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INFLUENCE OF NITROGEN SUPPLY ON THE ACCUMULATION OF BIOMASS AND LIPOPHILIC COMPOUNDS ACUTODESMUS DIMORPHUS (TURPIN) TSARENKO

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The work is devoted to the study of the influence of nitrogen supply on the accumulation of lipophilic compounds of Acutodesmus dimorphus (Turpin) Tsarenko. Acutodesmus dimorphus (Turpin) Tsarenko is a species of green algae of the Scenedesmaceae family. These are cynobial freshwater algae, which are representatives of water bodies of moderate latitudes. Nitrate supply is a key factor in the algae cultivation process. The conjugation of cellular processes of algae will depend on the amount of nitrogen in the nutrient medium. Nitrogen content is the dominant factor in the accumulation of lipophilic compounds in algae biomass. The effect of nitrate supply on the accumulation of biomass, the amount of proteins, lipids and carotenoids of A. dimorphus was determined. To simulate nitrogen deprivation, Yanovsky's medium with a reduced NaNO3 content was used. Janovsky's control medium contained 80 mg/L NaNO3 (100% nitrogen supply). In experimental versions of the environment, the amount of NaNO3 was reduced to 75%, 50% and 25%, respectively. A decrease in available nitrogen in the nutrient medium for A. dimorphus leads to an increase in the amount of triacylglycerols in algae cells. The highest indicator of the number of neutral lipids was noted at 50% and 25% nitrogen supply of A. dimorphus culture. Under conditions of nitrogen starvation, a decrease in the amount of total protein in A. dimorphus cells was noted, but this trend is not directly related to an increase in the amount of triacylglycerols. In order to obtain lipophilic compounds of A. dimorphus, it is recommended to use Yanovsky's medium with 25% NaNO3 content. Under these conditions, we obtain A. dimorphus biomass with an optimal content of triacylglycerols, carotenoids and protein.

Ключові слова: green algae, lipids, carotenoids, nitrogen supply

Introduction. Green microscopic algae is a promising object of biotechnology for obtaining valuable metabolites. Products based on algae biomass are widely used in medicine, cosmetology, food industry and energy (Ghafari et al., 2016). One of these energetically valuable compounds is triacylglycerols, which can be used to produce biodiesel as an alternative energy source. As in all organisms, a number of complex enzymatic processes are responsible for the biosynthesis of certain compounds (Fan et al., 2011). And green microscopic algae are no exception (Choksmi et al., 2015).

Lipids produced by most freshwater algae are not considered a high-value food ingredient due to their low levels of unsaturated fatty acids. However, they are a potential substitute resource for obtaining biodiesel (Hockin et al., 2012; Hu et al., 2008).

Algae lipids are always deposited in the cell, but under unfavorable conditions, lipids begin to be synthesized more actively (Alishah Aratboni et al., 2019; Chiu et al., 2009). Most algae can accumulate different amounts of lipids depending on environmental conditions. Such representatives of green algae as *Chlorella* sp., *Botryococcus braunii* and *Dunaliella salina* can accumulate about 20% of lipids, however, under adverse environmental conditions, the amount of lipids increases to 40-50% (Li-Beisson et al., 2015; Goodson et al., 2011; Widjaja et al., 2009). The physico-chemical parameters of cultivation and the content of nutrients in the environment affect both the productivity of biomass and the biochemical composition of cells (Zhu et al., 2016; Wang et al., 2009).

During stress, algae begin to actively synthesize not only lipids, but also various pigments (α, β, γ - carotenoids), chlorophyll, etc (Zolotareva et al., 2008). The amount of lipids can vary in different genera and even species of algae. It is known that the content of lipids in the biomass of green algae can be significantly increased by adding organic carbon to the medium or by reducing the nitrogen supply. Cultivation at altered temperatures and pH levels leads to the accumulation of unsaturated fatty acids and an increase in the amount of eicosopentaenoic acid among them (Mimouni et al., 2018; Park et al., 2015; Sharma et al., 2012). That is, the physicochemical parameters of cultivation are the main factor that determines the amount of accumulated triacylglycerols and free fatty acids in algae biomass (Solovchenko et al., 2007).

Nitrogen starvation is one of the key factors in the cultivation of algae producing lipophilic compounds. The conjugation of cellular processes is an important condition for the vital activity of all living cells, including algae cells, therefore the influence of nitrogen concentration occurs not only on the ways of synthesis of triacylglycerols, but also on the synthesis of other valuable compounds. During nitrogen starvation in green microscopic algae, the following changes occur: increase in de novo synthesis of TAG from acyl-CoA, recycling of acyl fragments from degraded membrane lipids and increase in carbon flow towards glycerol-3-phosphate and acyl-CoA for the synthesis of fatty acids (Klok et al., 2013; Sasaki et al., 2004). Also, during starvation, the flow of atmospheric carbon, recorded with the help of photosynthesis, and from sources of organic carbon entering the environment is redirected to the synthesis of TAG. Algae *Acutodesmus dimorphus* (Turpin) Tsarenko is a thermotolerant, freshwater green alga that forms coenobia (Guiry et al., 2020). Its ability to grow at temperatures higher than 25 °C allows predicting increased biosynthetic activity with respect to triacylglycerols.

The aim of the work was to study the effect of nitrogen supply on the accumulation of lipophilic compounds in *Acutodesmus dimorphus* (Turpin) Tsarenko.

Materials and methods. The collection culture of *Acutodesmus dimorphus* (Turpin) Tsarenko (IBASH-A), obtained from the collection of the Kholodny Institute of Botany of the National Academy of Sciences of Ukraine, for which we express our gratitude to it.

Acutodesmus dimorphus (Turpin) Tsarenko – colonies with 2-4 or more cells; cells are cylindrical with conical pointed ends (Guiry et al., 2020).

Algae were cultivated under sterile conditions on Janovsky's medium. Cultivation was carried out in Erlenmeyer flasks with a volume of 500 ml at a temperature of 21 ± 2°C, illuminated by fluorescent lamps of about 2500 lux and a 16-hour photoperiod, for 14 days (Zolotareva et al., 2008).

The effect of nitrogen supply on the accumulation of lipophilic compounds by cells of *Acutodesmus dimorphus* was studied. Janovsky's control medium contained 80 mg/L $NaNO_3$ (100% nitrogen supply). In experimental versions of the environment, the amount of $NaNO_3$ was reduced to 75%, 50% and 25%, respectively.

In the process of cultivation, the amount of green algae biomass was determined indirectly by optical density every 2 days. It was measured at 750 nm on a CaryWin UV 60 spectrophotometer (Agilent, USA) (Hevorhyz et al., 2008).

Upon completion of cultivation, biochemical analysis of the selected samples was carried out. The mass fraction of protein and lipids (%) was determined by the spectrophotocolorimetric method.

To separate algae cells from the liquid medium, the culture was centrifuged for 10 min at 5000 rpm on a Micromtd CM-3M centrifuge. 1 M phosphate buffer pH 7.6 was added to the selected algae biomass. Biomass was disintegrated by ultrasound using the Ultrasonic Cleaner CE-7200A ultrasonic bath. Determination of the total protein content was carried out according to the Lowry method (Lowry et al., 1951).

Lipids were extracted by the Folch method (Folch et al., 1957). For this purpose, the sediment obtained after protein isolation was used. It was filled with a mixture of chloroform and methanol (2:1). This suspension was left for 1 day in the dark, then centrifuged for 10 minutes at 3000 rpm. The amount of lipids was determined in the presence of sulfuric acid and phosphoric vanillin reagent.

For the extraction of carotenoids, 100% acetone was used in a ratio of 1:3 (Sanhez et al., 2008). To quantify the content of carotenoids, the optical density (D) of the obtained extracts was measured at 450 nm. All results were calculated on absolutely dry biomass.

The statistical processing of the obtained results was carried out using one-way analysis of variance (ANOVA), with further assessment of reliability by the difference between means according to Tukey's test.

Results and discussion. The successful growth of *A. dimorphus* algae depends on the balance of light, temperature, and the content of micro- and macroelements in the nutrient medium. *A. dimorphus* is a promising species for the synthesis of valuable compounds, namely lipids. These green algae grow quickly, are small compared to other algae, are heat-resistant and halophilic, which allows creating stressful conditions without the threat of cell death. It is also an advantage that these algae capture CO2 more efficiently than others (Choksmi et al., 2015). This characteristic has a positive effect on photosynthesis, and therefore on the growth of cell biomass. Nitrogen starvation is one of the powerful factors that can affect lipid synthesis.

Algae *A. dimorphus* were grown on Yanovsky's medium for 14 days. The advantages of this nutrient medium over others is the ease of creation, it includes 14 components, while, for example, Fitzgerald's medium requires 18 components. Another advantage is the easy control of the amount of nitrogen in the environment, by regulating the content of only one component, namely $NaNO_3$.

4 different environments were made, the control contained 80g of $NaNO_3$. In other nutrient media, the amount of sodium nitrate was reduced to 75%, 50%, and 25%, respectively.

The growth of all organisms depends on the availability of mineral nutrients, one of the most important of which is nitrogen. It is necessary for the construction of protein molecules, chlorophyll, nitrogenous bases. In terms of dry mass of cells, the amount of nitrogen is 7-10%.

Most photosynthetic algae can grow using inorganic nitrogen sources such as nitrate or ammonium ions. Moreover, ammonium nitrogen is primarily consumed, while nitrate is often not utilized as long as ammonium salts are present in the environment (Ghafari et al., 2016). Addition of ammonium to the nutrient medium of algal cultures leads to a sharp and complete inhibition of nitrate assimilation. This is because NH^{4+} is the final product of nitrate reduction and inhibits the process in a feedback type. NH^{4+} is converted into organic forms, and its assimilation is associated with the loss of intracellular hydrocarbon reserves (Park et al., 2015).

When ammonium is used as the main source of nitrogen, the pH of the medium can quickly decrease and reach a value of 3.0. A change in pH values can be the reason for slowing down the growth observed for some algae with an increase in the concentration of ammonium in the medium, and subsequently, an increase in the intracellular pH level due to the absorption of weakly dissociating ammonium hydroxide molecules. Consumption of nitrate ions leads to an increase in pH (Alishah Aratboni et al., 2019).

First of all, we noted the effect of nitrate starvation on the accumulation of biomass of *A. dimorphus* (Fig. 1.). The biomass obtained on the control medium was characterized by the following parameters (Table 1.)

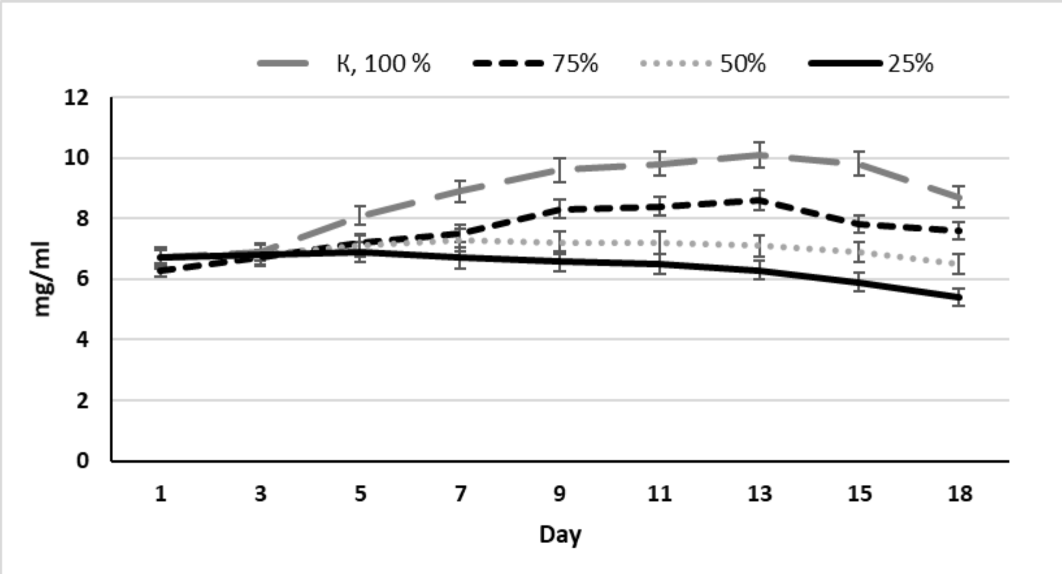

Fig. 1. Amount of biomass of A. dimorphus under different nitrogen supply

Table 1.

Biomass composition of A. dimorphus
(M±m, n=3, p≤0,05)

Criterion	Amount
protein, %	45,4 ± 3,12
carbohydrates,%	16,6 ± 0,99
lipids, %	21,0 ± 1,38
chlorophyll *a*, mg/g	11,23 ± 0,1
chlorophyll *b*, mg/g	7,01 ± 0,21
carotenoids, mg/g	11,98 ± 0,21

Nitrogen starvation had a positive effect on lipid synthesis. This is confirmed by the theoretical part and proved by us experimentally on the example of the algae *A. dimorphus* (Fig. 2).

Algae *A. dimorphus* not only survive in conditions of reduced nitrogen concentrations, but also begin to synthesize more energetically valuable compounds. Thus, at 100% nitrogen supply, the amount of triacylglycerols in the cells of the studied algae is 26%. Reducing the amount of available nitrogen in the composition of the nutrient medium allows to increase the amount of triacylglycerols in the cells of A. dimorphus. In the presence of 75% of nitrogen, the amount of lipids was 32.1%, i.e., the environment with this nitrogen supply is not profitable to use to increase the amount of TAG.

For 50 and 25% nitrogen supply, twice as many of these compounds are produced. A similar experiment was conducted with a different species, *Chlamydomonas reinhardtii* (Li-Beisson et al., 2015; Fan et al., 2011; Goodson et al., 2011). It was also confirmed that nitrogen starvation affects the de novo synthesis of TAG, by redirecting acetyl-CoA to the synthesis of fatty polyunsaturated acids from the pathways of starch synthesis.

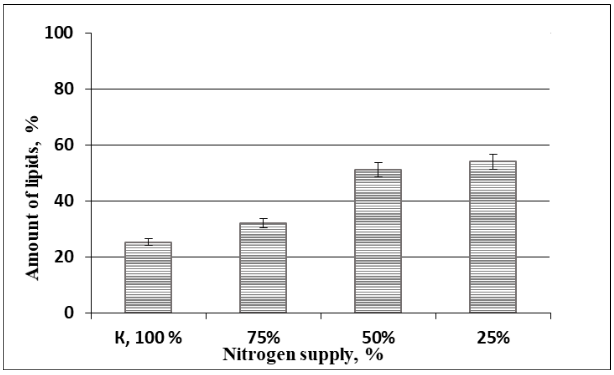

Fig. 2. The amount of lipids of A. dimorphus under different nitrogen supply

Differences in the amount of lipids for 50% and 25% nitrogen supply are insignificant and reliably do not differ from each other. This fact can be used economically and spend less sodium nitrate for the preparation of nutrient medium when the target product of cultivation is triacylglycerols. It is known that triacylglycerols from algae can become one of the main components of biodiesel due to their high energy value.

There is also a direct relationship between nitrogen concentration and the amount of synthesized protein (Fig. 3). A decrease in the amount of total protein was noted in those variants of the experiment, where a significant increase in the amount of lipids was recorded - 50% and 25% of nitrogen supply. However, the decrease in the amount of protein in the cells of *A. dimorphus* is not as pronounced as the increase in the amount of triacylglycerols.

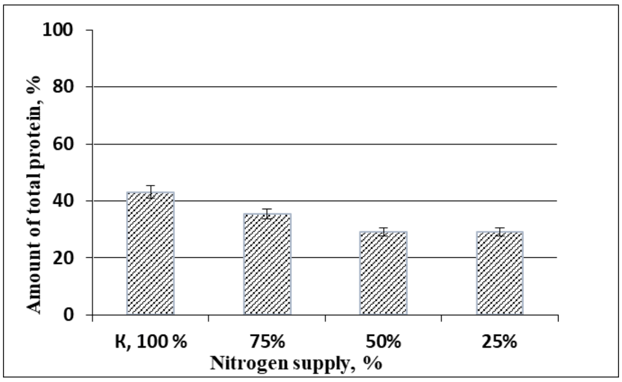

Fig. 3. The number of proteins of A. dimorphus under different nitrogen supply

Nitrogen is one of the main elements that is needed for the formation of the protein structure, it is logical to assume that a decrease in this element will lead to a decrease in protein, which is what our research proves. However, the decrease in the amount of protein is not critical in comparison with the control, and if we compare the amount of protein when supplied with nitrogen 50% and 25%, then no significant difference was found.

The next stage of our work was to determine the amount of carotenoids in the acetone extract. It is known that the concentration of mineral components of the nutrient medium has a significant influence on the content of the main photosynthetic pigments, in particular chlorophylls a, b and carotenoids. The formation of the photosynthetic apparatus and the speed of its renewal in the process of functioning depend on the number of available elements of mineral nutrition.

Under stressful conditions, green algae cells usually produce more carotenoids. However, it is known that the lack of a sufficient amount of nitrogen-containing compounds can lead to a decrease in the synthetic activity of cells. A slight decrease in the number of carotenoids was noted under different nitrogen supply of A. dimorphus cells (Fig. 4).

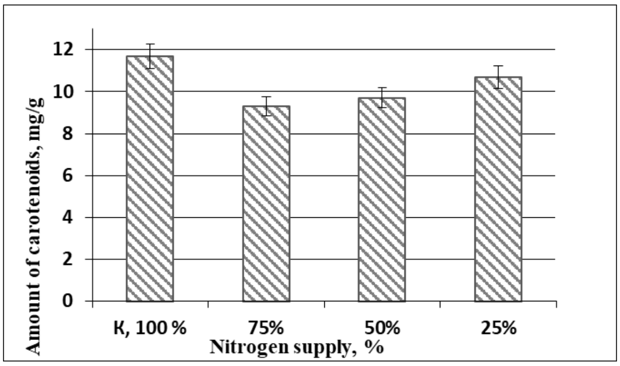

Fig. 4. Amount of carotenoids of A. dimorphus under different nitrogen supply

At 100% nitrogen supply, the content of carotenoids in algal cells was 11.7 mg/g. At 75% nitrogen supply, this indicator was about 9 mg/g, and the further decrease of nitrogen was not so critically reflected in the amount of carotenoids. It was noted that in the presence of only 25% of nitrogen from the control value, the amount of carotenoids did not significantly differ from the indicators of the control environment and was about 11 mg/g.

Algae carotenoids are used as biologically active additives in aquaculture. For example, astaxanthin can be used to improve the quality of fish roe, increase the profitability of aquaculture, and improve the reproductive function of fish.

Therefore, nitrogen is an important element that affects the synthesis of various compounds: carbohydrates, proteins, lipids, and carotenoids. This proves the importance of this element and its close relationship with the entire vital activity of the cell.

Our conducted research allows us to recommend the optimal composition of the medium for obtaining lipophilic compounds of *A. dimorphus* - where the supply of nitrogen in the medium is the lowest, that is, reduced to 25%. The difference between 50% nitrogen concentration and 25% was not reliably detected, which avoids the expense of sodium nitrate for the preparation of the medium.

Therefore, by regulating the concentration of nitrogen in the environment, namely reducing it to small amounts of 50% and 25%, we can increase the synthesis of neutral lipids (NTL). Also, low nitrogen concentration does not affect the amount of carotenoids in the cells of *A.dimorphus* algae. This allows the use of green algae *A. dimorphus* in bioenergetics as a promising species for the synthesis of lipophilic compounds.

Conclusions.

A decrease in available nitrogen in the nutrient medium for A.dimorphus leads to an increase in the amount of triacylglycerols in algae cells. The highest indicator of the number of neutral lipids was noted at 50% and 25% nitrogen supply of *A.dimorphus* culture.

Under conditions of nitrogen starvation, a decrease in the amount of total protein in A.dimorphus cells was noted, but this trend is not directly related to an increase in the amount of triacylglycerols.

In order to obtain lipophilic compounds of *A. dimorphus*, it is recommended to use Yanovsky's medium with 25% $NaNO_3$ content. Under these conditions, we obtain A.dimorphus biomass with an optimal content of triacylglycerols, carotenoids and protein.

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ВПЛИВ НІТРОГЕННОГО ЗАБЕЗПЕЧЕННЯ НА НАКОПИЧЕННЯ БІОМАСИ ТА ЛІПОФІЛЬНИХ СПОЛУК ACUTODESMUS DIMORPHUS (TURPIN) TSARENKO

Л. М. Чебан, А. Г. Середюк

Робота присвячена вивченню впливу нітрогенного забезпечення на накопичення ліпофільних сполук Acutodesmus dimorphus (Turpin) Tsarenko. Acutodesmus dimorphus (Turpin) Tsarenko – вид зелених водоростей родин Scenedesmaceae. Це цинобіальні прісноводні водорості, що є представниками водойм помірних широт. Нітратне забезпечення – це ключовий фактор у процесі культивування водоростей. Від кількості нітрогену у живильному середовищі буде залежати спряженість клітинних процесів водоростей. Вміст нітрогену є домінуючим фактором при накопиченні ліпофільних сполук біомаси водоростей. Визначали вплив нітратного забезпечення на накопичення біомаси, кількість білків, ліпідів та каротиноїдів A. dimorphus. Для моделювання депривації за нітрогеном використовували середовище Яновського зі зменшеним вмістом $NaNO_3$. Контрольне середовище Яновського містило 80 мг/л $NaNO_3$ (100 % забезпечення нітрогену). У дослідних варіантах середовища зменшували кількість $NaNO_3$ відповідно до 75%, 50 % та 25 %. Зменшення доступного нітрогену у складі живильного середовища для A. dimorphus призводить до збільшення кількості триацилгліцеролів у клітинах водоростей. Найвищий показник кількості нейтральних ліпідів відмічений при 50% та 25% забезпеченні нітрогеном культури A. dimorphus. За умови азотного голодування відмічене зменшення кількості загального білка у клітинах A. dimorphus, проте ця тенденція немає прямої залежності зі збільшенням кількості триацилгліцеролів. З метою отримання ліпофільних сполук A. dimorphus рекомендовано застосовувати середовище Яновського зі 25 % вмістом $NaNO_3$. За цих умов отримуємо біомасу A. dimorphus з оптимальним вмістом триацилгліцеролів, каротиноїдів та білка.

Keywords: зелені водорості, ліпіди, каротиноїди, забезпечення нітрогеном

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