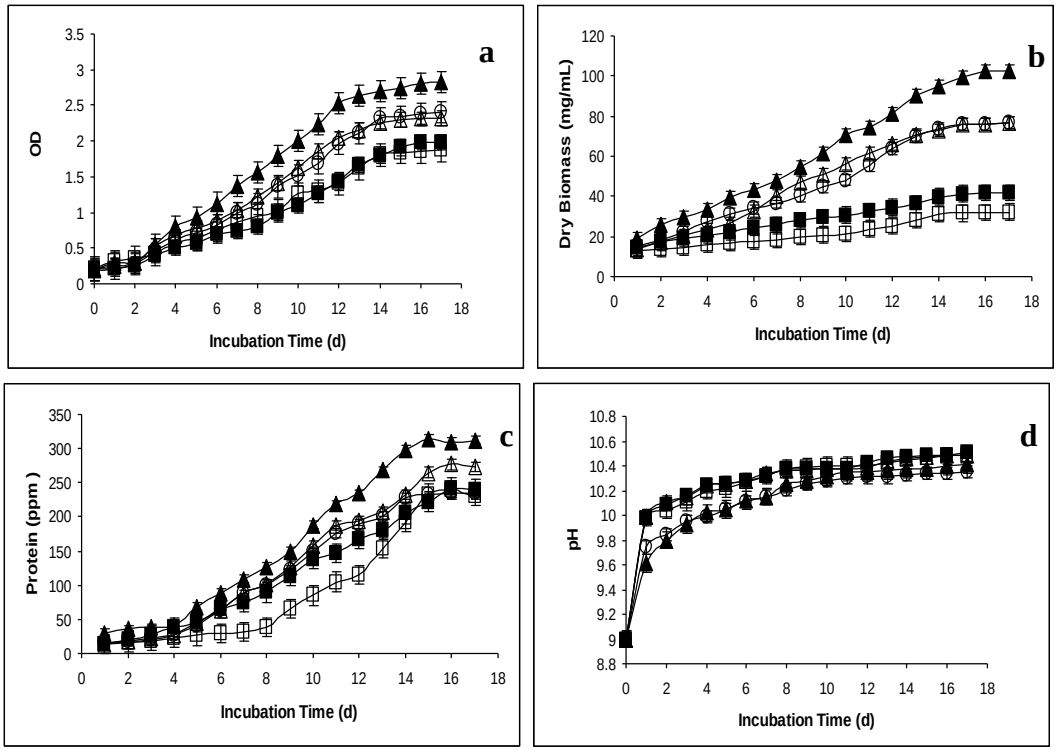

Figure 3.2 Variations of a) OD_{600}, b) dry biomass, c) protein, and d) pH levels in *S.platensis* depending the incubation period in media containing different sodium nitrate concentrations [10 mM (□); 30 mM (■); 60 mM (Δ); 100 mM (▲); 180 mM (o)]. The values are the mean ± SD for experiments of three separate experiments.

3.1.3 Variations on the Pigment Productions Depending on Sodium Nitrate Concentrations in S. platensis

Chlorophyll a and total carotenoids contents of *S. platensis* depending on different sodium nitrate concentrations are depicted in Figure 3.3. The highest chlorophyll a content was determined in the medium containing 100 mM nitrate as 106.3±5.2 μg g^{-1} on the 14th day of incubation period (Figure 3.3a). It was almost 1.56-fold higher than control ($p<0.05$). As depicted in Figure 3.3b, the highest total carotenoids content of *S. platensis* was estimated as 33.002±1.3 μg g^{-1} on the 14th day in the medium containing 100 mM sodium nitrate. It was approximately 1.36 times higher ($p<0.05$). 180 mM sodium nitrate inhibited total carotenoids production and the maximum total carotenoids content was determined as 24.86±1.0 μg g^{-1} on the 14th day of incubation period. However 10 mM sodium nitrate did not provide enough nitrogen for chlorophyll a synthesis. In this medium, the maximum chlorophyll a and total carotenoids contents were determined as 60.99±0.65 μg g^{-1} and 21.552±0.21 μg g^{-1}, respectively, on the 14th day of incubation period.

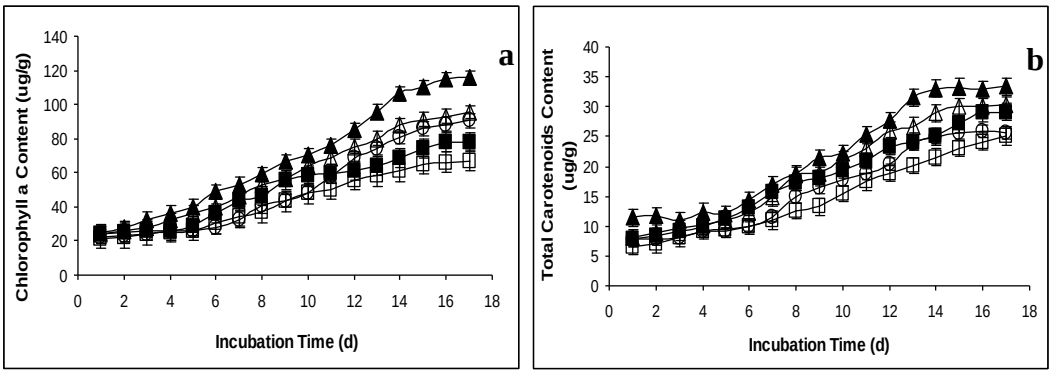

Figure 3.3 Variations of a) chl a and b) total carotenoids contents in *S. platensis* depending the incubation period in media containing different sodium nitrate concentrations [10 mM (□); 30 mM (■); 60 mM (Δ); 100 mM (▲); 180 mM (o)]. The values are the mean ± SD for experiments of three separate experiments.

C-phycocyanin (CPC), allophycocyanin (APC) and phycoeritrin (PE) contents of *S. platensis* were determined depending incubation period in the growth media of *S. platensis*. As depicted in Figure 3.4, the highest CPC, APC and PE contents determined as 1.406±0.05 mg g^{-1}, 0.47±0.01 mg g^{-1} and 222±8.7 μg g^{-1}, respectively,

in the medium containing 100 mM sodium nitrate on the 12th day of incubation period. The highest CPC content was determined in the medium containing 100 mM sodium nitrate on the 12th day of incubation period and it was 1.37 times higher than that's of control ($p<0.05$). Insufficient sodium nitrate concentration (10 mM) was inhibited phycobiliprotein production when compared with the control. The maximum CPC, APC and PE contents were determined as 0.865±0.02 mg g^{-1}, 0.298±0.01 mg g^{-1}, and 65.625±2.6 µg g^{-1}, respectively, in *S. platensis*. The results were almost 1.2, 1.19, and 1.18 times lower respectively, when compared with the control ($p<0.05$).

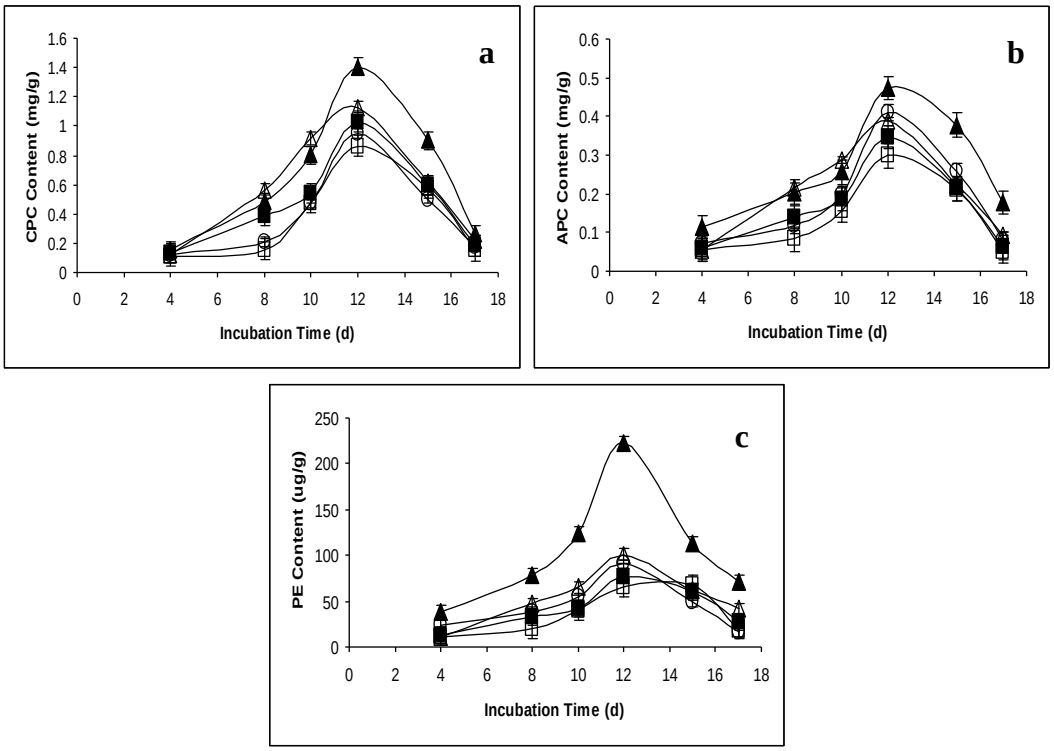

Figure 3.4 Variations of a) CPC, b) APC, and c) PE contents in *S. platensis* depending the incubation period in media containing different sodium nitrate concentrations [10 mM (□); 30 mM (■); 60 mM (Δ); 100 mM (▲); 180 mM (o)]. The values are the mean ± SD for experiments of three separate experiments.

According to the results; pigment contents (phycobiliproteins, total carotenoids and chlorophyll a) of the medium containing 10 mM sodium nitrate was almost lower when compared with the control medium. This situation shows that 10 mM sodium nitrate concentration did not provide enough nitrogen for algal pigment

production. The insufficient production of pigments in the presence of 10 mM sodium nitrate can be associated with the structures of pigments. Porphyrin rings of the chlorophyll a structures contain nitrogen atom as ringed groups of phycoerythrobilin and allophycocyanin. Thus pigment production requires enough nitrogen in the growth media. In addition, 180 mM sodium nitrate usage as nitrogen source resulted in reduction of phycobiliproteins, chlorophyll a and total carotenoids contents for all the tested concentrations when compared with control. This situation can be explained by the nitrate toxicity in biological components. Nitrate ions convert nitrite ions and nitrite ions oxidize the iron atoms and they are unable to carry oxygen (Kim-Shapiro, Gladwin, Patel & Hogg, 2005) and this is known as lack of oxygen generally. Thus this causes damages on growth, enzymatic and metabolic activities of organisms.

3.1.4 Variations on the Proline, Pyruvate and Total Carbohydrate Contents Depending on Sodium Nitrate Concentrations in S. platensis

Proline is an imino acid. Nitrogen concentration of the growth medium is highly important for its biosynthesis. It derives from L-glutamate with a group of reactions (Nelson, & Cox, 2005). This shows that nitrogen concentration and assimilation is very important for proline production. Proline contents of *S. platensis* depending on different nitrate concentrations with respect to incubation time are shown in Figure 3.5a. According to the results, the highest proline content was obtained in the medium containing 100 mM nitrate. It was obtained as 36.5±1.3 μmol g^{-1} on the 12^{th} day of incubation period. It was almost 2.41 times higher than control condition ($p<0.05$). This can be related with the optimum biomass production in the medium containing 100 mM nitrate. The highest biomass content of organism had the highest amount of structural components such as aminoacids. However, the maximum proline content was determined as 10.38±0.4 μmol g^{-1} in the medium containing 10 mM sodium nitrate on the 12^{th} day of incubation period. It was almost 1.5-fold lower than control ($p<0.05$).

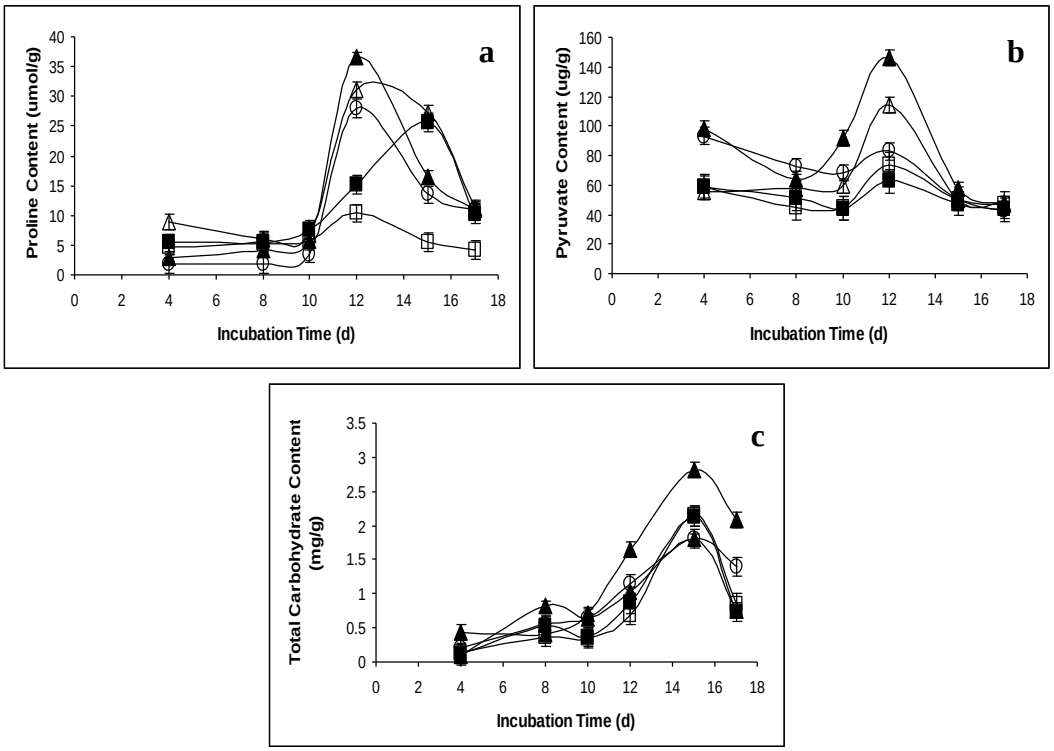

Figure 3.5 Variations of a) proline, b) pyruvate, and c) total carbohydrate contents in *S. platensis* depending the incubation period in media containing different sodium nitrate concentrations temperature [10 mM (□); 30 mM (■); 60 mM (Δ); 100 mM (▲); 180 mM (o)]. The values are the mean ± SD for experiments of three separate experiments.

Total carbohydrate and pyruvate contents affect nitrate assimilation. Carbon and nitrogen metabolisms are strongly linked with each other. The energy provided from TCA cycle is used for the enzymatic reactions as electron transport. Additionally, pyruvate supports TCA cycle thus affects 2-OG concentration. 2-OG transforms glutamate with transamination reaction and this affects GS/GOGAT pathway (Inokuchi, Kuma, Miyata, & Okada, 2002). It can be a conclusion that 2-OG and pyruvate play as allosteric regulators for GS and GOGAT activities thus nitrate assimilation metabolism. Pyruvate and total carbohydrate contents of *S. platensis* depending on different nitrate concentrations with respect to incubation time are shown in Figure 3.5. The highest pyruvate content was obtained on the 12th day of incubation period as 146±7.0 μg g^{-1} (Figure 3.5b) in the medium containing 100 mM nitrate. It was almost 2.35 times higher than control ($p<0.05$). The highest total carbohydrate content was determined as 2.824±0.1 mg g^{-1} in the same medium on the

15th day of incubation time. However, 10 mM sodium nitrate was insufficient for metabolite production and the lowest total carbohydrate and pyruvate contents of *S. platensis* were determined in this medium.

3.1.5 Variations on Some Nitrogen Assimilation Enzyme Activities Depending on Sodium Nitrate Concentrations in S. platensis

Nitrate is primary nitrogen source and has an important effect on growth and metabolism of cyanobacteria. Differences at sodium nitrate concentrations affect enzymatic activities in nitrate assimilation in cyanobacteria (Sood, Chanda, & Singh, 2002; Ali, Sivakami, & Raghuram, 2007; Jha, Ali, & Raghuram, 2007; Logeswaran, Kohli, & Vani, 2011). In nitrate assimilation processes, nitrate ions are transported into the cytoplasm and then reduced to ammonium ions. The first enzyme of nitrate assimilation pathway is NR (Perez-Garcia, Escalante, de-Bashan, & Bashan, 2011). Its activity was measured depending incubation period in the growth media of *S. platensis* containing different concentrations of sodium nitrate. The highest NR activity was obtained in the medium containing 100 mM sodium nitrate on the 12th day as 74.67±2.65 U mL^{-1} (Figure 3.6a). It was 2.2 times higher when compared to control condition ($p<0.05$). However 180 mM sodium nitrate inhibited NR activity and it caused approximately 2.71 times decreasing when compared with the activity in the medium containing 100 mM sodium nitrate ($p<0.05$). NiR is the second enzyme that is responsible for transportation of nitrate to ammonium ions. Maximum NiR activities were determined on the 10th day in all growth media (Figure 3.6b). The highest NiR activity was determined as 600.83±19.5 U mL^{-1} in the medium containing 100 mM sodium nitrate. This activity was approximately 1.2 times higher than that of control condition ($p<0.05$).

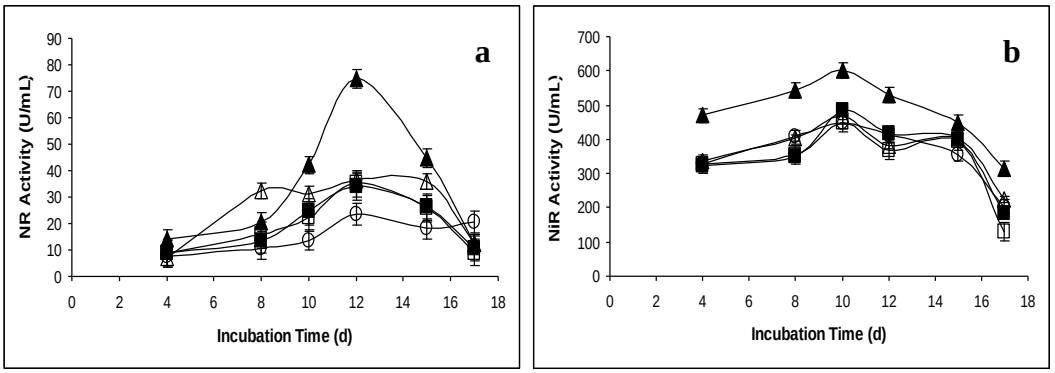

Figure 3.6 Variations of a) NR and b) NiR activites in *S. platensis* depending the incubation period in media containing different sodium nitrate concentrations [10 mM (□); 30 mM (■); 60 mM (Δ); 100 mM (▲); 180 mM (o)]. The values are the mean ± SD for experiments of three separate experiments.

Figures of specific activities were generated depending on maximum volume activities of NR and NiR (12^{th} day and 10^{th} day, respectively) with different sodium nitrate concentrations (Figure 3.7). The highest specific activity of NR was obtained as 32.09±1.13 U mg^{-1} protein in the medium containing 100 mM sodium nitrate. It was almost 1.78-fold higher than control ($p<0.05$). Insufficient sodium nitrate concentration caused inhibition on the specific activity of NR. The lowest specific activity of NR was estimated in the medium containing 10 mM sodium nitrate as 13.38±0.8 U mg^{-1} protein (Figure 3.7a). It was almost 1.46-fold lower than control ($p<0.05$). Similarly, 100 mM sodium nitrate concentration stimulated specific activity of NiR. The highest specific activity of NiR was determined as 107±3.7 U mg^{-1} protein (Figure 3.7b). It was almost 1.9 times higher than control ($p<0.05$). 10 mM sodium nitrate caused inhibition on the specific activity of NiR. The lowest specific activity of NiR was determined in this medium.

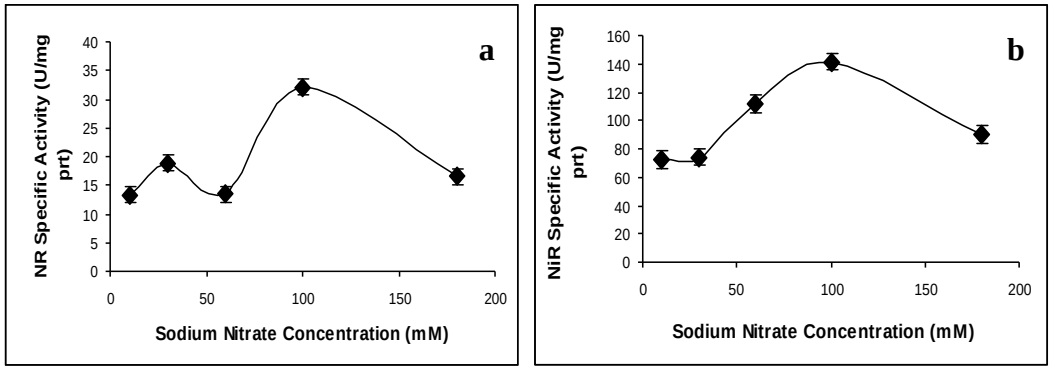

Figure 3.7 Variations of the specific activities of a) NR and b) NiR depending different sodium nitrate concentrations. The values are the mean ± SD for experiments of three separate experiments.

Logeswaran, Kohli and Vani (2011) used Zarrouk's medium containing 30 mM sodium nitrate as control condition and determined changings in nitrate assimilatory 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 our experiments, NR and NiR activities were several folds higher in the medium containing 100 mM sodium nitrate. This can be a conclusion that 100 mM sodium nitrate is optimum concentration for NR and NiR activities among the tested sodium nitrate concentrations.

After the reduction of nitrate, assimilation of ammonium is catalyzed by GS/GOGAT pathway. GS produces glutamine and GOGAT produces glutamate (Lu, Yuan, Zhang, Ou, Zhou, & Lin, 2005; Vanoni, & Curti, 2005). GS is responsible for initial assimilation of ammonium. According to the results, GS activity raised with the increasing sodium nitrate concentrations until 180 mM (Figure 3.8). 100 mM sodium nitrate stimulated GS activity when compared with 10, 30 and 60 mM sodium nitrate. The highest GS activity was determined in this medium on the 12th day of incubation period as 0.1084±0.01 U mL^{-1} (Figure 3.8a). It was 1.2 times higher when compared with the control ($p<0.05$). 180 mM sodium nitrate had an inhibitory effect on GS activity. GOGAT is the last enzyme of GS/GOGAT pathway. GOGAT activity was measured during the incubation period with different sodium nitrate concentrations (Figure 3.8b). The highest GOGAT activity was obtained in the medium containing 100 mM sodium nitrate as 34.726±1.3 U mL^{-1} on the 10th day

of the incubation period. This activity was 1.6 times higher than control medium ($p<0.05$). Excess sodium nitrate concentration (180 mM) caused inhibition on GOGAT activity. The maximum GOGAT activity was obtained on the 10th day of incubation period as 23.14±1.0 U mL^{-1} in the medium containing 180 mM sodium nitrate. In addition, in the presence of 10 mM sodium nitrate, on the 10th day of incubation period, the maximum GOGAT activity was almost 1.15-fold lower when compared with the control ($p<0.05$). After the 10th day, the activity was started to decrease in all growth media. Studies with GOGAT activity of radish cotyledons (Sood, Chanda & Singh, 2002) demonstrated that increasing nitrate concentrations stimulated enzyme activity as our results. Proline is an imino acid which is derived from glutamate. GOGAT activities showed that the highest activity was on the 10th day and the highest glutamate production was obtained after that day. The results demonstrated that the highest proline content was on the 12th day of incubation period. Therefore, glutamate production may start before proline production. According to the results, GS was correlated with GOGAT, positively ($r=0.951$, $p<0.05$). GS was also in a positive correlation with NR ($r=0.934$, $p<0.05$). In addition, it was shown a positive correlation with between GOGAT and NR ($r=0.968$, $p<0.05$), also GOGAT and NiR ($r=0.924$, $p<0.05$).

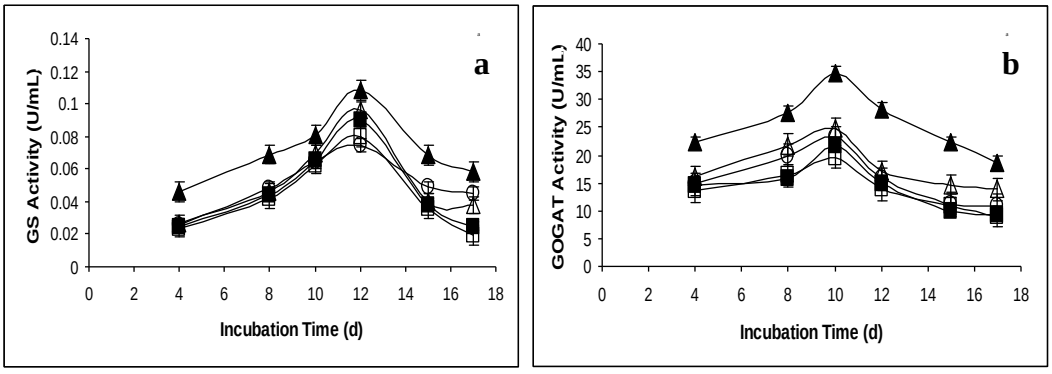

Figure 3.8 Variations of a) GS and b) GOGAT activities in *S. platensis* depending the incubation period in media containing different sodium nitrate concentrations [10 mM (□); 30 mM (■); 60 mM (Δ); 100 mM (▲); 180 mM (o)]. The values are the mean ± SD for experiments of three separate experiments.

Figures of specific activity were generated depending on maximum volume activities of GS and GOGAT (12th day and 10th day, respectively) with different

nitrate concentrations (Figure 3.9). Figure 3.9a shows the specific activity of GS. It was stimulated by 100 mM sodium nitrate concentration and the highest specific activity was determined as 0.0206±0.001 U mg^{-1} protein. For the specific activity of GOGAT, 100 mM sodium nitrate had stimulating effect but 180 mM sodium nitrate had inhibitory effect. In the medium containing 100 mM sodium nitrate, specific activity of enzyme was determined as 8.97±0.5 U mg^{-1} protein, while in the medium containing 180 mM sodium nitrate, the activity was determined as 5.36±0.3 U mg^{-1} protein (Figure 3.9b).

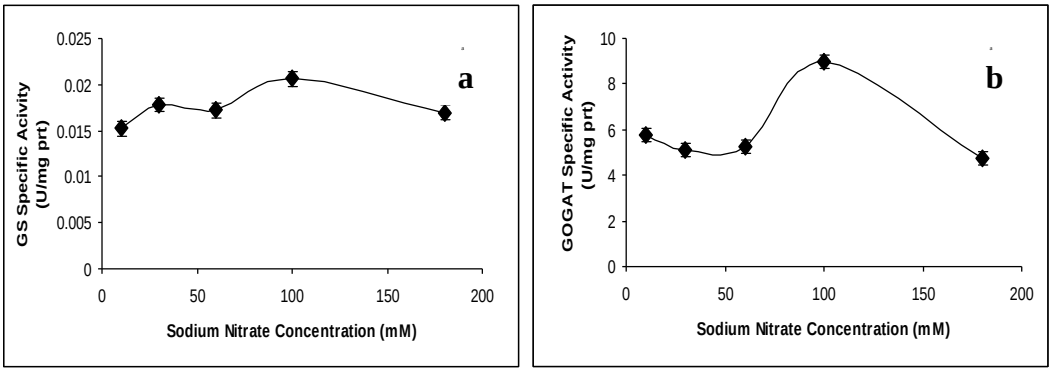

Figure 3.9 Variations of maximum specific activities of a) GS and b) GOGAT enzymes depending different sodium nitrate concentrations. The values are the mean ± SD for experiments of three separate experiments.

3.2 Effects of Different Ammonium Nitrate Concentrations on Some Nitrogen Assimilation Enzymes and Metabolites in *S. platensis*

Because of having of ammonium and nitrate different pathways in nitrogen assimilation process, ammonium nitrate is used as nitrogen source in this study. Thus we could examine both effects of ammonium and nitrate on nitrate/ammonium uptake levels, OD_{600}, dry biomass, pH, protein, CPC, APC, PE, chlorophyll a, total carotenoids, proline, pyruvate and total carbohydrate contents and some nitrate assimilation enzyme activities in *S. platensis* in the Zarrouk's medium with different ammonium nitrate (5-60 mM) concentrations (instead of sodium nitrate) during the incubation period. One of the reasons of ammonium nitrate preference as a nitrogen source is being more economic when compared with sodium nitrate. When examining the costs of ammonium nitrate and sodium nitrate, the cost of 500 g of

ammonium nitrate was about 53 € whereas the cost of 500 g of sodium nitrate was approximately 80 €. Standard Zarrouk's medium is used for *S. platensis* studies and contains 30 mM sodium nitrate concentration.

3.2.1 Variations at Nitrate/Ammonium Uptake Levels Depending on Ammonium Nitrate Concentrations in S. platensis

Nitrogen assimilation begins with uptake the nitrogen source into the cytoplasm and this is the basic step. Nitrate uptake is an active process. It is firstly transported into the cytoplasm by the specific transporters. It is mediated with the co-transport of two protons via proton-anion carrier mechanism. In the process, large amounts of energy, carbon and protons are consumed (Devriese, Tsakaloudi, Garbayo, León, Vílchez, & Vigara, 2001; Konnerup, & Brix, 2010). Ammonium nutrition is an advantage due to the lower energy costs associated with uptake and assimilation of it compared to nitrate. If ammonium is preferred for the nitrogen source, it is taken up by a uniport carrier system following the electrochemical gradient across the cytoplasm membrane (Piwpuan, Zhai, & Brix, 2013).

In the growth media containing various concentrations (5-60 mM) of ammonium nitrate, nitrate uptake rate was shown in Figure 3.10a. On the 10^{th} day of incubation period, concentrations of extracellular nitrate depletion in the growth media containing 5, 10, 30 and 60 mM ammonium nitrate were obtained as 84.68±4.2; 93.73±4.6; 66.306±3.1; 42.5±2.1%, respectively, whereas it was determined as 77.43±3.6% at the control condition. According to the results, the highest nitrate uptake rate was determined in the medium containing 10 mM ammonium nitrate. After the 14^{th} day of the incubation period, nitrate consumption from the external medium was decreased. In the media containing 30 and 60 mM ammonium nitrate, it was not consumed completely whereas, it was almost consumed completely and external nitrate concentrations were 0.109±0.01 and 0.130±0.01 mM, respectively, in the media containing 5 and 10 mM ammonium nitrate, on the 17^{th} day of incubation period.

In the growth media containing various concentrations (5-60 mM) of ammonium nitrate, ammonium uptake rate was shown in Figure 3.10b. On the 10^{th} day of incubation period, concentrations of extracellular ammonium depletion in the growth media containing 5, 10, 30 and 60 mM ammonium nitrate were obtained as 87.96±4.2; 94.5±4.6; 78.3±3.6 and 65.16±3.1%, respectively. In the medium containing 60 mM ammonium nitrate, nitrate uptake rate was almost 1.83-fold lower when compared with the control ($p<0.05$). The highest ammonium uptake rate was obtained in the medium containing 10 mM nitrate and it was 1.45-fold higher when compared with the medium containing 60 mM ammonium nitrate ($p<0.05$). In the media containing 5 and 10 mM ammonium nitrate, ammonium was almost consumed completely and external ammonium concentrations were 0.078±0.01 and 0.101±0.01 mM, respectively, on the 17^{th} day of incubation period.

Nitrate transportation is much more difficult and expensive than ammonium transportation as mentioned previously. When ammonium is used for the nitrogen source, its transport is a cheaper process when compared with nitrate. It can transport with monocomponent permeases (Lanquar et al., 2009). Studies also showed that when ammonium and nitrate are used together, ammonium inhibits nitrate transportation. Ammonium is directed to be used by the cells (Kronzucker, Glass, & Siddiqi, 1999; Muro-Pastor, Reyes & Florencio, 2001). Our results were in accordance with these studies. Nitrate uptake was inhibited with increasing ammonium concentrations. However ammonium uptake rates weren't affected by nitrate uptake processes.

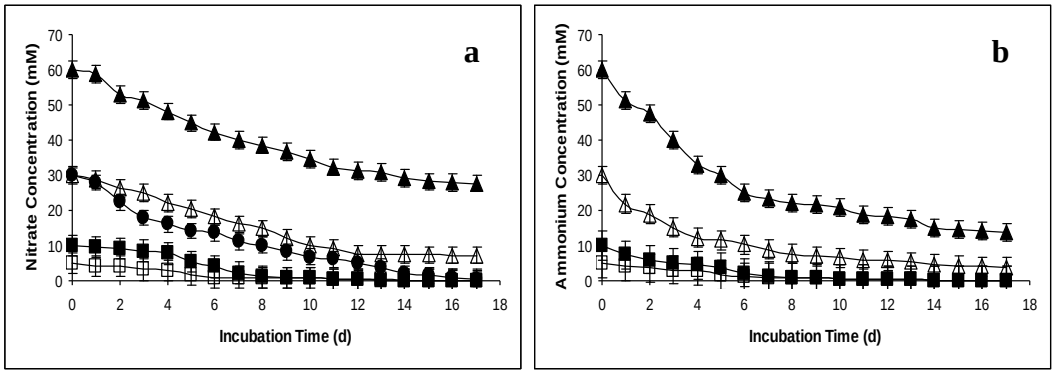

Figure 3.10 Variations of a) nitrate and b) ammonium uptake levels in *S. platensis* depending the incubation period in media containing different ammonium nitrate concentrations [5 mM (□); 10 mM (■); 30 mM (Δ); 60 mM (▲); Control (●)]. The values are the mean ± SD for experiments of three separate experiments.

3.2.2 Variations at the Growth, Dry Biomass, Protein and pH Levels Depending on Ammonium Nitrate Concentrations in S. platensis

In the growth media containing different ammonium nitrate concentrations, growth rate of *S. platensis* determined by OD_{600}, dry biomass, pH and protein levels depending incubation time (Figure 3.11). As depicted in Figure 3.11a, the highest growth of organism was observed on the 14^{th} day in the medium containing 10 mM ammonium nitrate while 60 mM ammonium nitrate was strongly inhibited it. Increasing of OD_{600} can be related with dry biomass and protein levels rise. Nitrate is an essential nutrient for algal growth and protein structure; so it had a stimulant effect on biomass and protein content of *S. platensis*. The highest dry biomass was determined on the 14^{th} day as 49.12±2.1 mg mL^{-1} in the medium containing 10 mM ammonium nitrate (Figure 3.11b). It was approximately 1.24-fold higher when compared with control ($p<0.05$). In the presence of 60 mM ammonium nitrate, it was almost 1.6-fold low at the same day ($p<0.05$). Control results of dry biomass were in accordance with the results of Tarko, Duda-Chodak and Kobus (2012) whereas in the presence of 10 mM ammonium nitrate, our results were almost 1.5-2-fold higher ($p<0.05$). The growth of the organism was showed a linear increasing until the 14^{th} day of incubation period. After that day, the growth parameters did not show any important change and the stationary phase was started. The highest protein level was obtained on the 14^{th} day in the same medium as 262.3±9.5 ppm (Figure 3.11c). In

the media containing 5 mM ammonium nitrate and the control had almost similar protein and dry biomass values and they were lower when compared with the medium containing 10 mM ammonium nitrate. As seen in Figure 3.11 dry biomass, protein and OD_{600} values started to increase after the 8^{th} day of incubation period and 60 mM ammonium nitrate inhibited growth of *S. platensis* extremely. On the 14^{th} day of incubation period, dry biomass and protein values were determined as 24.89±1.1 mg mL^{-1} and 122.4±5.7 ppm, respectively, in the medium containing 60 mM ammonium nitrate. After the 14^{th} day of incubation period, protein content of *S. platensis* did not change in all the tested media. There were not important pH differences among the tested ammonium nitrate concentrations (Figure 3.11d).

It can be concluded that 10 mM ammonium stimulated algal growth whereas 60 mM caused dramatically inhibition. This situation was in accordance with the studies of Costa, Cozza, Oliviera and Magagnin (2001); Madkour, Kamil and Nasr (2012). Their studies showed that low ammonium nitrate (5 and 10 mM) concentrations stimulated *S. platensis* growth while high concentrations of ammonium nitrate (such as 30, 50 mM) caused inhibiton on the growth.

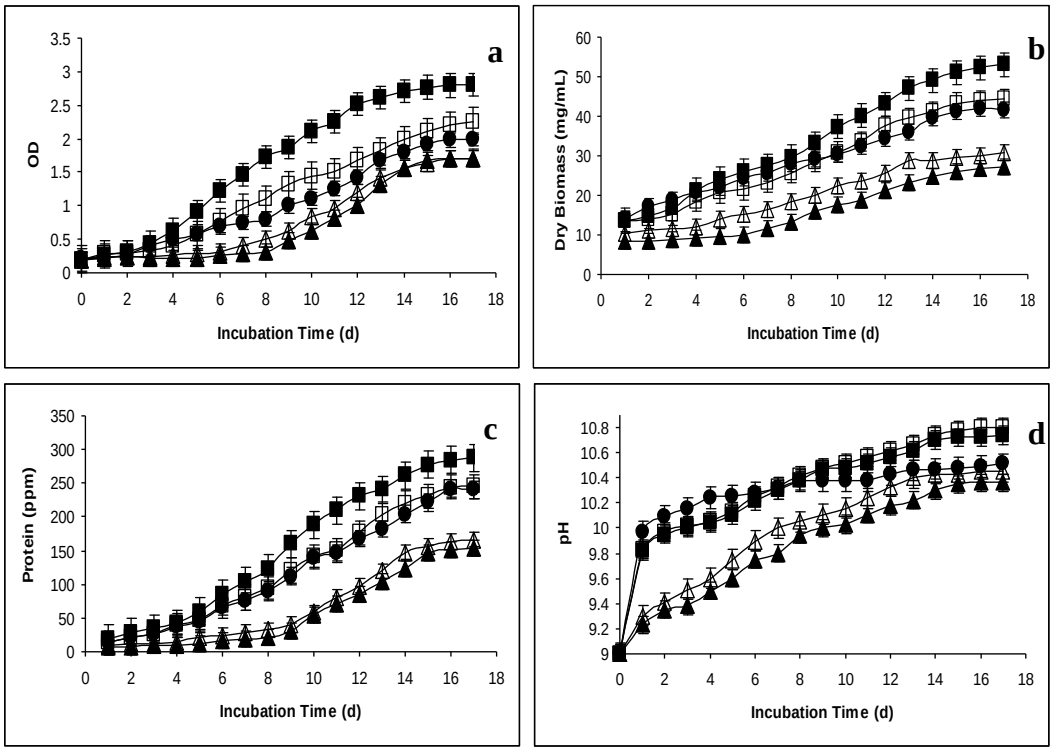

Figure 3.11 Variations of a) OD_{600}, b) dry biomass, c) protein, and d) pH levels in *S. platensis* depending the incubation period in media containing different ammonium nitrate concentrations [5 mM (□); 10 mM (■); 30 mM (Δ); 60 mM (▲); Control (●)]. The values are the mean ± SD for experiments of three separate experiments.

3.2.3 Variations on the Pigment Productions Depending on Ammonium Nitrate Concentrations in S. platensis

According to the results; pigment contents (phycobiliproteins, total carotenoids and chlorophyll a) of the media containing 30 and 60 mM ammonium nitrate were similar and almost lower when compared with the control condition. 30 and 60 mM ammonium nitrate concentrations resulted in reduction of phycobiliproteins, chlorophyll a and total carotenoids contents when compared with control concentration. The highest pigment production was obtained in the presence of 10 mM ammonium nitrate. Chlorophyll a and total carotenoids contents of *S. platensis* depending on different ammonium nitrate concentrations are depicted in Figure 3.12. The highest chlorophyll a and total carotenoids contents were determined in the medium containing 10 mM ammonium nitrate as 85.268±4.2 μg g^{-1} and 31.158±1.4 μg g^{-1}, respectively, on the 14^{th} day of incubation period. In the media containing 30

and 60 mM ammonium nitrate, chlorophyll a and total carotenoids contents were almost low when compared with control. For example, in the presence of 60 mM ammonium nitrate, it was 1.55-fold lower at the same day when compared with the control ($p<0.05$). This can be explained by the toxicity of excess concentrations of ammonium (Britto, & Kronzucker, 2002).

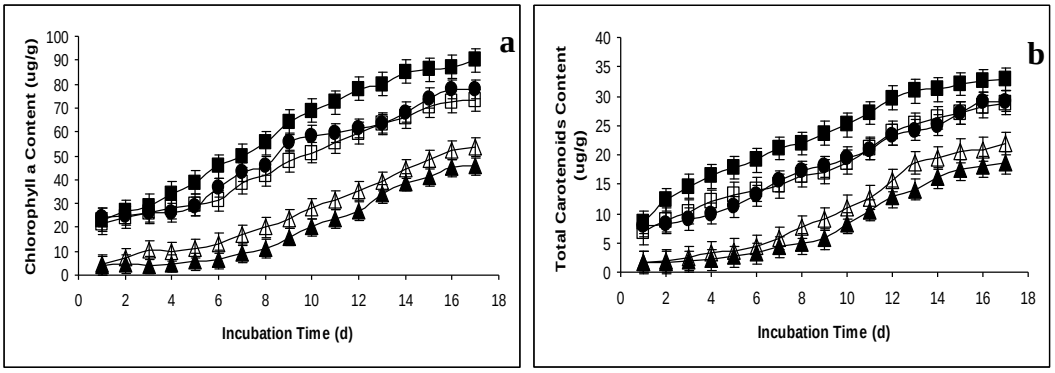

Figure 3.12 Variations of a) chl a and b) total carotenoids contents in *S. platensis* depending the incubation period in media containing different ammonium nitrate concentrations [5 mM (□); 10 mM (■); 30 mM (Δ); 60 mM (▲); Control (●)]. The values are the mean ± SD for experiments of three separate experiments.

C-phycocyanin (CPC), allophycocyanin (APC) and phycoeritrin (PE) contents of *S. platensis* were determined depending on different ammonium nitrate concentrations during the incubation time. As seen in Figure 3.13, the maximum CPC, APC and PE contents of all the tested media were determined on the 12th day of incubation period. On the 12th day of incubation period; CPC contents of the media containing 5 mM ammonium nitrate and control were similar while the media containing 30 and 60 mM ammonium nitrate were similar and approximately 1.97 and 3.13-fold lower when compared with control ($p<0.05$). The highest CPC, APC and PE contents were determined as 1.388±0.06 mg g^{-1}, 0.451±0.02 mg g^{-1} and 106.3±0.01 µg g^{-1}, respectively, in the medium containing 10 mM ammonium nitrate. The highest CPC content was almost 1.35-fold higher when compared with the control ($p<0.05$). In the media containing 30 and 60 mM ammonium nitrate, CPC, APC and PE contents of *S. platensis* were similar and almost lower when compared with the control condition.

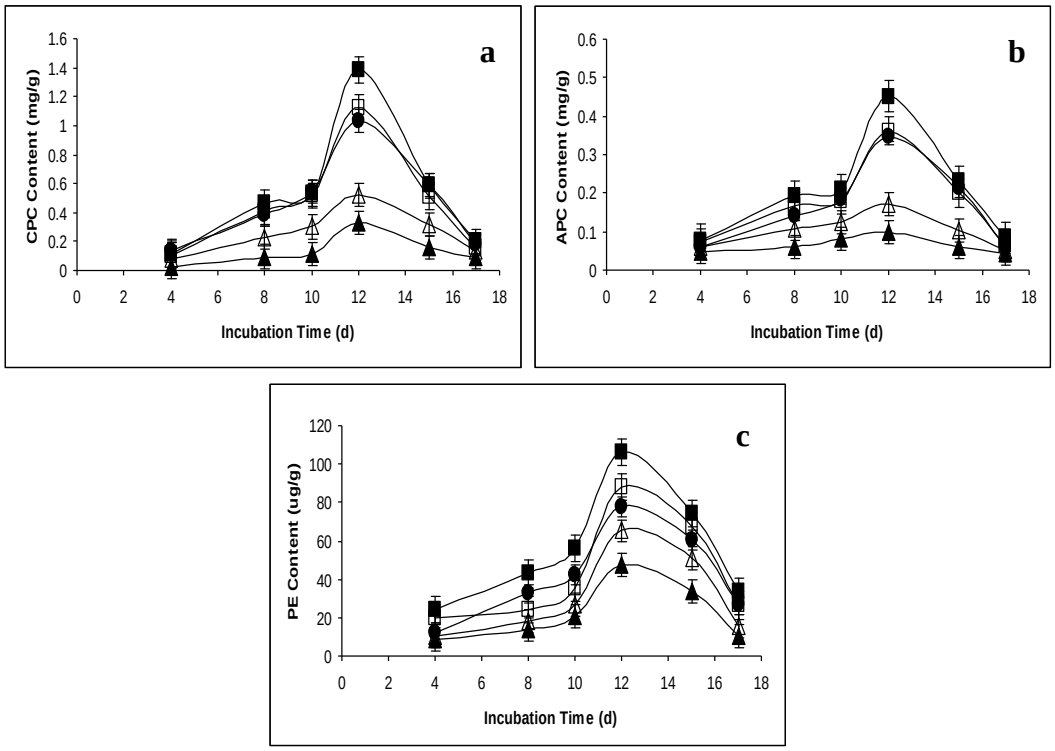

Figure 3.13 Variations of a) CPC, b) APC, and c) PE contents in *S. platensis* depending the incubation period in media containing different ammonium nitrate concentrations [5 mM (□); 10 mM (■); 30 mM (Δ); 60 mM (▲); Control (●)]. The values are the mean ± SD for experiments of three separate experiments.

3.2.4 Variations on the Proline, Pyruvate and Total Carbohydrate Contents Depending on Ammonium Nitrate Concentrations in S. platensis

Proline, pyruvate and total carbohydrate contents of *S. platensis* depending on different ammonium nitrate concentrations with respect to incubation time are shown in Figure 3.14. The maximum proline and pyruvate contents were determined on the 12th day of incubation period while the maximum total carbohydrate contents were obtained on the 15th day of incubation period in all the tested media. The highest proline content was obtained as 30.89±1.3 µmol g^{-1} in the medium containing 10 mM ammonium nitrate (Figure 3.14a). It was significantly higher (2-fold) than control ($p<0.05$). This can show the relationship with optimum biomass and proline production in the medium containing 10 mM ammonium nitrate. The highest biomass may contain highest amount of structural components such as aminoacids.

Pyruvate and total carbohydrate contents of *S. platensis* are extremely important for nitrogen assimilation by electron transport system. Additionally, pyruvate is a major substrate for GS/GOGAT pathway in nitrogen assimilation process (Devriese, Tsakaloudi, Garbayo, León, Vílchez, & Vigara, 2001). Ammonium as a nitrogen source has a stimulatory role for pyruvate kinase and this causes higher amounts of pyruvate production (Schweizer, & Erismann, 1985). Thus, it may play supporter for the nitrogen assimilation enzymes. The highest pyruvate and total carbohydrate contents of *S. platensis* were determined in the presence of 10 mM ammonium nitrate and they were 2.54 and 1.18 times higher, respectively, when compared with control ($p<0.05$).

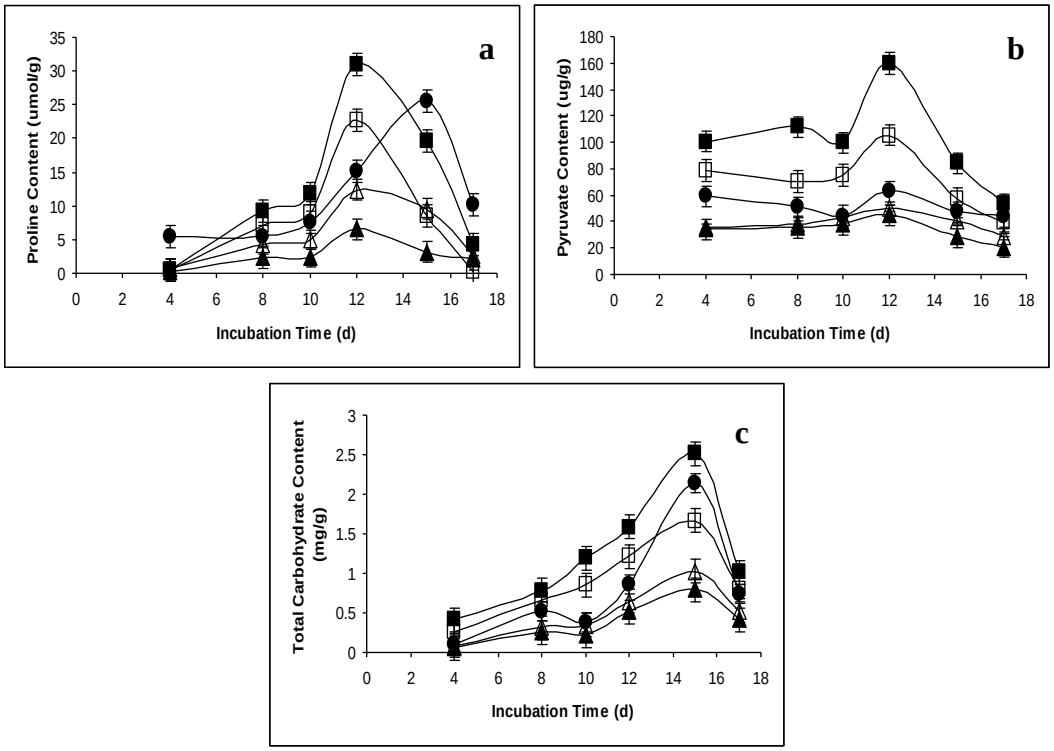

Figure 3.14 Variations of a) proline, b) pyruvate and c) total carbohydrate contents in *S. platensis* depending the incubation period in media containing different ammonium nitrate concentrations [5 mM (□); 10 mM (■); 30 mM (Δ); 60 mM (▲); Control (●)]. The values are the mean ± SD for experiments of three separate experiments.

3.2.5 Variations on Some Nitrogen Assimilation Enzyme Activities Depending on Ammonium Nitrate Concentrations in S. platensis

The first enzyme of nitrogen assimilation pathway is NR. Its activity was measured depending incubation period in the growth media of *S. platensis* containing different ammonium nitrate concentrations. The maximum NR activities of all the tested media were obtained on the 12^{th} day of incubation period. The highest NR activity was determined in the medium containing 10 mM ammonium nitrate as 40±2.2 U mL^{-1} (Figure 3.15a). It was almost 1.2-fold higher than control ($p<0.05$). 60 mM ammonium nitrate inhibited NR activity and the maximum activity was determined as 20.5±1.1 U mL^{-1} in this medium. NiR is the second enzyme that is responsible for transportation of nitrate to ammonium ions. The maximum NiR activities were determined on the 10^{th} day in all growth media (Figure 3.15b). The highest NiR activity was determined as 572±20.4 U mL^{-1} in the medium containing 10 mM ammonium nitrate. This activity was approximately 1.2 times higher than the control condition ($p<0.05$). 60 mM ammonium nitrate showed inhibitory effect on NiR activity. The maximum NiR activity was determined as 329±14.5 U mL^{-1} in the medium containing 60 mM ammonium nitrate. According to the results, the highest NR and NiR activities were obtained in the medium containing 10 mM ammonium nitrate. Increasing ammonium nitrate concentrations (30 and 60 mM) inhibited enzymatic activities. In this study, NR and NiR were in a positive correlation ($r=0.988$, $p<0.05$) with respect to the varying ammonium nitrate concentrations.

Logeswaran, Kohli and Vani (2011) used Zarrouk's medium containing 30 mM nitrate as control condition and determined 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 environmental conditions, but when used 10 mM ammonium nitrate, NR and NiR activities were several folds higher.

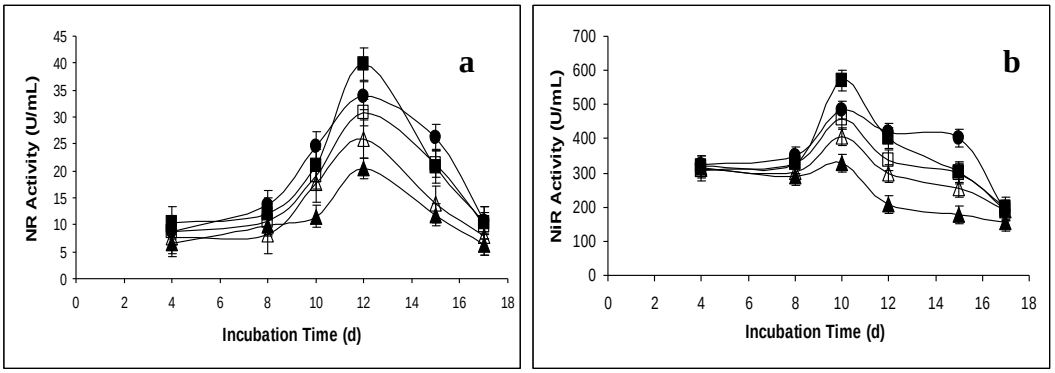

Figure 3.15 Variations of a) NR and b) NiR activities in *S. platensis* depending the incubation period in media containing different ammonium nitrate concentrations [5 mM (□); 10 mM (■); 30 mM (Δ); 60 mM (▲); Control (●)]. The values are the mean ± SD for experiments of three separate experiments.

Figures of specific activities were generated depending on the maximum volume activities of NR and NiR (12^{th} day and 10^{th} day, respectively) with different ammonium nitrate concentrations (Figure 3.16). The highest specific activity of NR was obtained as 25±1.2 U mg^{-1} protein in the medium containing 10 mM ammonium nitrate. The lowest specific activity of NR was estimated in the medium containing 60 mM ammonium nitrate as 11.97±0.5 U mg^{-1} protein (Figure 3.16a). 10 mM ammonium nitrate concentration also might stimulate specific activity of NiR. The highest specific activity of NiR was determined as 102.9±4.9 U mg^{-1} protein in the medium containing 10 mM ammonium nitrate as obtained volume activity (Figure 3.16b). It was approximately 1.28 times higher when compared with the control ($p<0.05$).

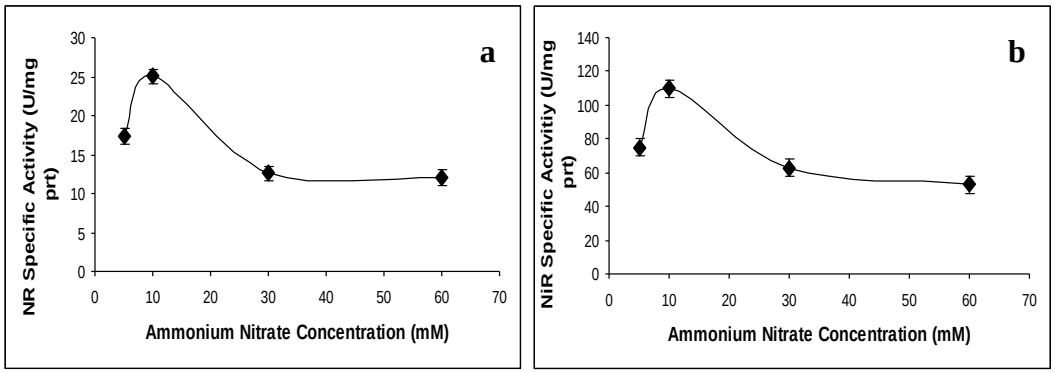

Figure 3.16 Variations of maximum specific activities of a) NR and b) NiR depending different ammonium nitrate concentrations. The values are the mean ± SD for experiments of three separate experiments.

The ammonium formed after the nitrate reduction or used as nitrogen source is catalyzed by GS/GOGAT pathway. GS is responsible for initial assimilation of ammonium. 10 mM ammonium nitrate stimulated GS activity when compared with 5, 30 and 60 mM ammonium nitrate. 30 and 60 mM ammonium nitrate had inhibitory effect on GS activity. The maximum GS activities in all the tested media were obtained on the 12th day of incubation period. The highest GS activity was determined in the medium containing 10 mM ammonium nitrate as 0.115±0.01 U mL^{-1} (Figure 3.17a). It was almost 1.3 times higher when compared with control ($p<0.05$). GOGAT is the last enzyme of GS/GOGAT pathway. The maximum GOGAT activities in all the tested media were obtained on the 10th day of incubation period (Figure 3.17b). The highest GOGAT activity was determined in the medium containing 10 mM ammonium nitrate as 35±1.4 U mL^{-1}. It was almost 1.6-fold higher than the control ($p<0.05$). After the 10th day, the activity was started to decrease in all growth media. The highest enzyme stimulation was obtained on GOGAT because of the support of pyruvate and ammonium. With relation to the GOGAT activity, the highest proline production started after the highest activity of the enzyme and it was determined on the 12th day of incubation period. According to the results, GS was correlated with GOGAT, positively ($r=0.973$, $p<0.05$). GS was also in a positive correlation with NR ($r=0.999$, $p<0.01$) and NiR ($r=0.988$, $p<0.05$). In addition, it was shown a positive correlation with between GOGAT and NR ($r=0.968$, $p<0.05$), and GOGAT and NiR ($r=0.99$, $p<0.05$).

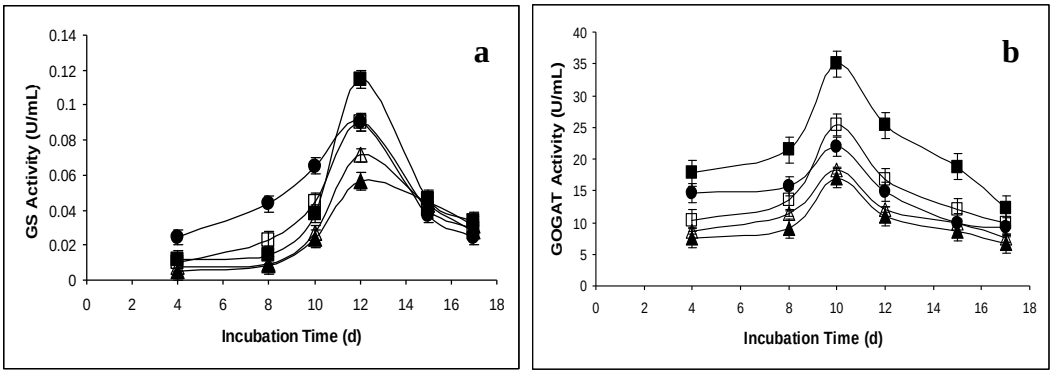

Figure 3.17 Variations of a) GS and b) GOGAT activities in *S. platensis* depending the incubation period in media containing different ammonium nitrate concentrations [5 mM (□); 10 mM (■); 30 mM (Δ); 60 mM (▲); Control (●)]. The values are the mean ± SD for experiments of three separate experiments.

Figures of specific activity were generated depending on maximum volume activities of GS and GOGAT (12^{th} day and 10^{th} day, respectively) with different ammonium nitrate concentrations (Figure 3.18). Figure 3.18a shows the specific activities of GS. The enzyme is also stimulated by 10 mM ammonium nitrate concentration. It was determined as 0.025 U mg^{-1} protein. For the specific activity of GOGAT, 10 mM ammonium nitrate had stimulating effect, but 30 and 60 mM ammonium nitrate concentrations had inhibitory effect. In the medium containing 10 mM ammonium nitrate, specific activity of GOGAT was determined as 6.22±0.3 U mg^{-1} protein, while in the media containing 30 and 60 mM ammonium nitrate, enzyme activities were determined as 3.27±0.12 and 3.09±0.12 U mg^{-1} protein, respectively (Figure 3.18b). Ammonium nitrate concentrations had the similar effect on the specific activities of GS and GOGAT. Low ammonium nitrate concentrations (5 and 10 mM) had positive effect on these specific enzyme activities when compared with high concentrations (30 and 60 mM).

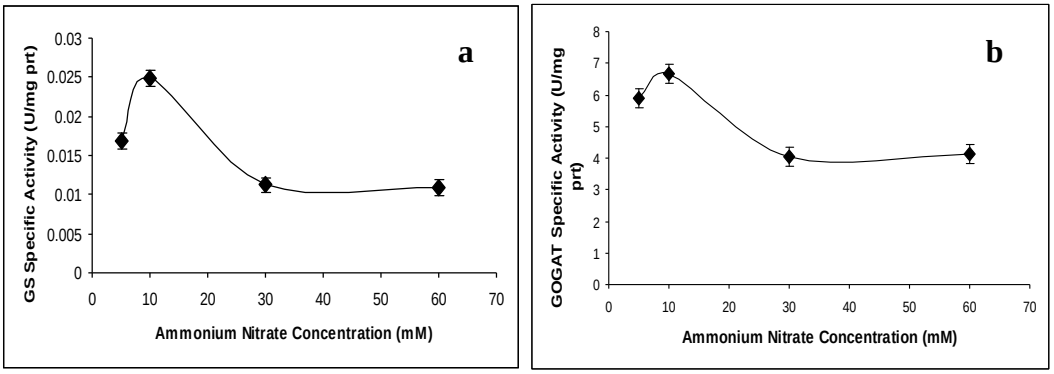

Figure 3.18 Variations of maximum specific activities of a) GS and b) GOGAT enzymes depending different ammonium nitrate concentrations. The values are the mean ± SD for experiments of three separate experiments.

According to the results, excess amounts of ammonium nitrate (30 and 60 mM) caused dramatically inhibition on the growth, production of the metabolites and pigments and some nitrogen assimilatory enzymes. This can be explained by the toxicity of excess concentrations of ammonium with causing chlorosis. In addition higher ammonium concentrations can cause disorder of ion balance in the growth medium (Britto, & Kronzucker, 2002). The highest yields of ammonium uptake and enzyme activities were reached in the medium containing 10 mM ammonium nitrate. This concentration resulted in the highest biomass production with incorporated structural components in *S. platensis*. Additionally, the highest yield of the growth, metabolite and pigment production can be related with the optimum biomass production.

In this study, effects of different concentrations of sodium nitrate (10-180 mM) and ammonium nitrate (5-60 mM) on the growth, production of some metabolites and pigments such as chlorophyll a, total carotenoids, proline, pyruvate, total carbohydrate and phycobiliprotein (CPC, APC and PE) contents and some nitrate assimilation enzymes such as NR, NiR, GS and GOGAT in *S. platensis* were investigated depending on incubation period. When preferred sodium nitrate as nitrogen source; the optimum growth, the highest amount of metabolite production and enzyme activities were reached in the medium containing 100 mM sodium nitrate. However, when preferred ammonium nitrate as nitrogen source, the optimum concentration for these parameters was obtained as 10 mM. According to the results,

if 10 mM ammonium nitrate and 100 mM sodium nitrate is compared, it can be concluded that the highest yield of growth, metabolite production and enzyme activities were reached in the presence of 100 mM sodium nitrate. The reason of this situation may be related with the structures of ammonium nitrate and sodium nitrate. In general, nitrogen is found in oxidized forms in nature. Nitrate is most widely preferred nitrogen sources by living organisms because of being oxidized form. Ammonium is an inorganic molecule and one of the major nitrogen sources with being the most reduced form (Muro-Pastor, & Florencio, 2003; Perez-Garcia, Escalante, de-Bashan, & Bashan, 2011). However being oxidized form can be the reason of the preference of nitrate by *S. platensis*.

CHAPTER FOUR
CONCLUSION

Nitrogen is a vital element with being the most important structural member of macromolecules such as proteins and nucleic acids and some antioxidant featured pigments such as chlorophylls and phycobiliproteins. Cyanobacteria can utilize different kinds of nitrogen sources such as N_2, nitrate, nitrite, ammonium, urea and aminoacids. The most widely used nitrogen sources in cyanobacteria are nitrate and ammonium. Nitrate is the nitrogen source which can be found in oxidized form in nature. This situation may be the reason for preference of it. Ammonium is the most reduced form of nitrogen. The usage of it gives advantage for spending less energy for assimilation reactions. Nitrogen is incorporated the carbon skeletons to inform aminoacids such as glutamine and glutamate via nitrogen assimilation processes. Nitrogen assimilation metabolism and carbon metabolism are strongly linked with each other. In nitrogen assimilation, the energy which is necessary for the enzymatic catalysis and 2-OG which is used as substrate are provided from TCA cycle. Glutamine and glutamate are produced by catalyzing enzymes such as NR, NiR, GS and GOGAT in nitrogen assimilation process. Glutamine and glutamate are the amino acids that have significant roles in biotechnology, biochemistry, and food and drug industry. For example; glutamate is used as food additive in food industry. Additionally, glutamine is used as food supplement for many cancer patients. The biomass of *S. platensis* cyanobacterium contains approximately 60-70% protein. Thus, nitrogen availability in the growth medium, its uptake into the cell and assimilation processes are extremely important for amino acid metabolism and the growth of *S. platensis*.

In this study various concentrations of sodium nitrate (10-180 mM) and ammonium nitrate (5-60 mM) (instead of 30 mM sodium nitrate) were used as the nitrogen sources in the Zarrouk's growth medium of *S. platensis* (Gamont) Geitler organism. Zarrouk's medium is optimum growth medium for *Spirulina* with being rich source of many minerals (Mg, Ca, Fe, Mn, Zn, Cu, Mo,...) and nutrients (NO_3^-, CO_3^-, PO_4^-,…). Standard Zarrouk's medium containing 30 mM sodium nitrate was

used as control condition in this study. The reason of the preference of Zarrouk's medium for *S. platensis* (Gamont) Geitler is using it for *Spirulina* growth in industry, generally (Gershwin, & Belay, 2008). For this purpose, different concentrations of two nitrogen sources as sodium nitrate and ammonium nitrate were chosen. Effects of different concentrations of sodium nitrate and ammonium nitrate was investigated on nitrate/ammonium uptake levels, OD_{600}, dry biomass, pH, protein, CPC, APC, PE, chlorophyll a, total carotenoids, proline, pyruvate and total carbohydrate contents and some nitrate assimilation enzyme activities such as NR, NiR, GS and GOGAT in *S. platensis*.

In the first part of this study, effects of different concentrations (10, 30, 60, 100 and 180 mM) of sodium nitrate were investigated on the growth, pigment and metabolite production and some nitrogen assimilation enzyme activities in *S. platensis*. According to the results; 100 mM sodium nitrate containing growth medium is optimum for nitrate assimilation in *S. platensis* (Gamont) Geitler organism. Nitrate assimilation starts with nitrate uptake into the cytoplasm. Our results demonstrated that nitrate consumption was faster between 6-12 days of incubation period. It may be associated with increasing nitrate uptake rate. The highest NR, NiR, GS and GOGAT activities were determined in the presence of 100 mM sodium nitrate. They were almost 2.2, 1.2, 1.2 and 1.6-fold higher, respectively, when compared with the control condition ($p<0.05$). The highest NR and GS activities were obtained on the 12^{th} day whereas the highest NiR and GOGAT activities were reached on the 10^{th} day of incubation period. 30 mM nitrate containing Zarrouk's medium as control wasn't enough for achieving the highest nitrate assimilation enzyme activities, some metabolites and growth parameters. 10 mM sodium nitrate had insufficient effect on algal pigments production such as chlorophyll a, total carotenoids, CPC, APC and PE. Similarly, the lowest proline and pyruvate contents were determined in the presence of 10 mM sodium nitrate in the growth medium of *S. platensis*. The highest pigment and metabolite production was achieved in the medium containing 100 mM sodium nitrate.

In the second part of the study, effects of different concentrations (5, 10, 30 and 60 mM) of ammonium nitrate were investigated on the growth, pigment and metabolite production and NR, NiR, GS and GOGAT activities in *S. platensis*. The studies showed that excess ammonium nitrate concentrations had highly inhibitory effect on the growth, pigment production and activities of nitrogen assimilation enzymes such as NR, NiR, GS and GOGAT. In this study, the highest NR, NiR, GS and GOGAT activities were found in the presence of 10 mM ammonium nitrate. They were almost 1.2, 1.2, 1.3 and 1.6-fold higher, respectively, when compared with the control condition ($p<0.05$). Additionally, the best growth and antioxidant featured pigment production such as chlorophyll a, total carotenoids; CPC, APC and PE and nitrate/ammonium uptake rates were achieved in the medium containing 10 mM ammonium nitrate. Similarly, the highest proline, pyruvate and total carbohydrate contents were achieved in the same medium. *S. platensis* was sensitive to higher ammonium nitrate concentrations (30 and 60 mM) and they caused growth and metabolic damages on the organism. 60 mM ammonium nitrate decreased growth of *S. platensis*. Until the 8^{th} day of incubation period, the dry biomass and OD_{600} values didn't increase linearly in this medium. 60 mM ammonium nitrate had highly inhibitory effect on NR, NiR, GS and GOGAT activities. Additionally, the lowest pigment and metabolite production was determined in this medium. The reason of this inhibition may be related with the toxicity of excess ammonium concentrations in the growth media of cyanobacteria. Ammonium nitrate usage as nitrogen source had advantage with being more economic than sodium nitrate. When examining the costs of ammonium nitrate and sodium nitrate, the cost of 500 g of ammonium nitrate was about 53 € whereas the cost of 500 g of sodium nitrate was approximately 80 €. Moreover, high amount of ammonium nitrate usage was an expensive way to cultivate to the organism. Thus, 10 mM ammonium nitrate usage has advantage with more economic. In addition, it was investigated that both effects of ammonium and nitrate on the growth and nitrogen metabolisms in *S. platensis*.

In conclusion, the best growth, pigment and metabolite production and NR, NiR, GS and GOGAT activities were obtained in the presence of 100 mM sodium nitrate and 10 mM ammonium nitrate in *S. platensis*. If 10 mM ammonium nitrate and 100

mM sodium nitrate is compared, it can be concluded that the highest yield of growth, metabolite production and enzyme activities were reached in the presence of 100 mM sodium nitrate. The reason of achieving best results in the present of sodium nitrate may arise from nitrate structure. However ammonium is one of the most preferred nitrogen sources by cyanobacteria with being the most reduced form, nitrate is most widely used and found nitrogen source in nature due to being oxidized form. Thus, the best growth, pigment and metabolite production and nitrogen assimilatory enzyme activities were achieved in the presence of sodium nitrate as nitrogen source.

Consequently; 100 mM sodium nitrate containing Zarrouk's growth medium was suitable for *S. platensis* (Gamont) Geitler organism in growth parameters, production of pigments and metabolites and the activities of some nitrogen assimilation enzymes. Higher enzyme activities may cause high amounts of amino acid production which has biotechnological, industrial and biochemical importance such as glutamine and glutamate. On the other hand, there is a link between nitrogen assimilation and carbon metabolism. The best growth, pigment and metabolite production and enzymatic activity may support carbon metabolism of *S. platensis*. It can be concluded that high content of the pigments, carbonic compounds and protein may make *S. platensis* a rich food source.

REFERENCES

Ali, A., Sivakami, S., & Raghuram, N. (2007). Effect of nitrate, nitrite, ammonium, glutamate, glutamine and 2-oxoglutarate on the RNA levels and enzyme activities of nitrate reductase and nitrite reductase in rice. *Physiology and Molecular Biology of Plants, 13,* 17-25.

Ayehunie, S., Belay, A., Baba, T. W., & Ruprecht, R. M. (1998). Inhibition of HIV-1 replication by an aqueous extract of *Spirulina platensis* (*Arthrospira platensis*). *Journal of Acquired Immune Deficiency Syndromes & Human Retrovirology, 18*(1), 7-12.

Bartzatt, R., & Donigan, L. (2004). The colorimetric determination of nitrate anion in aqueous and solid samples utilizing an aromatic derivative in acidic solvent. *Toxicological & Environmental Chemistry, 86,* 75–85.

Bates, L. S., Waldren, R. P., & Teare I. D. (1973). Rapid determination of free proline for water-stress studies. *Plant and Soil, 39,* 205-207.

Becker, E. W. (2007). Micro-algae as a source of protein. *Biotechnology Advances, 25,* 207-210.

Berteli, F., Corrales, E., Guerrero, C., Ariza, M. J., Pilego, F., & Valpuesta, V. (1995). Salt stress increases ferredoxin-dependent glutamate synthase activity and protein level in the leaves of tomato. *Physiologia Plantarum, 93,* 259–264.

Bradford, M. M. (1976). A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72,* 248–254.

Britto, D. T., & Kronzucker, H. J. (2002). NH_4^+ toxicity in higher plants: A critical review. *Journal of Plant Physiology, 159,* 567 –584.

Chen, F. L., & Cullimore, J. V. (1988). Two isoenzymes of NADH-dependent glutamate synthase in root nodules of *Phaseolus vulgaris*: Purification, properties and activity changes during nodule development. *Plant Physiology, 88,* 1411–1417.

Costa, J. A. V., Cozza, K. L., Oliviera, L., & Magagnin, G. (2001). Different nitrogen sources and growth responses of *Spirulina platensis* in microenvironments. *World Journal of Microbiology and Biotechnology, 17,* 439-442.

Crawford, N. M. (1995). Nitrate: Nutrient and signal for plant growth. *The Plant Cell, 7,* 859-868.

Devriese, M., Tsakaloudi, V., Garbayo, I., León, R., Vílchez, C., & Vigara, J. (2001). Effect of heavy metals on nitrate assimilation in the eukaryotic microalga *Chlamydomonas reinhardtii*. *Plant Physiology and Biochemistry, 39,* 443−448.

Dey, S., & Rathod, V. K. (2013). Ultrasound assisted extraction of β-carotene from *Spirulina platensis*. *Ultrasonics Sonochemistry, 20,* 271–276.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry, 28,* 350–356.

Eisenberg, D., Gill, H. S., Pfluegl, G. M. U., & Rotstein, S. H. (2000). Structure–function relationships of glutamine synthetases. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology, 1477,* 122–145.

Fernandez, E., & Galvan, A. (2007). Inorganic nitrogen assimilation in *Chlamydomonas*. *Journal of Experimental Botany, 58,* 2279-2287.

Ficner, R., & Huber, R. (1993). Refined crystal structure of phycoerythrin from *Porphyridium cruentum* at 0.23-nm resolution and localization of the γ subunit. *European Journal of Biochemistry, 218,* 103–106.

Fiore, C. L., Jarett, J. K., Olson, N. D., & Lesser. M. P. (2010). Nitrogen fixation and nitrogen transformations in marine symbioses. *Trends in Microbiology, 18,* 455–463.

Flores, E., Frias, J. E., Rubio, L. M., & Herrero, A. (2005). Photosynthetic nitrate assimilation in cyanobacteria. *Photosynthesis Research, 83,* 117-133.

Friedeman, E., & Haugen, G. E. (1943). Pyruvic acid II: The determination of keto acids. *The Journal of Biological Chemistry, 147,* 415-442.

Gemma, C., Mesches, M. H., Sepesi, B., Choo, K., Holmes, D. B., & Bickford P. C. (2002). Diets enriched in foods with high antioxidant activity reduce age-induced decreases in cerebellar beta-adrenergic function in proinflammatory cytokines. *The Journal of Neuroscience, 22,* 6114-6120.

Gershwin M., & Belay, A. (2008). Spirulina (Arthrospira): Production and quality assurance. In *Spirulina in Human Nutrition and Health* (1-26). Taylor & Francis Group, London.

Glass, J. B., Wolfe-Simon, F., & Anbar, A. D. (2009). Coevolution of metal availability and nitrogen assimilation in cyanobacteria and algae. *Geobiology, 7,* 100–123.

Glazer, A. N. (1989). Light guides. Directional energy transfer in a photosynthetic antenna. *The Journal of Biological Chemistry, 264,* 1-4.

Granbom, M., Chow, F., Lopes, P. F., Oliveira, M. C., Colepicolo, P., Paula, E. J. et al. (2004). Characterisation of nitrate reductase in the marine macroalga *Kappaphycus alvarezii* (Rhodophyta). *Aquatic Botany, 78,* 295–305.

Henrikson, R. (Ed). (2010). *Spirulina world food* (7th ed.). Hawaii: Ronore Enterprises Inc.

Herrero, A., & Guerrero, M. G. (1986). Regulation of nitrite reductase in the cyanobacterium *Anacystis nidulans*. *Journal of General Microbiology, 132,* 2463-2468.

Herrero, A., Muro-Pastor, A. M., & Flores, E. (2001). Nitrogen control in cyanobacteria. *Journal of Bacteriology. 183,* 411-425.

Hirahashi, T., Matsumoto, M., Hazeki, K., Saeki, Y., Ui, M., & Seya, T. (2002). Activation of the human innate immune system by *Spirulina*: augmentation of interferon production and NK cytotoxicity by oral administration of hot water extract of *Spirulina platensis*. *International Immunopharmacology, 2,* 423-424.

Hirata, T., Tanaka, M., Ooike, M., Tsunomura, T., & Sakaguchi, M. (2000). Antioxidant activities of phycocyanobilin prepared from *Spirulina platensis*. *Journal of Applied Phycology, 12,* 435–439.

Inokuchi, R., Kuma, K., Miyata, T., & Okada, M. (2002). Nitrogen-assimilating enzymes in land plants and algae: Phylogenic and physiological perspectives. *Physiologia Plantarum, 116*(1), 1–11.

Jha, P., Ali, A., & Raghuram, N. (2007). Nitrate-induction of nitrate reductase and its inhibition by nitrite and ammonium ions in *Spirulina platensis*. *Physiology and Molecular Biology of Plants, 13,* 163-167.

Kerfeld, C. H. A. (2004). Water-soluble carotenoid protein of cyanobacteria. *Archives of Biochemistry and Biophysics, 430*(1), 2-9.

Kim-Shapiro, D. B., Gladwin, M. T., Patel, R. P., & Hogg, N. (2005). The reaction between nitrite and hemoglobin: the role of nitrite in hemoglobin-mediated hypoxic vasodilation. *Journal of Inorganic Biochemistry, 99,* 237-246.

Konnerup, D., & Brix, H. (2010). Nitrogen nutrition of *Canna indica*: Effects of ammonium versus nitrate on growth, biomass allocation, photosynthesis, nitrate reductase activity and N uptake rates. *Aquatic Botany, 92,* 142-148.

Kronzucker, H. J., Glass, A. D. M., & Siddiqi, M. Y. (1999). Inhibition of nitrate uptake by ammonium in barley. Analysis of component fluxes. *Plant Physiology, 120,* 283-292.

Lichtenthaler, H. K., & Wellburn, A. R. (1983). Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. Biochemical *Society Transactions, 11,* 591-592.

Lanquar, V., Loque´, D., Hörmann, F., Yuan, L., Bohner, A., Engelsberger, W. R. et al. (2009). Feedback inhibition of ammonium uptake by a phospho-dependent allosteric mechanism in Arabidopsis. *The Plant Cell, 21,* 3610-3622.

Logeswaran, P. K., Kohli, V., & Vani, B. (2011). Interplay between calcium influx and nitrate asimilation in *Spirulina platensis*. *African Journal of Microbiology Research, 5*, 5438-5443.

Losada, M., & Paneque, A. (1971). Nitrite reductase. *Methods Enzymology, 23,* 487–491.

Lu, B., Yuan, Y., Zhang, C., Ou, J., Zhou, W., & Lin, Q. (2005). Modulation of key enzymes involved in ammonium assimilation and carbon metabolism by low temperature in rice (Oryza sativa L.) roots. *Plant Sciences, 169,* 295-302.

Madkour, F. F., Kamil, A. E., & Nasr, H. S. (2012). Production and nutritive value of *Spirulina platensis* in reduced cost media. *Egyptian Journal of Aquatic Research, 38,* 51-57.

Mathew, B., Sankaranarayanan, R., Nair, P. P., Varghese, C., Somanathan, T., Amma, B. P. et al. (1995). Evaluation of chemoprevention of oral cancer with spirulina fusiformis. *Nutrition and Cancer, 24,* 197-202.

Mendiola, J. A., Jaime, L., Santoyo, S., Reglero, G., Cifuentes, A., Ibanez, E. et al. (2007). Screening of functional compounds in supercritical fluid extracts from *Spirulina platensis*. *Food Chemistry, 102,* 1357-1367.

Moreno-Vivian, C., Cabello, P., Martinez-Luque, M., Blasco, R., & Castillo, F. (1999). Prokaryotic nitrate reduction: Molecular properties and functional distinction among bacterial nitrate reductases. *Journal of Bacteriology, 18,* 6573–6584.

Muro-Pastor, M. I., & Florencio, F. J. (2003). Regulation of ammonium assimilation in cyanobacteria. *Plant Physiology and Biochemistry, 41,* 595–603.

Muro-Pastor, M. I., Reyes, J. C., & Florencio, F. J. (2001). Cyanobacteria perceive nitrogen status by sensing intracellular 2-oxoglutarate levels. *The Journal of Biological Chemistry, 276,* 38320-38328.

Murphy, M. J., Siegel, L. M., Tove, S. R., & Kamin, H. (1974). Siroheme: A new prosthetic group participating in six-electron reduction reactions catalyzed by both sulfite and nitrite reductases. *Proceedings of the National Academy of Sciences of the United States of America, 71,* 612-616.

Nelson, D. L. & Cox, M. M. (2008). *Lehninger Principles of Biochemistry.* (5th ed.). New York.

Öztürk Ürek, R., & Tarhan, L. (2012). The relationship between the antioxidant system and phycocyanin production in *Spirulina maxima* with respect to nitrate concentration. *Turkish Journal of Botany, 36,* 369-377.

Perez-Garcia, O., Escalante, F. M. E., de-Bashan, L. E., & Bashan, Y. (2011). Heterotrophic cultures of microalgae: Metabolism and potential products. *Water Research, 45,* 11-36.

Piwpuan, N., Zhai, X. & Brix, H. (2013). Nitrogen nutrition of *Cyperus laevigatus* and *Phormium tenax*: Effects of ammonium versus nitrate on growth, nitrate reductase activity and N uptake kinetics. *Aquatic Botany, 106,* 42-51.

Ravasio, S., Dossena, L., Martin-Figueroa, E., Florencio, J. F., Mattevi, A., Morandi, P. et al. (2002). Properties of the recombinant ferredoxin-dependent glutamate synthase of *Synechocystis* PCC6803. Comparison with the *Azospirillum brasilense* NADPH-dependent enzyme and its isolated alpha subunit. *Biochemistry, 41,* 8120–8133.

Raven, P. H., Evert, R. F., & Eichhorn, S. E. (Ed.). (2005). *Biology of Plants* (7th ed.) W.H. Freeman and Company Publishers.

Redinbaugh, M. G., & Campbell, W. H. (1985). Quaternary structure and composition of squash NADH:nitrate reductase. *The Journal of Biological Chemistry, 260,* 3380-3385.

Schwartz, J., & Shklar, G. (1987). Regression of experimental hamster cancer by beta-carotene and algae extracts. *Journal of Oral and Maxillofacial Surgery, 45,* 510-515.

Schweizer, P., & Erismann, K. H. (1985). Effect of nitrate and ammonium nutrition on nonnodulated *Phaseolus vulgaris* L. on phosphoenolpyruvate carboxylase and pyruvate kinase activity. *Plant Physiology, 78,* 455-458.

Singh, S., Kate, B. N., & Banerjee, U. C. (2005). Bioactive Compounds from Cyanobacteria and Microalgae: An Overview. *Critical Reviews in Biotechnology, 25,* 73-95.

Sood, C. R., Chanda, S. V., & Singh, Y. (2002). Effect of different nitrogen sources and plant growth regulators on glutamine synthetase and glutamate synthase activities of radish cotyledons. *Bulgarian Journal of Plant Physiology, 28,* 46–56.

Suda, D., Schwartz, J., & Shklar, G. (1986). Inhibition of experimental oral carcinogenesis by topical beta carotene. *Carcinogenesis, 7,* 711-715.

Sun, L., Wang, S., & Qiao, Z. (2006). Chemical stabilization of the phycocyanin from cyanobacterium *Spirulina platensis. Journal of Biotechnology, 121,* 563–569.

Tarko, T., Duda-Chodak, A., & Kobus, M. (2012). Influence of growth medium composition on synthesis of bioactive compounds and antioxidant properties of selected strains of Arthrospira cyanobacteria. *Czech Journal of Food Sciences, 30,* 258-267.

Vanoni, M. A., & Curti, B. (1999). Glutamate synthase: A complex iron-sulfur flavoprotein. *Cellular and Molecular Life Sciences, 55,* 617–638.

Vanoni, M. A., & Curti, B. (2005). Structure-function studies on the iron-sulfur flavoenzyme glutamate synthase: An unexpectedly complex self-regulated enzyme. *Archives of Biochemistry and Biophysics, 433,* 93-211.

Vincent, W. F. (2009). Algae (Including cyanobacteria): Planktonic and attached. In G. E. Likens, (Ed.). *Plankton of inland waters* (1st ed.) (142-148). United Kingdom: Elsevier Inc.

Weatherburn, M. W. (1967). Phenol-hypochlorite reaction for determination of ammonia. *Analytical Chemistry, 39,* 971-974.

Woodward, R. B., Ayer, W. A., Beaton, J. M., Bickelhaupt, F., Bonnett, R., Buchschacher, P. et al. (1990). The total synthesis of chlorophyll a. *Tetrahedron, 46,* 7599–7659.

Zahroojiana, N., Moraveja, H., & Shivazada, M. (2011). Comparison of marine algae (*Spirulina platensis*) and synthetic pigment in enhancing egg yolk colour of laying hens. *British Poultry Science, 52,* 584-588.

Zarrouk, C. (1966). *Contribution à l'étude d'une cyanophycée. Influence de divers facteurs physiques et chimiques sur la croissance et la photosynthèse de Spirulina maxima.* Ph.D. Thesis, Université de Paris, France.