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ДІЯ ФІЗИЧНИХ ФАКТОРІВ НА БІОЛОГІЧНІ ОБ'ЄКТИ

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TEMPERATURE-SALT STRESS INCREASES YIELD OF VALUABLE METABOLITES AND SHELF LIFE OF MICROALGAE

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Background: Microalgae are very important for production of some chemicals industrially, such as carbohydrates, peptides, lipids, and carotenoids. There are many ways by which the yield of the valuable chemicals can be improved. They may include the reduction of cultivation temperature and change in the composition of growth media.

Objectives: study adaptive mechanisms of *Dunaliella salina* Teodoresco and *Chlorococcum dissectum* Korshikov to low temperature and to develop the method for their hypothermic storage.

Materials and methods: The objects of research were unicellular green microalgae *D. salina* and *Ch. dissectum*. Cold adaptation (for 24 hours) and hypothermic storage (for 3–30 days) of cultures were performed at 4 °C without lighting. Light and confocal microscopy methods were used to determine the viability and pigment composition of cells. The Alamar Blue (AB) test was used as an express method for assessing the metabolic activity of cells before and after cold adaptation.

Results: The study has showed that lowered cultivation temperature and increased salinity of the growth medium increase the fluorescence of the NR dye in *D. salina* cells and do not affect this indicator in *Ch. dissectum*. The 24 h exposition at 4 °C does not lead to a significant decrease in the relative fluorescence units according to the AB test. Storage the algae at 4 °C does not result in their loss of viability and motility for up to 30 days.

Conclusions: Incubation of *D. salina* at 4 °C for 24 hours increase carotenoid production compared to the intact culture, while it has no effect on *Ch. dissectum*, regardless of the growth medium composition. The short-term effect of low temperatures does not lead to a significant decrease in the metabolic activity of *D. salina* and *Ch. dissectum*. Storage of museum collection of *D. salina* and *Ch. dissectum* is possible for a period of 30 days at 4 °C without significant loss of metabolic activity, motility and cell concentration. These results also demonstrate that a combination of high salt and low temperature stresses increase the yield of valuable metabolites.

KEY WORDS: microalgae; salt stress; temperature stress; *Dunaliella salina*; *Chlorococcum dissectum*.

Microalgae today are a significant part of the bio-business, which is developing rapidly and gaining global scale. Various types of microalgae under certain conditions are able to synthesize valuable metabolites such as carbohydrates, peptides, lipids, and carotenoids [1–6].

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Halotolerant microalgae *Dunaliella salina* is one of the richest sources of natural carotenoids. They can accumulate carotenoids under conditions that are sub-optimal for their growth high light intensity, low temperatures, nutrient limitation, high salt concentrations. Under the conditions carotenoids may make up 10% of their dry biomass. *Chlorococcum dissectum* is another species of interest that may change its production of lipids and carotenoids depending on changes in the composition of the growth medium, making microalgae a potential source of important bio-business resources [7].

One of the primary environmental drivers of algal productivity is temperature. During mass cultivation in open ponds, temperature fluctuations can stress or temporarily distort metabolic processes affecting growth, overall productivity, and biomass composition [8–10]. As reported for several taxa, cold stress, either individually or in some combination with another environmental stress, reduces growth/productivity. However, it can stimulate lipid production [11, 12] which is of industrial interest. Thus, the fundamental study of the mechanisms of resistance of various microalgae to temperature stress is the basis for the targeted synthesis of industrially important compounds.

The ability of algae to survive under extreme conditions has formed during evolution. Recent metabolic analyses show that plants have developed a number of strategies to reorganize their metabolism in adverse conditions, but the full extent of their adaptation mechanisms is unknown [13, 14]. The cellular responses used by chlorophytes and other microorganisms to survive at low temperatures have only been extensively studied in recent years, with a focus on psychrophilic species [15, 16]. For different types of algae there are obviously common genetically determined protective processes taking plays during the stress response phase. The processes enable them to survive during short-term exposure to various chemical and physical factors such low temperature [17, 18]. The processes include the synthesis of carotenoids, cold shock proteins, and activation of the pentose phosphate pathway [6, 19]. At the same time, it has been shown that even a short-term temperature decrease to 3 °C can lead to death of vegetative cells of blue-green algae *Anacystis nidulans* and *Chlamydomonas reinhardtii* [20, 21]. Thus, the resistance of microalgae to low temperatures depends on their taxonomic and morphofunctional properties. It is of high priority to investigate the effects of cold temperature and other factor on certain types of algae. On the one hand, this may allow to increase the production of metabolites, on the other hand, to avoid the death of cell in culture because of too harsh condition.

The aim of this study was to examine adaptive mechanisms of *Dunaliella salina* and *Chlorococcum dissectum* to low temperature and to develop the method for their hypothermic storage.

MATERIALS AND METHODS

Microalgae culture

Unicellular green microalgae *Dunaliella salina* Teodoresco and *Chlorococcum dissectum* Korshikov were included in this study.

Ch. dissectum is a freshwater biflagellate. Its cell wall consists mainly of glycoproteins (hydroxyproline). *Ch. dissectum* has a massive chloroplast.

D. salina is a biflagellate, halophilic, unicellular photosynthetic microalga that does not have rigid cell wall, which makes the alga sensitive to osmotic pressure.

Cultures of *D. salina* and *Ch. dissectum* were obtained from the collection of microalgae cultures of the Department of Botany, V. N. Karazin Kharkiv National University.

Cultural medium and growth measurements

Cultures of *D. salina* and *Ch. dissectum* were grown under passive aeration conditions before the stationary growth phase. Microalgae were cultured at 25±2 °C (normothermia).

Biomass accumulation was performed in culture vials (TPP, Switzerland) with a volume of 40 ml, under round-the-clock illumination with white fluorescent light 52.84 μmol photons $m^{-2}s^{-1}$ or 3 kLux [22, 23].

Ch. dissectum was cultured in growth medium BG-11 [24] and BG-11 with the addition of sodium chloride at final concentrations of 0.06 M NaCl or 0.2 M NaCl.

D. salina was grown in two media containing different amounts of sodium chloride: Ramaraj (Rm) [25] and Ramaraj with a high sodium chloride (Rm_{NaCl}) concentration of 4 M NaCl.

Cold adaptation and hypothermic storage

Samples of all tested cultures (20 ml) in the stationary growth phase were placed in culture vials (TPP, Switzerland) with a volume of 40 ml. Cold adaptation (for 24 hours) and hypothermic storage (for 3-30 days) of cultures were performed at 4°C without lighting.

Viability and pigment analysis

Growth dynamics of all cultures, the amount of *D. salina* cells and their motility were controlled by counting cells in a hemocytometer [26] using a light microscope. The preservation of *D. salina* cells was assessed based on morphological properties: cell integrity, chloroplast structure, motility, presence or loss of flagella. Additionally, *D. salina* samples were cultivated in a liquid growth media.

Viability of *Ch. dissectum* cells was determined by controlling the number of colony-forming units (CFU) for 10 days at 25 °C and round-the-clock lighting on agar medium BG-11 having an appropriate content of sodium chloride (n=3) [27].

The Alamar Blue (AB) test was used as an express method for assessing the metabolic activity of *Ch. dissectum* and *D. salina* cells before and after cold adaptation [28]. The initial concentration of cells in all samples was about 1.5×10^8 cells/ml. Control are cells under normal cultivation conditions in the same growth media. Optical density was measured using a Tecan Genios Microplate Reader (Tecan Inc, Australia) at a wavelength of 550 nm and an emission of 590 nm. The obtained data were processed using XFLUOR4 v.4.50 and expressed in relative fluorescence units (RFU).

To evaluate the intracellular synthesis of lipids before and after cold adaptation, Nile Red (NR) dye (Sigma-Aldrich) was used, which was added at a ratio of 10 μl per 1 ml of culture [29, 30].

The fluorescence of total chlorophyll and NR in *D. salina* cells was determined using a LSM-510 Meta laser confocal microscope (Carl Zeiss, Germany) under excitation with a diode laser at 405 and 543 nm. The fluorescence intensity was determined using the AimImageBrowser program.

Ch. dissectum cells were visualized by confocal microscopy using an Olympus FV10i-LIV laser scanning confocal microscope (Olympus, Japan). The sensitivity of the detectors and the intensity of the lasers in all the studied samples were the same. The size of the confocal aperture was 2.0. Relative fluorescence intensity measurements in each individual cell and image deconvolution were performed using the Olympus CellSens Dimension Desktop software.

All data were processed by Statistica 6.0 package for Windows (Tulsa, OK, USA), and the results were expressed as means and standard deviation. Comparisons were tested using Student's t-test with Bonferroni correction. Values of $p<0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

When studying the effect of short-term adaptation at 4°C, certain regularities were revealed: within 24 hours, cell viability, and motility of *D. salina* and *Ch. dissectum* cultures

did not change compared to control samples (normothermia). Despite this, structural changes took place in *D. salina* culture, namely, an increase in the number and size of lipid globules containing carotenoids (Fig. 1). NR fluorescence was also elevated compared to intact culture (Fig. 2).

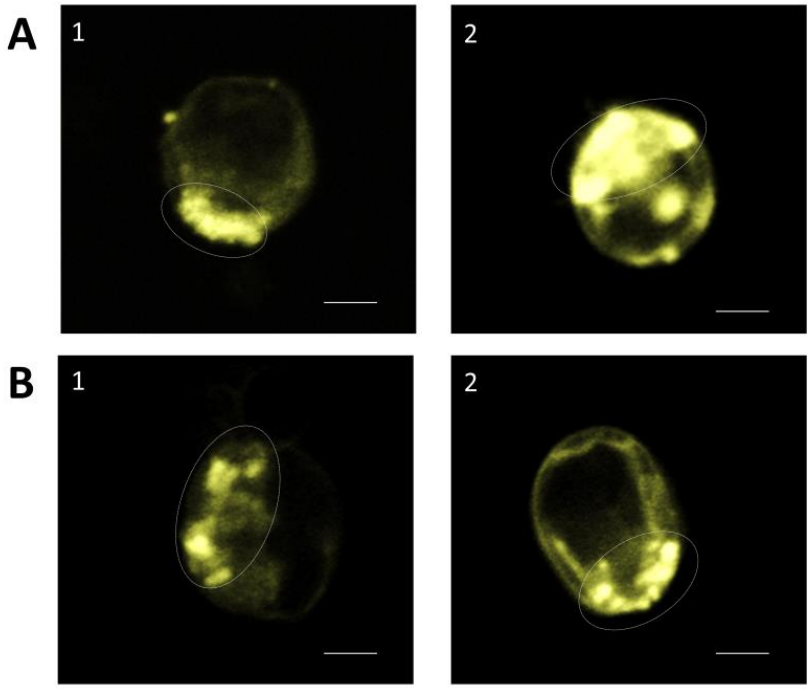

Fig. 1. Visualization of lipid droplets in *D. salina* cells by confocal microscopy during cultivation in Rm (A) and Rm_{NaCl} (B) media depending on the cultivation temperature: 1 — control, 2 — after incubation at 4 °C for 24 hours. Yellow is NR fluorescence (scale bars =10 μm).

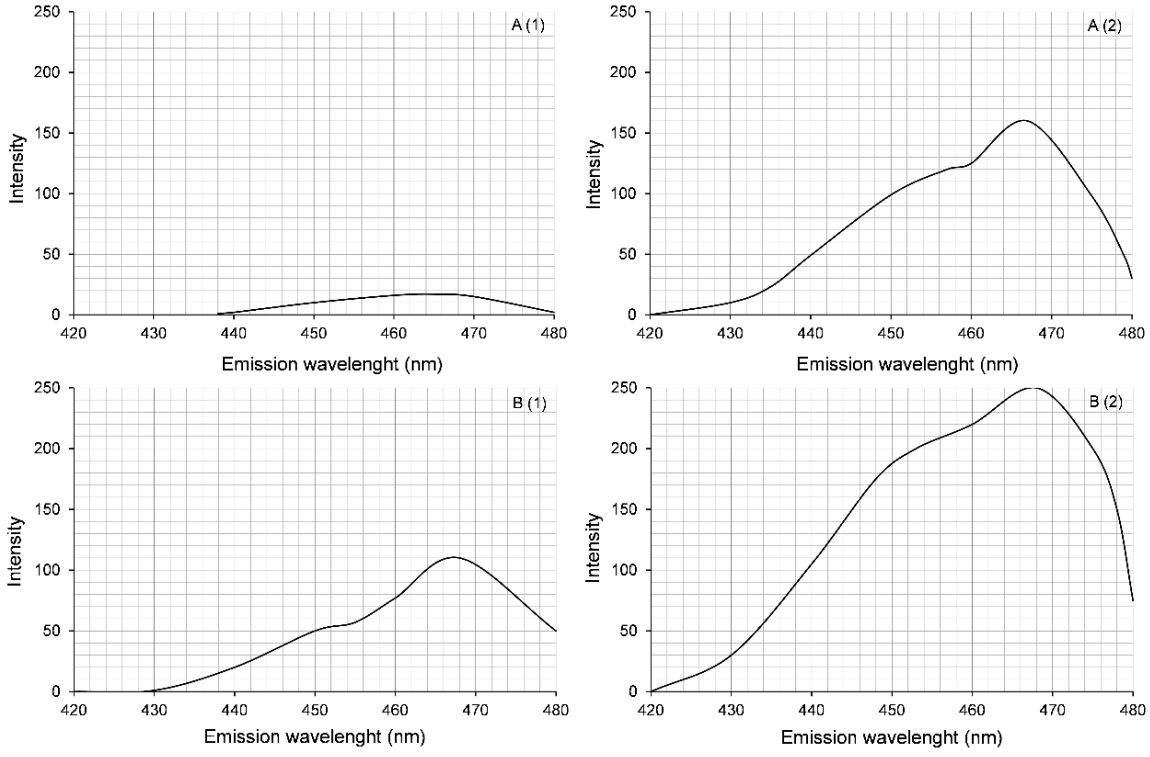

Fig. 2. NR fluorescence of *D. salina* cells during cultivation in Rm (A) and Rm_{NaCl} (B) media depending on the cultivation temperature: 1 — control, 2 — after incubation at 4°C for 24 hours.

The composition of the culture-adaptation medium also influenced the lipid synthesis. NR fluorescence was higher in the medium with high levels of sodium chloride. The results indicate the benefits of using a combination of low temperature and high salinity in a two-step culture process to maximize the synthesis of valuable metabolites.

Measurement of the intensity of intrinsic fluorescence of total chlorophyll in *D. salina* cells showed that short-term incubation at 4 °C and deprivation of light did not cause degradation of chlorophyll in the cells regardless of culture medium composition (Fig. 3).

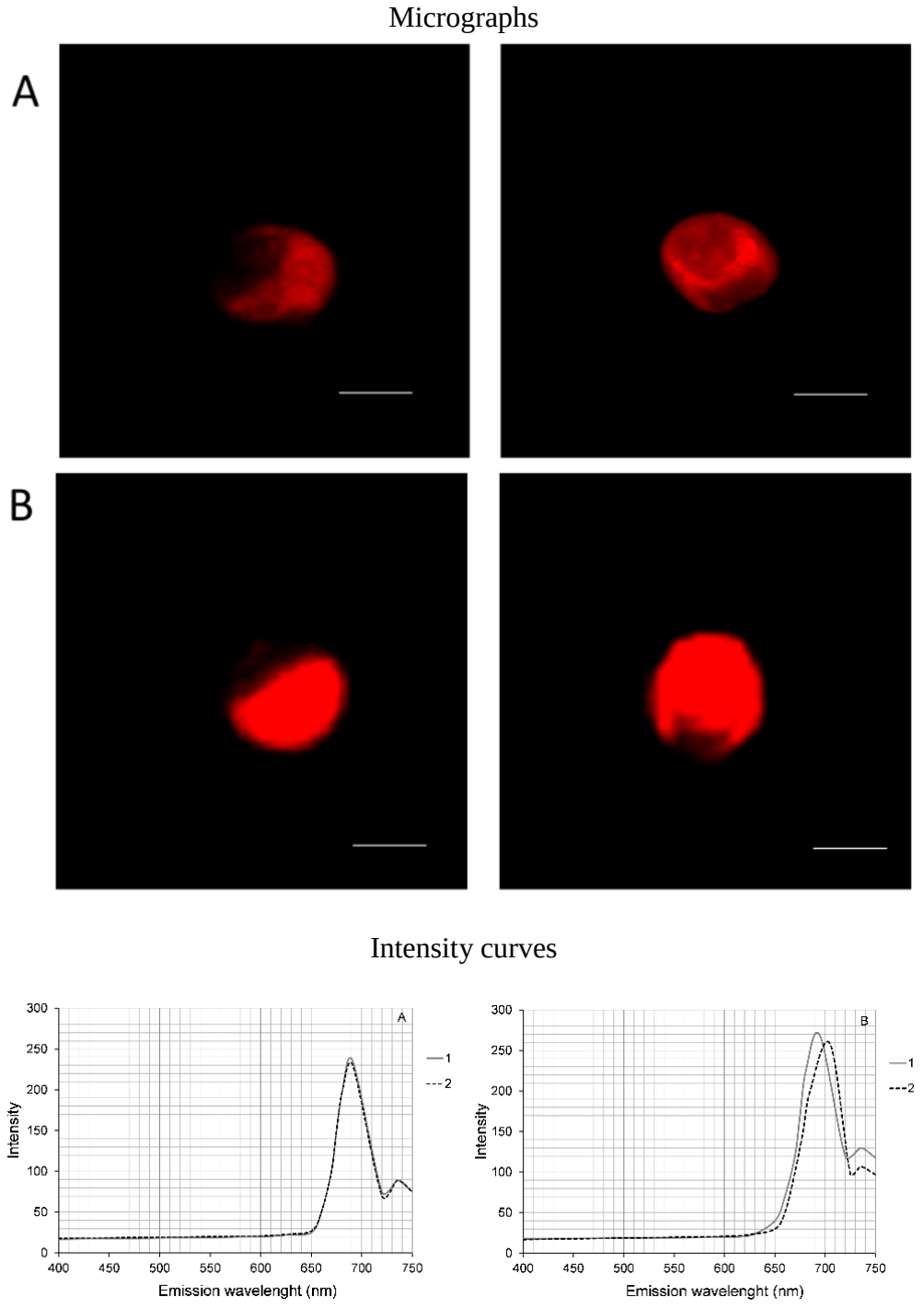

Fig. 3. Fluorescence of chlorophyll (red color) (micrographs and intensity curves) in *D. salina* cells in Rm (A) and Rm_{NaCl} (B): 1 — control, 2 — after incubation at 4°C for 24 hours (scale bars = 10 μm).

Incubation of *Ch. dissectum* cells at 4 °C for 24 hours did not lead to regular changes in the fluorescence intensity of chlorophyll or NR regardless of the content of sodium chloride in the growth medium (Fig. 4).

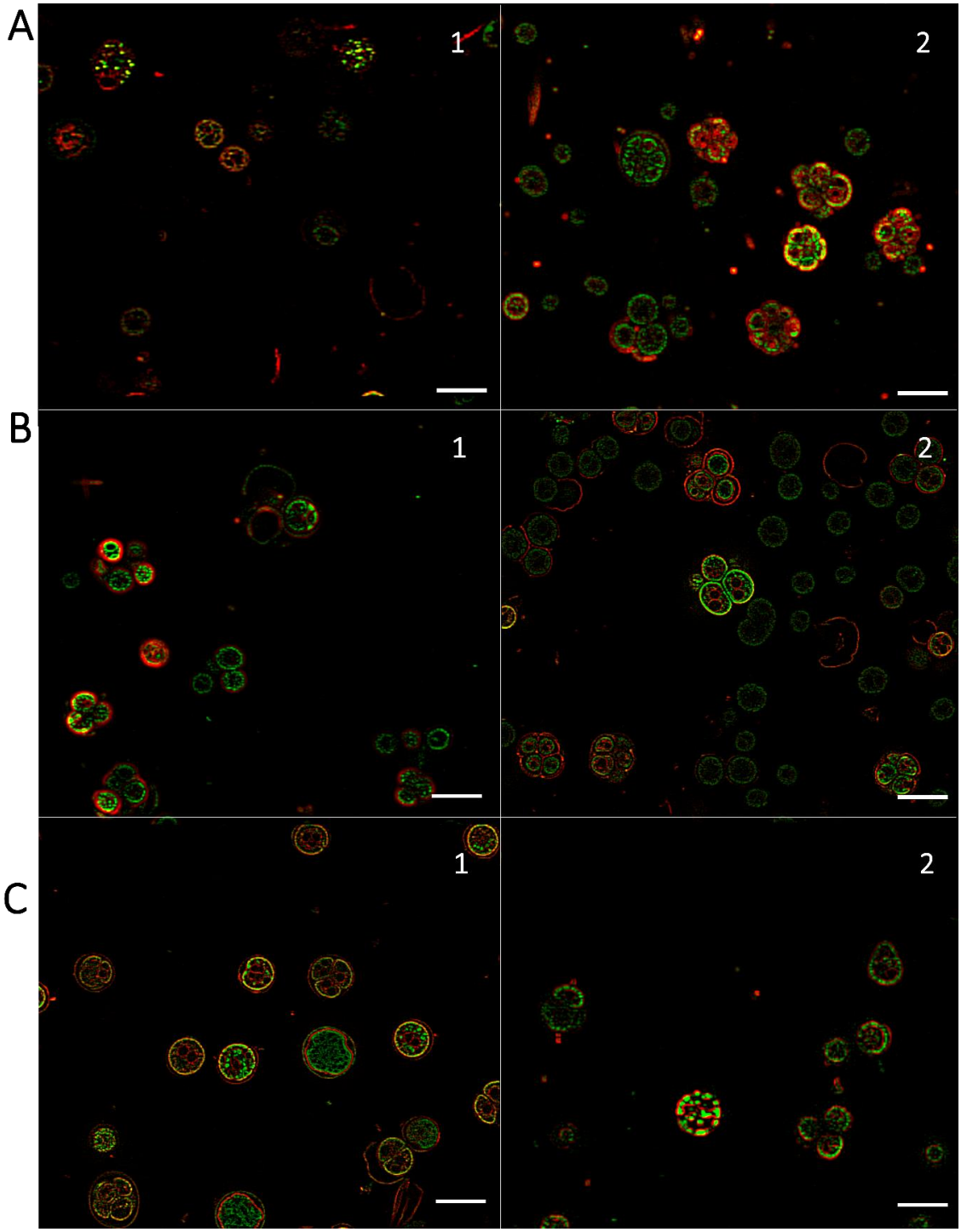

Fig. 4. Fluorescence of chlorophyll (green) and NR dye (yellow) in *Ch. dissectum* microalgae cells in the growth media with different sodium chloride content: 0 M (A), 0.06 M (B), 0.2 M (C) before (1) and after (2) incubation at 4 °C for 24 hours (scale bar=10μm).

The absence of regular changes in the pigment composition of *Ch. dissectum* cells after low temperature exposition and high salt stress may be species-specific. In contrast to

halotolerant *D. salina*, which is able to adapt to abrupt temperature and salinity changes, *Ch. dissectum* obviously lacks this rapid natural adaptation mechanism aimed at maintaining cell viability under unfavorable conditions.

It was shown that the values of RFU of *D. salina* and *Ch. dissectum* cells slightly decreased before and after cold adaptation.

No significant inhibition of *D. salina* cells' metabolic activity was recorded (Fig. 5A).

The lowest RFU values (up to 35 %) compared to control group was observed in *Ch. dissectum* culture in the medium containing 0.2 M sodium chloride. In other samples metabolic activity decreased slightly or remained unchanged (Fig. 5B). This behavior of cells may be due to the fact that *Ch. dissectum* cells were subjected to double stress: salt and temperature. That would lead to slowdown in the respiratory chain electron transfer of the cells and inhibition of their metabolic activity.

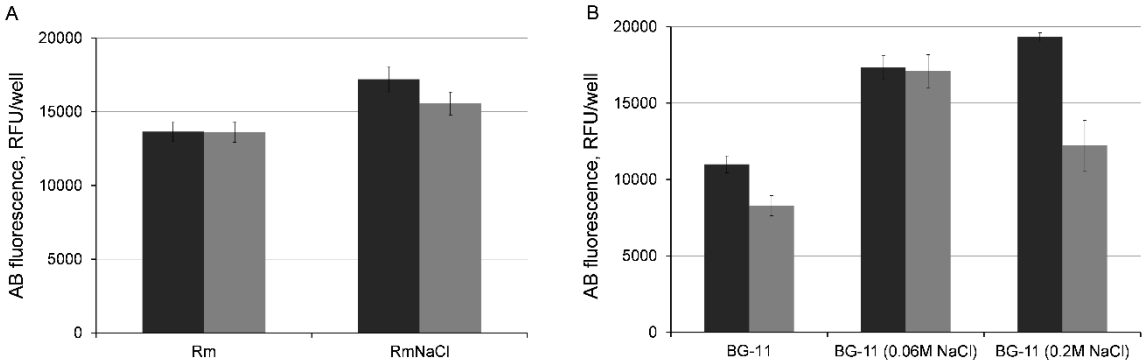

Fig. 5. AB fluorescence in ■ — control samples and ■ — samples after cold adaptation of *D. salina* (A) and *Ch. dissectum* (B) microalgae cells at 4 °C for 24 hours.

The effect of hypothermic storage on the viability and functional properties of *D. salina* and *Ch. dissectum* microalgae cells

Low temperature storage is widely used to preserve various types of microalgae. However, there is not enough information about the long-term effect of low temperatures on the stability of the cells of *D. salina* and *Ch. dissectum* and their functioning. Therefore, after studying the short-term effect of low temperatures on *D. salina* and *Ch. dissectum*, it was also expedient to study the effect of long-term hypothermic storage on these cultures to assess the survival and motility of the cells depending on the exposure time.

Our results showed that 30-day-exposure at 4 ºC had individual species-specific differences.

The long-term storage of *D. salina* cells at low temperatures did not affect the cell concentration during the entire period of observation (Fig. 5A). On day 30, there was a significant increase in the concentration of *D. salina* cells in the Rm medium compared to the control, which indicated an increase in biomass.

At the same time, hypothermic storage at 4 °C reduced cell motility (Fig. 6B). A significant decrease by 20% was obtained in Rm_{NaCl} medium by day 10. The decrease in Rm medium made up 40% by day 30. At the same time, the results indicate that such a decrease in mobility was not fatal for these cells: the cells continued to divide and their concentration grew.

The study of *Ch. dissectum* viability showed that 30-day hypothermic storage in BG-11, BG-11 (0.06 M NaCl) and BG-11 (0.2 M NaCl) at 4 °C increased the number of CFU compared to the corresponding samples, cultivated under normothermia (Fig. 7). At the same time, a more considerable increase in biomass was observed in BG-11 medium (0.06 M NaCl). These data

have indicated that the cell culture of microalgae *Ch. dissectum* can be stored at 4 °C for 30 days without their viability loss.

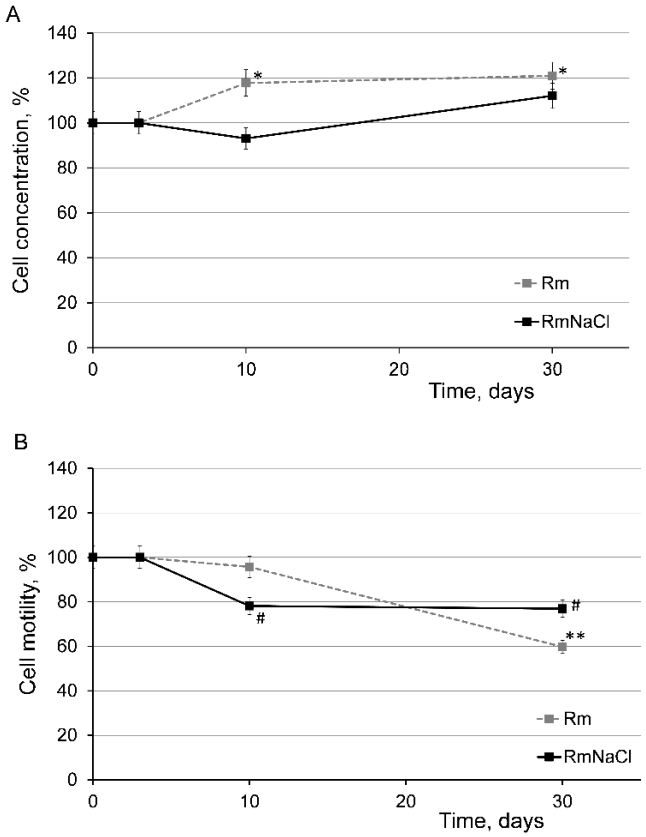

Fig. 6. Concentration (A) and motility (B) of *D. salina* cells during their incubation at 4 °C depending on the duration of exposition and culture media: --■-- — Rm, ■ — Rm_{NaCl}.

** — The difference was statistically significant compared to day 0 ($p<0.05$).*
*** — The difference was statistically significant compared to day 0 in the Rm medium ($p<0.05$).*
 # — compared to day 0 in the Rm_{NaCl} medium ($p<0.05$).

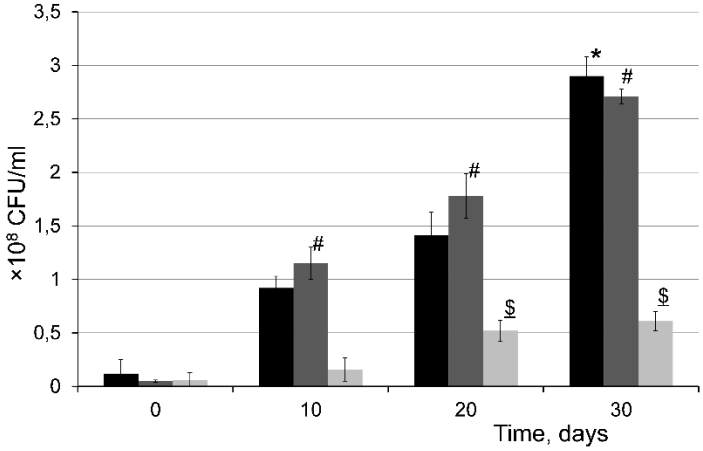

Fig. 7. Determination of *Ch. dissectum* viability by their ability to form colonies after they were deposited at 4 °C, depending on the type of growth medium and exposure time: ■ — BG-11, ■ — BG-11 (0.06 MNaCl); ■ — BG-11 (0.2 MNaCl).

** — The difference was statistically significant compared to day 0 value ($p<0.05$) for the environment BG-11.*
 # — compared to day 0 for medium BG-11 (0.06 M NaCl) ($p<0.05$).
$ — compared to day 0 for medium BG-11 (0.2 M NaCl) ($p<0.05$).

Thus, increasing the content of sodium chloride to 4 M for *D. salina* and 0.06 M for *Ch. dissectum* can promote the synthesis of protective compounds, increase the resistance of the cells to low temperatures, and prolong their hypothermic storage. Moreover, this may increase the yield of valuable metabolites from the cells.

CONCLUSIONS

Incubation of *D. salina* at 4 °C for 24 hours increase carotenoid production compared to the intact culture, while it have no effect on *Ch. dissectum*, regardless of the growth medium composition; the short-term effect of low temperatures does not lead to a significant decrease in the metabolic activity of *D. salina* and *Ch. dissectum*; storage of museum collection of *D. salina* and *Ch. dissectum* is possible for a period of 30 days at 4 °C without significant loss of metabolic activity, motility, and cell concentration. These results also demonstrate that a combination of high salt and low temperature stresses may increase the yield of valuable metabolites.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report. We certify that the submission is original work and is not under review at any other publication.

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ТЕМПЕРАТУРНО-СОЛЬОВИЙ СТРЕС ЯК СПОСІБ ПІДВИЩЕННЯ ВИХОДУ ЦІННИХ МЕТАБОЛІТІВ ТА ЗБІЛЬШЕННЯ ТЕРМІНІВ ЗБЕРЕЖЕННЯ МІКРОВОДОРОСТЕЙ

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Актуальність. Мікроводорості важливі для промислового виробництва деяких хімічних речовин, таких як вуглеводи, пептиди, ліпіди та каротиноїди. Є багато способів, за допомогою яких можна підвищити вихід цінних хімічних речовин з клітин мікроводоростей. Вони можуть включати зниження температури культивування та зміну складу середовища для росту.
Мета роботи. Вивчити механізми адаптації *Dunaliella salina* Teodoresco та *Chlorococcum dissectum* Korshikov до низької температури та розробити методику їх гіпотермічного зберігання.
Матеріали і методи. Об'єктом дослідження були одноклітинні зелені мікроводорості *D. salina* та *Ch. dissectum*. Культивування здійснювали відповідно до стандартних методик. Холодову адаптацію (протягом 24 годин) та гіпотермічне зберігання (упродовж 3–30 днів) культур здійснювали при 4 °С без освітлення. Життєздатність та пігментний аналіз клітин вивчали за допомогою методів світлової та конфокальної мікроскопії. Alamar Blue (AB) тест використовували в якості експрес-методу оцінки метаболічної активності *Ch. dissectum* та *D. salina* до та після холодової адаптації.
Результати. Дослідження показали, що знижена температура культивування та підвищена солоність середовища росту збільшує флуоресценцію барвника NR у клітинах *D. salina* та не впливає на цей показник в *Ch. dissectum*. Було встановлено, що 24-годинна експозиція суспензій обох культур при 4 °С не призводить до значного зниження відносних одиниць флуоресценції за АВ-тестом. Зберігання при 4 °С не спричиняє втрату життєздатності та рухливості досліджених мікроводоростей протягом 30 днів.
Висновки. Депонування *D. salina* при 4 °С протягом 24 годин збільшує виробництво каротиноїдів порівняно з інтактною культурою на відміну від *Ch. dissectum*, де не зафіксовано достовірних відмінностей, незалежно від складу середовища росту. Короткочасна дія низьких температур не призводить до істотного зниження метаболічної активності *D. salina* та *Ch. dissectum*. Зберігання музейної колекційної культури *D. salina* та *Ch. dissectum* можливо протягом 30 днів при 4 °С без значної втрати метаболічної активності, рухливості та концентрації клітин. Отримані результати демонструють, що поєднана дія підвищеної солоності та низьких температур може збільшити вихід цінних метаболітів.
КЛЮЧОВІ СЛОВА: мікроводорості; сольовий стрес; температурний стрес; *Dunaliella salina*; *Chlorococcum dissectum*.