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ACTION OF PHYSICAL AGENTS ON BIOLOGICAL OBJECTS

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EFFECTS OF SALT STRESS AND COOLING REGIMES ON THE PIGMENT COMPOSITION OF *DUNALIELLA SALINA* CELLS

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Background: Microalgae are able to produce a significant amount of biologically significant substances. In connection with the growing popularity of microalgae, it is important to develop effective methods for storing cultures and creating strain banks. This will not only meet the needs of science and biotechnology for viable and sustainable crops, but will also solve the problem of biodiversity conservation.
Objectives: study the effect of salt stress and cooling regimes on the pigment composition of microalgae *Dunaliella salina* cells in order to increase their safety after freezing-thawing.
Materials and methods: The objects of the study were the unicellular green microalgae *D. salina*. Cultivation was carried out according to the standard method on nutrient media with different amounts of NaCl and trace elements. Adaptation to low temperatures was carried out by exposure of samples in the dark at temperature 4°C for 24 hours. Freezing was performed by placing 1 ml of the cell suspension in a 1.8 ml polypropylene cryogenic vial (Nunc, Sigma-Aldrich), cooled at a rate of 1 deg/min using a Mr. Frosty with following regimes: to -10°C, -40°C, -40°C followed by immersion to liquid nitrogen or direct immersion to liquid nitrogen (-196°C). Thawing was carried out in a water bath (37°C) with continuous shaking for 1–2 min. Microscopic examinations were carried out on an LSM-510 Meta laser scanning microscope (Carl Zeiss, Germany) upon excitation by a diode laser with a wavelength of 405 nm and 573 nm using a Nile Red stain.
Results: It has been established that the formation of intracellular lipid globules and the synthesis of carotenoids in *D. salina* cells contribute to an increase in the concentration and number of motile cells after freezing-thawing. It has been shown that during rapid cooling, adaptive mechanisms do not have time to turn on in cells, and complete destruction of carotene-containing lipid globules occurs.
Conclusions: Cryopreservation of *D. salina* cells should be carried out at a rate of 1 deg/min to -40°C, followed by immersion in liquid nitrogen and a mandatory stage of precultivation at 4°C for 24 hours. This approach allows the cells to adapt to a decrease in temperature, which contributes to the best result after freezing-thawing.
KEY WORDS: microalga; salt stress; temperature stress; *Dunaliella salina*; cryopreservation; cryoresistance.

Microalgae are important for the development of modern biotechnology, as they are able to accumulate a significant amount of biologically important compounds. One of the promising strains is the unicellular halophilic green microalgae *Dunaliella salina*. Under certain conditions, it is capable of hypersynthesis of lipids, glycerol, proline, beta-carotene

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and stress proteins [1–7]. Accumulation usually occurs under conditions of stressful growth. Therefore, the content of valuable metabolites in the biomass of microalgae can be increased by programmed variation of their growing conditions, for example, changing the composition of the cultivation medium, increasing salinity, or decreasing temperature [8–10].

The increasing popularity of microalgae for biotechnology [11, 12] increases the relevance of finding effective ways to preserve them for a long time. The development of low-temperature storage methods aims to minimize the time and cost involved, prevent contamination, and maintain genetic stability that is compromised by conventional serial transfers.

It is known that glycerol is widely used as a cryoprotectant to increase the safety of cells after cryopreservation [13–15]. Therefore, it can be assumed that *Dunaliella salina* cells containing an increased amount of glycerol may be more resistant to low temperatures.

It was also shown [16, 17] that preliminary cold hardening of plant cells can increase the percentage of preservation after cryopreservation due to the launch of adaptive cellular mechanisms.

In a previous work, we have shown that a decrease of cultivation temperature [18], microelement deficiency in the growth medium, and preliminary hardening [19] of *Dunaliella salina* increase the content of lipid globules in cells. In this work, we investigated how temperature and salt stress together, the concentration of sodium chloride in the culture medium, and the choice of cooling mode affect the ability of cells to produce lipid globules and resistance of microalgae to low temperatures.

Therefore, the aim of the work was to study the effect of salt stress and cooling regimes on the pigment composition of *Dunaliella salina* cells in order to increase their safety after freezing-thawing.

MATERIALS AND METHODS

Microalga *D. salina* Teod. obtained from the collection of the Department of Botany and Plant Ecology, V. N. Karazin Kharkiv National University.

D. salina cultures were grown without aeration until the stationary phase of growth (21 days). Microalgae were cultivated at 25±2°C (normothermia). The accumulation of biomass was carried out in culture flasks (TPP, Switzerland) with a volume of 40 ml under round-the-clock illumination with white fluorescent light of 52.84 μmol photons $m^{-2}s^{-1}$ [20, 21]. *D. salina* was grown on different media: Ramaraj [22] 1.5 M (Rm) and 4 M (Rm_{NaCl}) sodium chloride, Artari (Ar) [23] without trace elements.

Nile Red (NR) (Sigma-Aldrich) stain was used to visualize the accumulation of intracellular lipid globules according to the method described in [9].

Precultivation to low temperatures was carried out by exposure of samples in the dark at temperature 4°C for 24 hours.

Freezing was performed by placing 1 ml of the cell suspension in a 1.8 ml cryogenic vial (Nunc, Sigma-Aldrich), cooled at a rate of 1 deg/min using a Mr. Frosty freezing container with following regimes: to -10°C, -40°C, -40°C followed by immersion to liquid nitrogen or direct immersion to liquid nitrogen (-196°C). Thawing was carried out in a water bath (37°C).

Microscopic studies were performed using an LSM-510 Meta laser scanning microscope (Carl Zeiss, Germany) when excited by a diode laser with a wavelength of 405 nm and 573 nm.

The Aim Image Examiner computer program (Carl Zeiss Micro Imaging) was used for a comprehensive assessment of the safety of cells of the microalgae *D. salina*.

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

Features of changes in the pigment composition of cells depending on the stage of culture growth

We found that in the process of cell growth, the ratio between carotenoids and chlorophylls changes, towards an increase in carotenoids and a decrease in the number of chlorophylls, Fig. 1. Changes in the pigment composition of cells are associated with the fact that as the culture grows, the amount of nutrients in the growth media decreases, metabolic products accumulate, and therefore the cells begin to function in a new mode, which contributes to the formation of adaptive reactions.

At the same time, the maximum increase in the number of lipid globules containing carotenoids was in the culture in the stationary phase of growth, grown under conditions of salt and nutrient (without trace elements) stress. This fact explains the greater resistance of microalgae cells to stress factors in the stationary phase of growth [24].

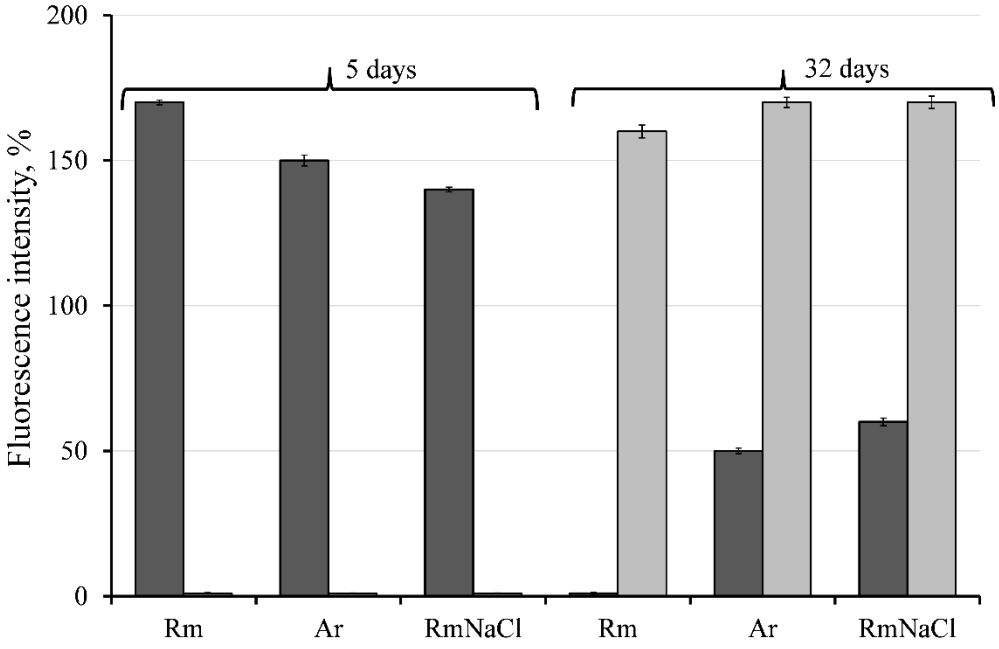

Fig. 1. Fluorescence intensity of *D. salina* cells depending on the growth stage and growth media at normothermia: ■ — chlorophyll, ■ — β-carotene.

Microphotographs also prove the fact that the pigment composition changes during the transition of cells from the exponential to the stationary phase of growth, Fig. 2. In the stationary phase of growth (21 day) the morphological state of the *D. salina* cells changes: increases size and the number of specific inclusions.

Effect of different freezing regimes on the change in the pigment composition of D. salina cells

We have previously shown that microalgae cells are capable of synthesizing glycerol and accumulating carotenoids not only under conditions of salt and nutrient stress, but also under

the influence of low temperatures [18]. It is known that carotenoids have an antioxidant effect, protect the membrane from damage by free radicals, and therefore can have a protective effect, increasing cell survival after freezing and thawing [25, 26]. Therefore, we studied the effect of different freezing regimes on the change in the pigment composition of *D. salina* cells after freezing-thawing, depending on the composition of the growth media.

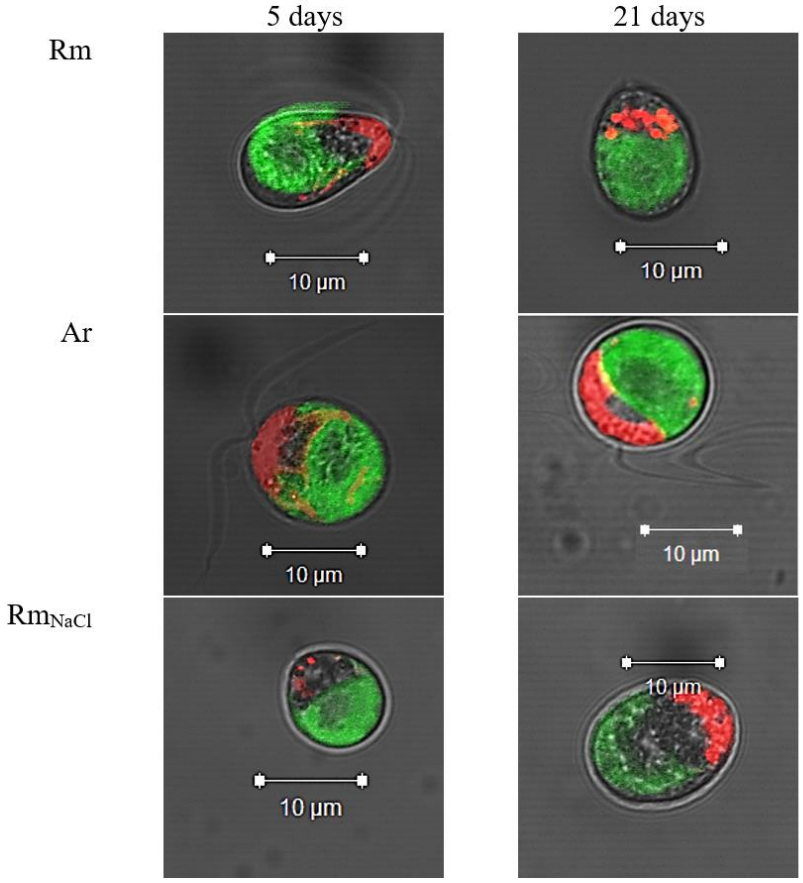

Fig. 2. Micrographs of *D. salina* cells in exponential (5 days) and stationary (21 days) growth phases. Green color — chlorophyll autofluorescence, red — Nile Red stain fluorescence.

It was found that 24-hour incubation of cells at 4°C leads to a significant increase in the intensity of NR fluorescence in cells, regardless of the growth media, Fig. 3 (2). At the same time, the intensity of chlorophyll autofluorescence did not change compared to the control when cultivated on the Rm and significantly decreased when cultivated on the Ar and Rm_{NaCl}, Fig. 3 (1). This may indicate that cells initially grown under stress conditions adapt more quickly to a decrease in temperature, while the change in the ratio between carotenoids and chlorophylls occurs more slowly in a culture grown under optimal conditions.

Slow freezing of cells to –40°C followed by immersion of samples in liquid nitrogen after thawing led to a decrease in the intensity of chlorophyll autofluorescence by 40% in a sample with Rm media, Fig. 3 (1). In the culture on the Ar, the decrease in autofluorescence was at the level of about 20%; no significant changes were recorded on the Rm_{NaCl}. At the same time, a significant increase in NR fluorescence by 10% compared with the control was recorded only in cells on the Rm_{NaCl}, Fig. 3 (2). The absence of obvious changes in the pigment composition during slow controlled freezing to -40°C followed by immersion in

liquid nitrogen indicates that the cells do not have time to undergo structural changes that would allow them to adapt to temperature stress.

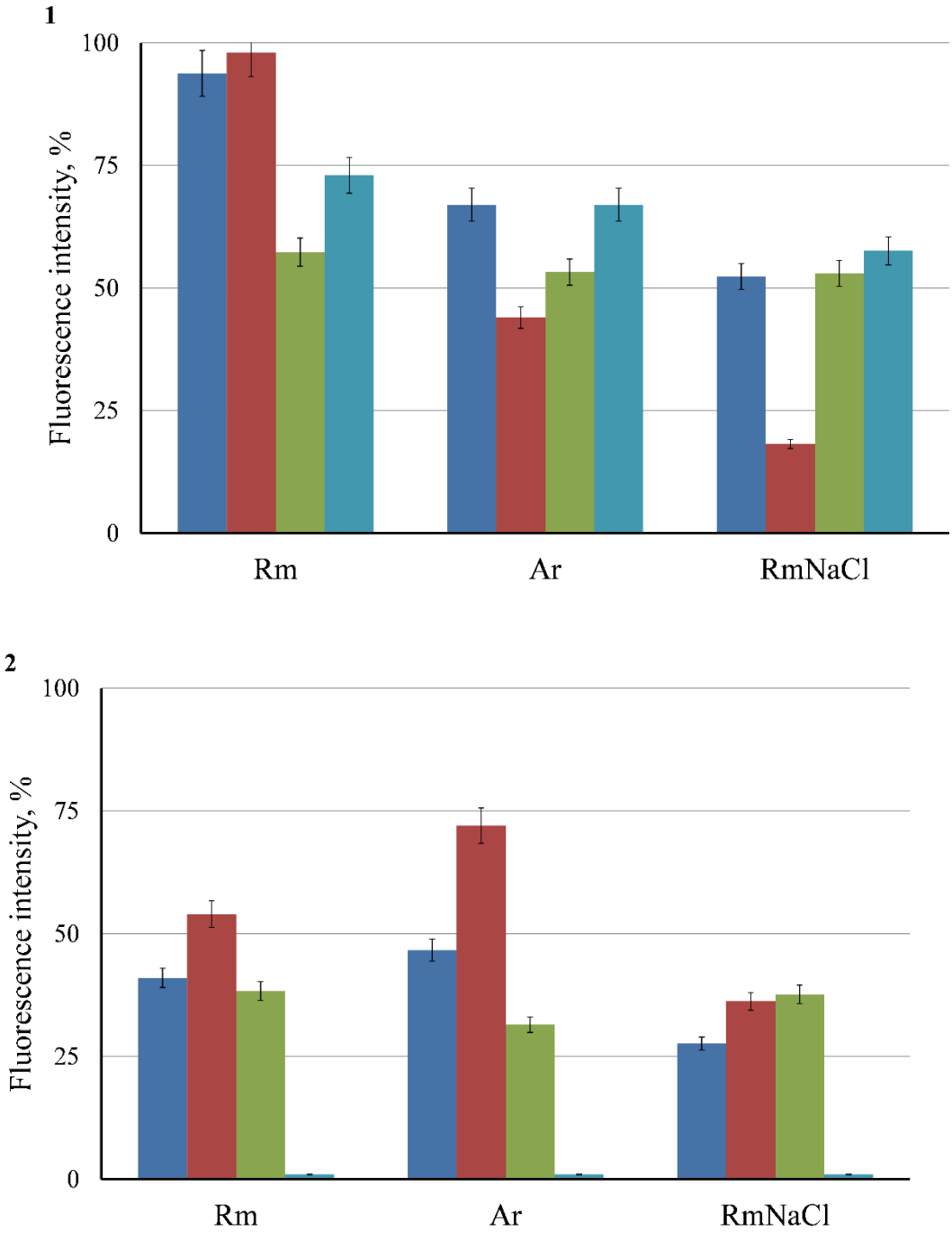

Fig. 3. Fluorescence intensity of chlorophyll (1) and Nile Red stain (2) in *D. salina* cells, depending on the cooling regimes and growth medium: ■ — control, ■ — precultivation at 4°C for 24 hours, ■ — freezing 1 deg/min, ■ — freezing by immersion in liquid nitrogen.

Rapid freezing by immersing samples in liquid nitrogen after thawing did not lead to a significant decrease in chlorophyll autofluorescence in cells, a significant decrease by 20% was only when microalgae were cultivated on Rm, Fig. 3 (1). Regarding NR fluorescence,

rapid cooling resulted in complete degradation of carotene-containing lipid globules in all samples examined, Fig. 3 (2).

Concentration of *D. salina* cells and their motility after freezing-thawing

It was found that the survival of microalgae depends on the final cooling temperature and the composition of the growth media. It was shown that cooling to -10°C at a rate of 1 deg/min did not reduce the concentration of cells after heating in comparison with the control. Cooling to -40°C at a rate of 1 deg/min led to a significant decrease in cell concentration, and the cells grown on the Rm_{NaCl} turned out to be more resistant, Fig. 4 (1).

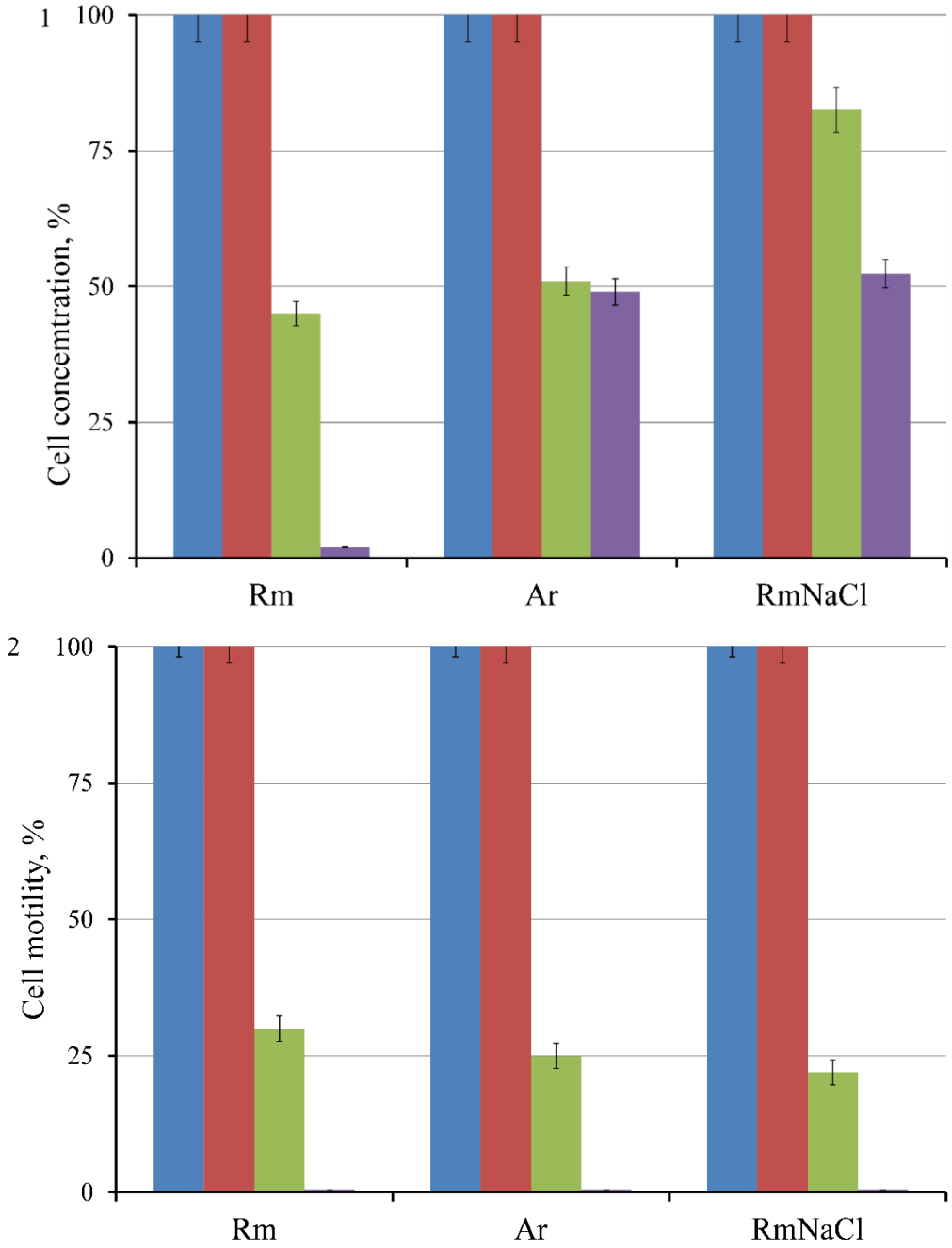

Fig. 4. Concentration (1) and motility (2) of *D. salina* cells after freezing/thawing depending on the cooling regimes: ■ — control; ■ — -10°C; ■ — -40°C; ■ — -40°C followed by immersion in liquid nitrogen.

Slow freezing to -40°C followed by immersion in liquid nitrogen showed that cells grown on a media with the optimal composition for growth (Rm) are the least resistant to cryopreservation. The concentration in cultures on the Ar and Rm_{NaCl} after heating decreased by 50% compared to the control, while in the Rm the cell concentration approached zero, Fig. 4 (1). It can be assumed that there is a direct correlation between the number of carotene-containing lipid globules in microalgae cells and their resistance to low temperatures.

The study of cell motility showed that freezing to -10°C does not reduce this indicator in comparison with the control in all studied samples. Upon cooling to -40°C, motility decreased by an average of 75% compared with the control; after freezing to -40°C followed by immersion in liquid nitrogen, no motile cells were found, Fig. 4 (2). It is likely that when cooled to temperatures below -40°C, the functional activity of contractile proteins decreases, which makes *D. salina* cells immobile.

CONCLUSION

It was shown that the pigment composition of *D. salina* cells changes in the stationary growth phase: the number of lipid globules containing carotenoids increases. It has been established that the accumulation of carotene in microalgae is induced by an increase in the concentration of sodium chloride in the growth media up to 4 M.

It has been established that the formation of intracellular lipid globules and the synthesis of carotenoids in *D. salina* cells contribute to an increase in the concentration and number of motile cells after freezing-thawing.

It has been shown that during rapid cooling, adaptive mechanisms do not have time to turn on in cells, and complete destruction of carotene-containing lipid globules occurs.

Therefore, cryopreservation of *D. salina* cells should be carried out at a rate of 1 deg/min to -40°C, followed by immersion in liquid nitrogen and a mandatory stage of precultivation at 4°C for 24 hours.

This approach allows the cells to adapt to a decrease in temperature, as well as to get the best result after freezing-thawing.

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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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ВПЛИВ СОЛЬОВОГО СТРЕСУ ТА РЕЖИМІВ ОХОЛОДЖЕННЯ НА ПІГМЕНТНИЙ СКЛАД КЛІТИН *DUNALIELLA SALINA*

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Актуальність. Мікроводорості здатні виробляти значну кількість біологічно значущих речовин. У зв'язку зі зростанням популярності мікроводоростей актуальна розробка ефективних методів зберігання культур та створення банків штамів. Це не тільки задовольнить потреби науки та біотехнології у життєздатних і стійких культурах, але й вирішить проблему збереження біорізноманіття.

Мета роботи. дослідити вплив сольового стресу та режимів охолодження на пігментний склад клітин мікроводорості *Dunaliella salina* з метою підвищення їх збереження після заморожування-відігрівання.

Матеріали і методи. Об’єктами дослідження були одноклітинні зелені мікроводорості *D. salina*. Культивування проводили за стандартною методикою на поживних середовищах з різною кількістю NaCl та мікроелементів. Адаптацію до низьких температур здійснювали витримкою зразків при температурі 4°C протягом 24 годин без освітлення. Заморожування проводили шляхом додавання 1 мл суспензії клітин у поліпропіленову кріогенну пробірку об’ємом 1,8 мл (Nunc, Sigma-Aldrich), охолоджували зі швидкістю 1 град/хв за допомогою Mr. Frosty з використання наступних режимів: до -10°C, -40°C, -40°C з наступним зануренням у рідкий азот або прямим зануренням у рідкий азот (-196°C). Відігрівання здійснювали на водяній бані (37°C) при безперервному струшуванні протягом 1–2 хв. Мікроскопічні дослідження проводили на лазерному скануючому мікроскопі LSM-510 Meta (Carl Zeiss, Німеччина) при збудженні діодним лазером з довжиною хвилі 405 нм і 573 нм з використанням барвника Nile Red.

Результати. Встановлено, що утворення внутрішньоклітинних ліпідних глобул і синтез каротиноїдів у клітинах *D. salina* сприяють збільшенню концентрації та кількості рухомих клітин після заморожування-відтавання. Показано, що при швидкому охолодженні в клітинах не встигають включатися пристосувальні механізми і відбувається повне руйнування каротиновмісних ліпідних глобул.

Висновки. Кріоконсервацію клітин *D. salina* слід здійснювати зі швидкістю 1 град/хв до -40°C з подальшим зануренням у рідкий азот та обов’язковим етапом прекультивації при 4°C протягом 24 годин. Такий підхід дозволяє клітинам адаптуватися до зниження температури, що сприяє найкращому результату після заморожування-відігрівання.

КЛЮЧОВІ СЛОВА: мікроводорості; сольовий стрес; температурний стрес; *Dunaliella salina*; кріоконсервування; кріорезистентність.