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Effect of nitrogen limitation on growth, total lipid accumulation and protein amount in *Scenedesmus acutus* as biofuel reactor candidate

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Abstract

Objective: in this study, the changes in growth, protein and lipid amounts of *Scenedesmus acutus* which considering as biofuel producer microorganism in nitrogen-limited liquid media were investigated.

Material and Methods: The microalgal strain of *Scenedesmus acutus* was used which isolated from the Keban Reservoir in Eastern Anatolia, Turkey. Microalgal strain was grown in Jaworsky's medium. Algal cells were calculated by measuring the optical density at 680 nm using a visible density spectrophotometer. The total lipid content was determined using the Bligh and Dyer method. The total protein content was determined by the Lowry method.

Results: The results have shown that there is an inverse relationship between cellular growth, lipid amount and nitrogen concentration. *S. acutus* survived all applied nitrogen concentrations and increases were observed in the amount of its cellular lipid. It was determined that there was enough nitrogen in the nitrogen-limited media to support protein synthesis and cell growth of *S. acutus* and that the amount of lipid in the 50% nitrogen-limited media was 19.48% higher than that in the control group.

Conclusion: *S. acutus* survived in all the nitrogen concentrations tested (25% and 50% limited nitrogen in medium) and increases were observed in the amount of its cellular lipid. Significant increase in the amount of lipid in *Scenedesmus acutus* subjected to nitrogen stress suggests the idea that the microalga in question can be one of the potential organisms that can be used to obtain biofuel.

Keywords: *Scenedesmus acutus*, nitrogen limitation, protein, lipid accumulation, biofuel

Introduction

In recent years there have been many studies conducted on the discovery of renewable energy sources and their availability in various areas of everyday life. Biodiesel is one of the main renewable energy sources which is obtained from biological mass and has the potential to replace diesel fuel. Trapping carbon in biomass and allowing the restoration of global carbon balance, biomass energy is a more environmentally friendly option than other renewable energy sources (1). Microalgae have high potential as a source of renewable energy due to their high lipid content, rapid growth and low space requirements for their production. To this end, current studies have focused on identifying suitable biomass-producing species that provide higher energy output than traditional fossil fuels (2-4). Target species of choice for biomass production are those with known life cycles, fast cell division, high amount of protein and rich metabolite content.

Microalgae are much richer organisms in terms of photosynthesis and lipid content than terrestrial plants.

One of the main reasons for the increased biotechnological use of microalgae in recent years is that their lipid rate ranges from 20 to 50 % in general and even in some species, the dry weight of the lipid rate increases up to 80% (2,5,6).

Microalgae are known to undergo some morphological and biochemical changes as a survival strategy, especially under stress conditions. Many studies have been conducted on achieving high growth rates in algae species (7-10). Nutrient stress can strongly affect microalgal metabolism such as lipid content and photosynthesis. It was reported that high growth rate resulted in an increase in the biomass of microalgae whereas there was a high increase in the lipid content of microalgae grown under stress conditions (9,10).

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It was determined that there was a decrease in the rate of photosynthetic proteins in the cells of the green alga Chlamydomonas reinhardtii in nitrogen-limited media (11).

Temperature, light intensity and nutrient limitation such as nitrogen, iron and silicon are important factors affecting the amount and content of lipid in algae. The factor studied most often is the effects of nitrogen starvation on the amount of lipid (5). Nitrogen, which is the essential component of many macromolecules such as DNA, RNA, chlorophyll and protein, is known to be one of the most important nutrients for microalgae. Many studies report that nitrogen starvation leads to a decrease in photosynthesis and protein synthesis while an increase in lipid and carbohydrate synthesis (10,12,13). It was reported that the amount of nitrogen was 40% higher in Chlorella vulgaris grown in low-nitrogen media than in those grown in control media (14,15). It was also reported that the amount of lipid increased up to 7.9% in Nannochloropsis oculata and 5.9% in Chlorella vulgaris when the amount of nitrogen in the medium was decreased to 75% (16).

Chlorophyta members constitute a significant part of the algae used in biotechnological studies. *Scenedesmus acutus*, a member of Chlorophyta, is a freshwater alga. High protein, vitamin, mineral and lipid content of *Scenedesmus acutus* have made it biotechnologically important. It is considered one of the potential renewable sources in the future for alternative fuel production due to its rapid and high growth rate and high lipid content. Thus, this study observed the changes in the growth, and protein and lipid content of the green alga *Scenedesmus acutus* grown at different nitrogen levels.

Material and Methods

Algal Cultures and Media

The microalgal strain of *Scenedesmus acutus* used in this study was isolated from the Keban Reservoir in Eastern Anatolia, Turkey was grown in Jaworsky's medium consisting of 80 mg NaNO3, 36 mg Na2HPO4.12H2O, 20 mg Ca(NO3)2.4H2O, 12.4 mg KH2PO4, 50 mg MgSO4. 7H2O, 2.25 mg EDTAFeNa, 2.25 mg EDTANa2, 2480 µg H3BO3, 15.9 mg NaHCO3, 1390 µg MnCl2.4H2O, 1000 µg (NH4)6Mo7.4H2O, 40 µg biotin, 40 µg cyanocobalamin (B12) and 40 µg thiamin (B1). All media were sterilized at 121 °C for 15 minutes at 1 atmosphere pressure.

Two groups (treatment and control) were used for the experiments. The treatment group was from Jaworsky's medium with 25% and 50% nitrogen limitations and the original Jaworsky's medium was used as the control group.

Scenedesmus acutus was inoculated into 250-ml Erlenmeyer flasks containing 100-ml Jaworsky's medium. The Erlenmeyer flasks were incubated in a climate cabinet at 23 ±1 °C, light density 55 µmol photon m-2s-1, for 16-h light followed by 6-h darkness.10-ml samples from the media that reached a certain density were inoculated into the control and nitrogen-limited media. The inoculated control and 25% and 50% nitrogen-limited media were incubated at 23 ±1 °C, light density 55 µmol photon m-2s-1, for 16-h light followed by 6-h darkness. The Erlenmeyer flasks were shaken three times a day without CO2 addition. The analyses of the number of *S. acutus*, and protein and lipid content of the control and 25% and 50% nitrogen-limited media were performed for ten days in three repetitions.

Growth Measurement

S. acutus was counted under a microscope using a plankton counting chamber. Meanwhile, the same samples were examined daily using a visible spectrophotometer at a wavelength of 680 nm. Algal cells were calculated by measuring the optical density at 680 nm using a visible density spectrophotometer. The measurements on the spectrophotometer were compared with microscopic counts. A standard curve relating optical density was generated and used to calculate the numbers of individuals based on optic density. The calculations were performed in three repetitions.

Determination of Total Lipid

The total lipid content was determined using the Bligh and Dyer method (17). A mixture consisting of 40-ml methanol and 80 ml chloroform was added onto a 0.2-gr sample on which 20-ml CaCl2 (0.4%) was then added. The mixture was filtered through a filter paper and kept overnight in the dark. The next day, methanol/water phase was removed using a separating funnel and chloroform was evaporated in a 60 °C water bath. The remaining mixture was kept in a 90ºC drying-oven for 1 hour to evaporate the chloroform completely and then weighed.

Determination of Total Protein

The total protein content was determined by the Lowry method (18). 0.1-ml DOC solution was added onto 1-ml sample and the sample was kept at room temperature for 10 minutes. Afterwards, 0.1-ml TCA was added onto the sample which was, then, centrifuged at 7500 rpm for 10 minutes. After the removal of the supernatant, 1-ml Lowry solution was added to the precipitate and the precipitate was kept at room temperature for 20 minutes. Later on, 1-ml folin reagent was added to the sample, which was, then, kept for 30 minutes. Lastly, a standard curve was made by plotting absorbance at 750 nm and the results were evaluated based on the standard curve.

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Results and Discussion

Effect of nitrogen limitation on growth of *S. acutus*

Studies on the determination of factors affecting the cellular growth and metabolite content of algae are important nowadays. This study investigated the effects of different nitrogen concentrations on the cellular growth, and protein and lipid content of *Scenedesmus acutus*. Figure 1 shows the growth of *S. acutus* in the modified Jaworsky's medium at different nitrogen concentrations. The 10 day observation period after the day of inoculation indicates that the rate of increase in cell number is 325.95%in the control group, 335.38% in the 25% nitrogen-limited media and 410.50% in the 50% nitrogen-limited media. Figure 1 demonstrates that the rate of increase in cell number in the 50% nitrogen-limited media is significantly higher than those in the other media. Nitrogen is known to be the most important nutrient element for microalgae since it is necessary for the synthesis of organic materials such as protein, chlorophyll and nucleic acids. This study yields that the nitrogen limitation changes the rate of cell division to some degree. It is stated that the growth rates of microalgae in nitrogen-limited media are high (14,19-21). The results of this study also reveal that the cell growth rate of *S. acutus* is higher in the nitrogen-limited media than in the other media and that there is an inverse relationship between the growth rate of *S. acutus* under nitrogen stress conditions and the nitrogen concentration.

The Effect of nitrogen limitation on the protein amount

Proteins are the basic building blocks of living organisms. Approximately 50% of the dry weight of *S. acutus* is composed of proteins. Figure 2 shows the changes in the amount of protein in the control group and the nitrogen-limited media. The amount of protein, which was 44.145 µg/ml in Jaworsky's medium (control group) on the day of inoculation, regularly increased and reached 72.503 µg/ml on day 10 (Figure 2). The amount of protein, which was 44.145 µg/ml in the 25% nitrogen-limited media on the day of inoculation, regularly increased and reached 67.95µg/ml on day 8, however, started to decrease the following days and reached 63.048µg/ml on day 10. A similar situation was observed in the 50% nitrogen-limited media as well. The amount of protein in the 50% nitrogen-limited media continued to increase until day 8 (64.60µg/ml) and then started to decrease and reached 61.30µg/ml on day 10 (Fig.2). The comparison of Jaworsky's medium and the nitrogen-limited media reveals that the increase in protein in the latter is lower than in the former. During the 10-day experiment, rates of increase in protein were 64.238%, 42.820% and 38.860% in the control group, 25% nitrogen-limited media and 50% nitrogen-limited media, respectively. There is approximately a 25% decrease in the amount of protein in the nitrogen-limited media compared to the control group. There is an inverse correlation between the amount of nitrogen in the liquid media and the amount of protein.

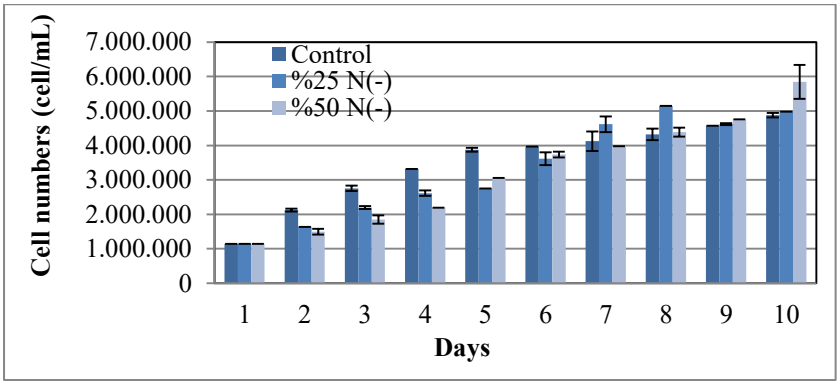

Figure 1: Effect of nitrogen limitation on growth of *S. acutus*

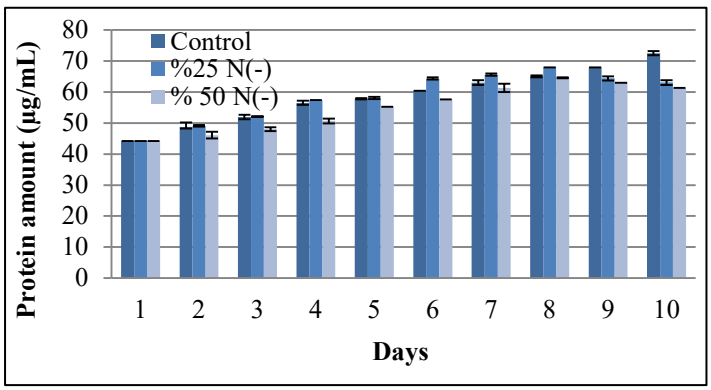

Figure 2: The effect of nitrogen limitation on protein amount of *S. acutus*

The effect of nitrogen limitation on the lipid amount

Lipid accumulation in microalgae can be significantly increased by nitrogen limitation in a culture medium (5,22,23). This study investigated the changes in the amount of lipid of *S. acutus* by nitrogen limitations in liquid nutrient medium. The amount of lipid, which was initially 7.7%, reached 11.4% in the control media at the end of the study period. The results of the experiment show that the increase in the amount of lipid in the control media is 48.05%. The amount of lipid of *S. acutus* grown in the 25% nitrogen-limited media was 7.7% on the day of inoculation and reached 12.7% on day 10 with a 64.93% rate of increase. However, the increase in the amount of lipid in the 50% nitrogen-limited media was significantly high. The amount of lipid in these media reached 12.9% and the rate of increase was 67.53% more than it was on the day of inoculation. The results indicate that the amount of nitrogen in the culture medium has a significant effect on the lipid content of *S. acutus* and that there is an inverse relationship between the amount of nitrogen in the culture medium and the amount of lipid (Fig.3).

This study shows that the biochemical composition of microalgae can be manipulated by changing the physical and chemical parameters of the medium. Many factors such as nitrogen deficiency in the medium, phosphate limitation, temperature and pH are known to affect lipid the content of microalgae (2,24,25). Nitrogen deficiency leads to stress conditions for all organisms because nitrogen is the main component of proteins and nucleic acids in living cells. When inoculated into a low-nitrogen containing medium, *Chlorella vulgaris* accumulate more total lipid in their cells. Widjaja *et al*. report that the ratio of total lipid and triglycerol in *C. vulgaris,* a freshwater microalga, significantly increased when grown in a nitrogen-depleted medium (26). Limited cell division, an increase in cell volume, and in lipid and carbohydrate synthesis were observed in *Chlamydomonas reinhardii* and *Scenedesmus subcapitatus* grown in nitrogen-limited media (27).

All studies demonstrate that the lipid content of microalga cells can be enhanced by nitrogen limitation.

The results of this study also confirm that the lipid content of *S. acutus* is significantly affected by the amount of nitrogen in the liquid media and that nitrogen deficiency leads to a decrease in the growth rate of and an increase in lipid accumulation in *S. acutus*. There are some proposals to account for the lipid accumulation of microalgae under nitrogen deficiency conditions. Botham and Ratledge argue that when nitrogen is depleted due to high energy load (ATP/AMP ratio), glucose conversion into lipids is triggered (28). Some researchers maintain that nitrogen deficiency promotes the conversion of excess glucose into lipid and leads to a higher rate of lipid transformation than that of cell division under autotrophic or heterotrophic culture conditions (29-31). One of the mechanisms suggested for the explanation of lipid accumulation in microalgae under nitrogen deficiency conditions is based on the premise that chloroplast nitrogen is transported using1.5-bifosfat carboxylase/oxygenase which leads to the mobilization of the lipid in chloroplast membranes (32). Another mechanism suggested for the explanation of lipid accumulation in microalgae grown under N deficiency conditions is attributed to mobilization of lipid from chloroplast membranes due to repositioning of the chloroplast nitrogen by using 1,5-bisphosphate carboxylase/oxygenase. Sheehan, et al. hypothesize that the reason for the increase in lipid content is that nitrogen depletion leads to the inhibition of cell division without a gradual decrease in lipid production which results in accumulation of fat in cells (33). Other researchers suggest that lipid accumulation may be related not only to high levels of lipid-synthesizing enzymes under nitrogen deficiency conditions but also to the inhibition of cell growth and reproduction by the operation of lipid-accumulating special enzymes (30,34). It is also maintained that many microalgae are able to adapt their metabolic pathways to store lipid in high amounts under nitrogen deficiency conditions (5,12,35,36).

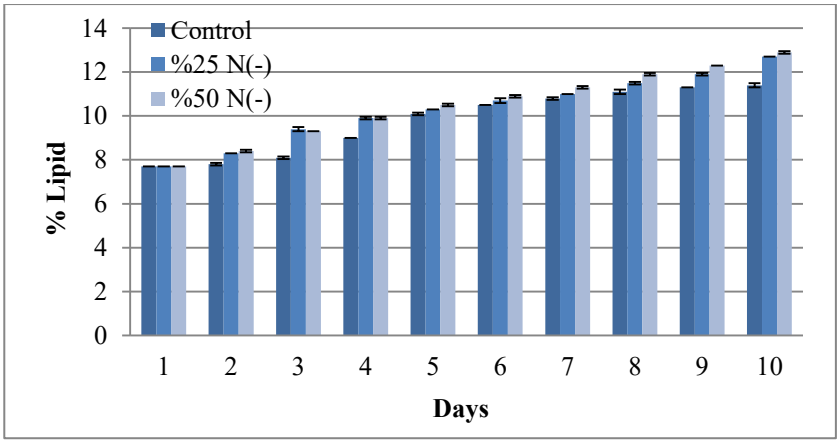

Figure 3: The effect of nitrogen limitation on lipid amount of *S. acutus*

Sheehan et al. reported that the nitrogen deficiency conditions led to a shift in the carbon flux from protein synthesis to lipid synthesis which resulted in an increase in lipid content in microalgae (33). However, nitrogen limitation does not always lead to lipid accumulation. *Achnanthes breviceps* and *Tetraselmis* sp. grown under nitrogen deficiency conditions were observed to have accumulated carbohydrates (28,37). This indicates that lipid accumulation due to nitrogen deficiency is species specific. The decrease in the amount of protein and the increase in the amount of lipid in *S. acutus* subjected to nitrogen stress indicate that the microalga in question adapts its metabolic pathways to store lipid under nitrogen deficiency conditions.

Conclusions

This study examined the changes in growth, and protein and lipid amounts of *S. acutus* grown in liquid media subjected to nitrogen stress. The results show that there is an inverse relationship between cellular growth, lipid amount and nitrogen concentration. *S. acutus* survived all the nitrogen concentrations tested and increases were observed in the amount of its cellular lipid. It was determined that there was enough nitrogen in the nitrogen-limited media to support protein synthesis and cell growth of *S. acutus* and that the amount of lipid in the 50% nitrogen-limited media was 19.48% higher than that in the control group. Significant increase in the amount of lipid in *Scenedesmus acutus* subjected to nitrogen stress suggests the idea that the microalga in question can be one of the potential organisms that can be used to obtain biofuel.

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Author's Contributions: NA, AKC: Collecting of data, writing and revision of article,

Ethical issues: All Authors declare that Originality of research/article etc... and ethical approval of research, and responsibilities of research against local ethics commission are under the Authors responsibilities. The study was conducted due to defined rules by the Local Ethics Commission guidelines and audits.

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