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FACTORS AFFECTING C-PHYCOCYANIN CONCENTRATION IN CELLS OF *LIMNOSPIRA PLATENSIS* (GOMONT) K. R. S. SANTOS & HENTSCHKE (*SPIRULINA*) AT DIFFERENT STORAGE PERIODS IN DEHYDRATED STATE

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Limnospira platensis (Gomont) K. R. S. Santos & Hentschke, 1973 (*Spirulina*) has a high nutritional value: it contains up to 70% of protein, as well as carotenoids, B vitamins, vitamin E, and other nutrients and minerals. Optimal storage of cyanobacteria is achieved by drying, and in this form, the biomass preserves its useful properties for a long time. Moreover, cells remain viable when entering the state of anhydrobiosis, and this is crucial for preserving the biodiversity of cyanoprokaryotes and microalgae in storages. Among the biochemical components of *L. platensis*, the pigment C-phycocyanin (C-PC) is of particular interest: it has antioxidant, immunomodulatory, and cancer-preventing properties, and it is a component of photosynthetic pigment complexes of cyanobacteria. The aim of this work was to determine C-PC content in *L. platensis* samples preserved in the state of anhydrobiosis within 1–19 years and to reveal the key factors affecting the decrease in its concentration. To analyze *L. platensis* biomass, standard methods (biochemical and optical ones) were used, and also microscopic technique was applied to control associated microflora. The maximum amount of C-PC was obtained at a temperature of +30 °C (5.6 and 3.4% for the 2nd and 12th years of storage, respectively). Dehydration at higher temperatures, +50 and +60 °C, led to a drop in pigment content (1.9 and 0.54% for the 2nd and 12th years of storage, respectively). No clear correlation between the level of C-PC concentration and residual moisture was revealed; however, in 23% of samples at high dehydration temperatures, increased residual moisture was recorded (12–16%). Storage for 19 years, in contrast to storage for 2 years, was characterized by the dominance of destroyed trichomes and the abundant development of associated bacteria. A decrease in C-PC concentration in the samples and a decline in variability of the indicator with increasing storage period were noted. Apparently, storage period, along with temperature and residual moisture, affected the drop in the pigment concentration. Probably, C-PC content can be considered as an indicator of *L. platensis* cell viability. When transferring cyanobacteria to the state of anhydrobiosis, it is most reasonable to carry out dehydration at a temperature of +30 °C and store samples at a residual moisture of 9–11% (for no more than 2 years). The results may be useful for preserving biodiversity and obtaining C-PC-rich biomass in anhydrobiotic collections of algae and cyanobacteria.

Keywords: cyanobacteria, anhydrobiosis, microalgae, dehydration, residual moisture, temperature, storage period

Among phototrophs which have adaptogenic effect on an organism, *Limnospira platensis* (Gomont) K. R. S. Santos & Hentschke (*Spirulina*) is of particular interest due to its exceptionally high nutritional value [Kedik et al., 2010; Soni et al., 2017; Spirulina platensis, 1996]. Along with protein in a high concentration (up to 70%), it contains an almost complete spectrum of carotenoids, B vitamins in a significant amount, vitamin E, essential gamma-linolenic acid, and a wide range of microelements [Becker, 1993; Choopani et al., 2016; Los, 2013; Petryakov, 2015; Spirulina platensis, 1996]. Among the biochemical components of *L. platensis*, certain attention is paid to C-phycocyanin (hereinafter C-PC): an accessory pigment and a protein-pigment complex of phycobiliproteins absorbing light, along with allophycocyanin and phycoerythrin [Chaouachi et al., 2024; Liu et al., 2016; Stadnichuk et al., 2015]. C-PC is often considered as an indicator of viability of cells, as its concentration may indirectly reflect the condition of cyanobacterial photosynthetic apparatus [Mróz et al., 2024; Spirulina platensis, 1996]. C-PC features antioxidant, immunomodulatory, and cancer-protective properties [Choopani et al., 2016; Los, 2013; Petryakov, 2015]; therefore, while storing *L. platensis* biomass, it is necessary to maintain conditions ensuring maximum preservation of the pigment. Dehydration is the most common technique for storing Cyanobacteria; it allows biomass of cyanoprokaryotes to retain its beneficial properties for a long time. Dehydrated *L. platensis* cells enter the state of anhydrobiosis, and this is crucial for preserving cultures of microorganisms. The analysis of the dependence between C-PC content in dehydrated *L. platensis* cells and storage period is highly relevant, while data available in literature is extremely fragmentary.

The aim of this work is to determine C-phycocyanin content in *Limnospira platensis* samples preserved in the state of anhydrobiosis for 1–19 years, and to reveal the key factors of a decline in the pigment concentration.

MATERIAL AND METHODS

Plant material. The subject of the study was a unialgal, non-axenic culture of *L. platensis* (strain IBBS-31) from the collection of anhydrobiotic cultures maintained by staff of IBSS Department of Biotechnology and Phytoresources. The culture was transferred to the state of anhydrobiosis in different years of the investigation. The longest storage period was 21 years. Samples were obtained both in a laboratory and under conditions of industrial cultivation. The Zarrouk's medium was used as a nutrient medium for the culture [Faucher et al., 1979]. In total, 34 samples of dehydrated cultures of *L. platensis* were analyzed.

Cultivation conditions in the laboratory in semi-industrial bioreactors. Cyanobacteria were batch-cultured under stable round-the-clock illumination [at the surface of the medium, illuminance was 15 klx (Yu-116 light meter, Russia)]. Consistent airflow was provided by Hailea ACO 308 air compressor (China); it allowed removing excessive oxygen from the nutrient medium and maintaining stable temperature of its layer. Temperature of the medium ranged from +25 to +29 °C. When *L. platensis* growth reached the stationary stage, trichomes were separated from the culture medium by filtration *via* gauze (100–105 PE), and then rinsed twice with two volumes of distilled water. The biomass was spread in a thin layer on a polyethylene film, placed in a thermostat (TS-80M-2, Russia), and dehydrated at temperatures of +30, +50, and +60 °C. Dehydration lasted for 6–20 h depending on temperature and amount of biomass. Dryness was considered achieved when edges of dehydrated biomass curled inward, and cyanobacteria mat was easy to separate from the underlying polyethylene film.

Conditions of sample storage. Dried samples were placed in polyethylene grip seal bags, and then into plastic containers. Those were placed in a storage for anhydrobiotic cultures (temperature was maintained at the level of +18…+20 °C). The samples were stored for 1–19 years.

Biochemical analysis and determination of residual moisture. C-PC was extracted from 0.01–0.05 g of dry biomass of cyanobacteria with 2 mL of distilled water; the procedure was repeated until the extract became clear. Pigment content was determined on a UV/Vis spectrophotometer SF-2000 (LOMO, Russia), and calculated by formula specified in [Gevorgiz, Nekhoroshev, 2017; Voronin et al., 2001]. C-PC content was expressed based on dry weight. Residual moisture was determined by standard technique of drying to constant weight [Metody, 1975].

Reactivation after the state of anhydrobiosis and preparation of *Limnospira platensis* suspension for examination under a scanning electron microscope. *Prior* to examination, all solutions were filtered through membrane filters with pore size of 0.2 μm (Sartorius, Germany); sterile labware was used. For reactivation of cyanobacteria, a 0.01–0.05-g sample of *L. platensis* was hydrated with 3 mL of the Zarrouk's medium diluted with distilled water (ratio 1 : 2) [Patent 2541452, 2015] and left for 1 h at a room temperature. An aliquot of *L. platensis* cell suspension (1–2 mL) was treated with glutaraldehyde for 60 min to a final concentration of 2.5%, and then precipitated on a 0.2-μm polycarbonate filter (manufactured by Joint Institute for Nuclear Research, Dubna, Russia). Subsequently, the samples were dehydrated in a graded ethanol series: from 20 to 100% [Bratbak, 1993]. A Leica EM CPD300 critical point dryer (Germany) was used to dry the samples at the critical point (1.5–2.5 h). A Leica EM ACE200 vacuum coater was used for sputter coating (Au/Pd, 0.5–1.0 min). The samples were viewed and micrographs were captured under a scanning electron microscope Hitachi SU3500 (Japan), at 4000× to 6000× magnification.

RESULTS AND DISCUSSION

Effect of temperature on C-phycocyanin content in *Limnospira platensis* samples. We analyzed fluctuations of C-PC concentration at different temperature regimes (+30, +50, and +60 °C). We chose the samples from one batch, which had been stored for 2 and 12 years.

For the culture that had been stored in the state of anhydrobiosis for 2 years, the highest C-PC content (5.6% of dry weight) was recorded for dehydration temperature of +30 °C. Apparently, with a rise to +50 and +60 °C, primary destruction of trichomes in cyanobacteria occurred, as we registered a statistically significant decrease (unpaired *t*-test, $p < 0.05$) by 2.3–2.9 times (down to 2.4 and 1.9%, respectively). For the culture that had been stored under anhydrobiosis condition for 12 years and dehydrated at +30 °C, C-PC concentration amounted to 3.4% of dry weight. At dehydration temperature of +50 °C, the value decreased by 1.3 times relative to the sample dehydrated at +30 °C (down to 2.6%), and at +60 °C, by 6.8 times (down to 0.5%). Thus, the samples stored for 2 and 12 years showed a trend of declining C-PC content as the dehydration temperature increased (Fig. 1a, b).

Samples stored for longer (for 17, 19, and 21 years) featured lower C-PC concentration: mean values were 1.86 and 1.97% for +30 and +50 °C, respectively. The difference between values was statistically non-significant (unpaired *t*-test, $p > 0.05$). At +60 °C, phycocyanin content decreased by 3.8 times, and the mean value was 0.54%.

As previously reported, to ensure the viability of cyanobacteria (after anhydrobiosis) and to properly preserve biochemical components, dehydration should be carried out at +30…+60 °C [Kharchuk, 2018; Patent 2541452, 2015]. However, for maximum preservation of C-PC, dehydration at +30 °C turned out

to be the most acceptable for samples stored for both 2 years (which corresponds to the shelf life of commercial preparations of *L. platensis*) and 12 years. Dehydration temperature of +30 °C is known to correspond to physiological optimum for cyanobacterial growth. For instance, samples dehydrated under such conditions had no changes in the lipid composition, whereas samples dehydrated at +60 and +70 °C exhibited higher content of unsaturated fatty acids, and this contributed to increased stability of membranes and probably was a response to the negative effect of temperature [Kharchuk, Kopitov, 2005]. According to [Efimov, 2007; Liu et al., 2016; Spirulina platensis, 1996], C-PC concentration declined as temperature increased to values above +50…+60 °C; at +70 °C, pigment degradation occurred, apparently due to destructive processes triggered in the protein segment of phycobiliproteins. This is consistent with our data.

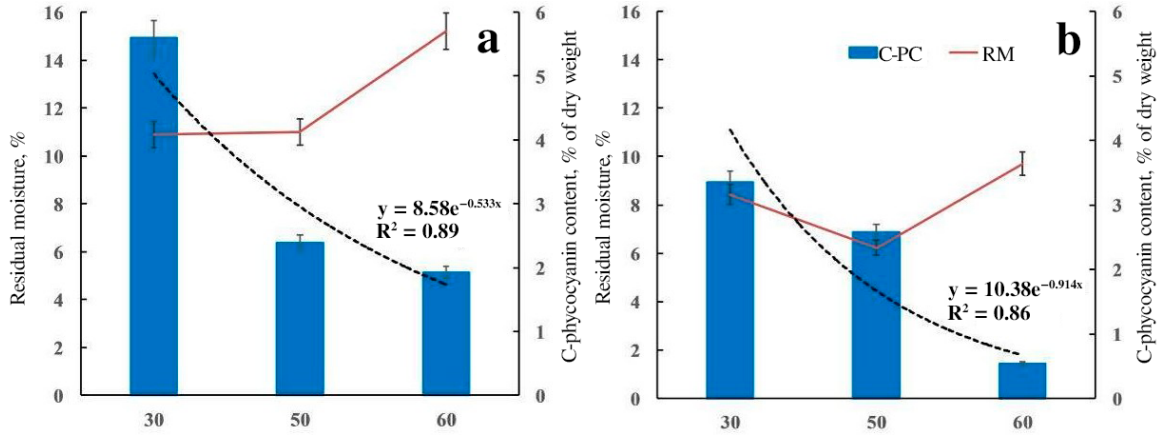

Fig. 1. Dependence of C-phycocyanin concentration (C-PC) and residual moisture (RM) on the dehydration temperature of *Limnospira platensis* samples at different storage periods: a, 2 years; b, 12 years. The dotted line shows the trend

Thus, we confirmed that dehydration temperature was the key mediator of C-PC content in *L. platensis* cells. Dehydration at +30 °C was optimal for C-PC preservation, whereas higher temperatures seemed to cause thermal degradation of the pigment.

Effect of residual moisture on C-phycocyanin content in *Limnospira platensis* samples. As noted above, the samples dehydrated at +60 °C and stored for 2 and 12 years, exhibited the highest values of residual moisture (hereinafter RM) (15.2 and 9.7%, respectively) and the lowest C-PC concentration (see Fig. 1). The samples stored for longer and dehydrated at the same temperature were characterized by high RM as well (9.16%). Over the entire period of our study, RM varied within 6.9–15.4%. No clear correlation was established between RM of stored cultures and dehydration temperature. However, for the data array, it was found as follows: elevated moisture levels (12–15.2%) were more frequently observed in samples dehydrated at higher temperatures (23% of the total sample) (Fig. 2). Interestingly, low RM (6.6–8.54%) was recorded in 47.4 % of samples stored for a long time (more than 12 years).

During dehydration, first, free water is removed, and concentration of intracellular components increases by 2–5 times. Subsequent dehydration eliminates bound (structural) water, which is the primary factor determining RM. Bound water passes into gel-like state and protects the cellular structures thus preventing complete degradation of photosystems I and II and, accordingly, ensuring C-PC preservation in cells [Bekker et al., 1981, 1990; Liu et al., 2016].

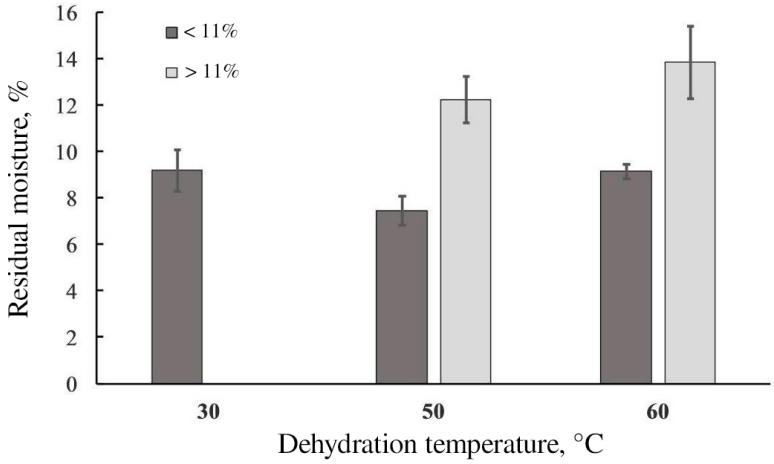

Fig. 2. Parameters of residual moisture in *Limnospira platensis* samples at different dehydration temperature

Limits of RM for preservation of certain cyanobacteria and microalgae in the state of anhydrobiosis without deterioration of their chemical and biochemical properties were determined previously. For example, successful reactivation of *Synechococcus elongatus* (Nägeli) Nägeli, 1849 and its recovery requires RM of 8–11%; *Phaeodactylum tricornutum* Bohlin, 1897, RM of 12–14%; and *L. platensis*, RM of 9–11% [Kharchuk, 2008a]. When the level of RM is below or above the optimal one, cells lose viability and do not enter the state of anhydrobiosis [Bekker et al., 1990; Liu et al., 2016].

Relative humidity is known to depend on temperature. The higher the temperature, the more water vapor air can hold, and therefore, the more moisture it can retain [Trockaya et al., 2014]. This dependence seems to underlie the phenomenon when higher RM is observed in samples dehydrated at higher temperatures. Besides, if samples subjected to high-temperature dehydration are packaged, this increases the likelihood of more intense condensate formation in storage containers. RM > 12% is critical for survival of *L. platensis* dried cells, and it triggers destruction of trichomes [Kharchuk, 2008b]. For instance, M. Bekker *et al.* [1981] reported that high RM, 20–24%, caused death of yeast cells. Apparently, high dehydration temperature and elevated RM in samples had a combined negative effect on cell condition. Trichomes were disintegrating and releasing metabolites, which contributed to the growing pool of organic substances thereby causing active development of associated microflora [Mogale, 2016; Tarhova, 2005; Vardaka et al., 2016].

Samples after 2 and 19 years of storage were reactivated and examined. In the first case, suspension contained relatively intact trichomes, and abundance of bacterial cells was low (Fig. 3a). In samples after a long-term storage, deformed and fragmented trichomes dominated, and abundance of bacterial cells was high (Fig. 3b, e). In the present study, we did not carry out a quantitative assessment of bacterial associations. Visually, bacterial abundance in samples after 19 years of storage was very high, especially on fragments of destroyed trichomes. Bacterial cells were found on the surface of mucilaginous sheath of cyanobacteria (Fig. 3a, d, g, white solid arrows), on the surface of disintegrated trichomes (Fig. 3e, f, black dashed arrows), inside the mucilaginous sheath of cyanobacteria (Fig. 3g, black solid arrows), and in the culture medium outside *L. platensis* cells (Fig. 3a–c, white dashed arrows). Dominance of irreversibly damaged and dead cells (65.8 and 34.2%) with low C-PC content (1.4%) in suspension after a long-term storage was reported previously [Kharchuk et al., 2022].

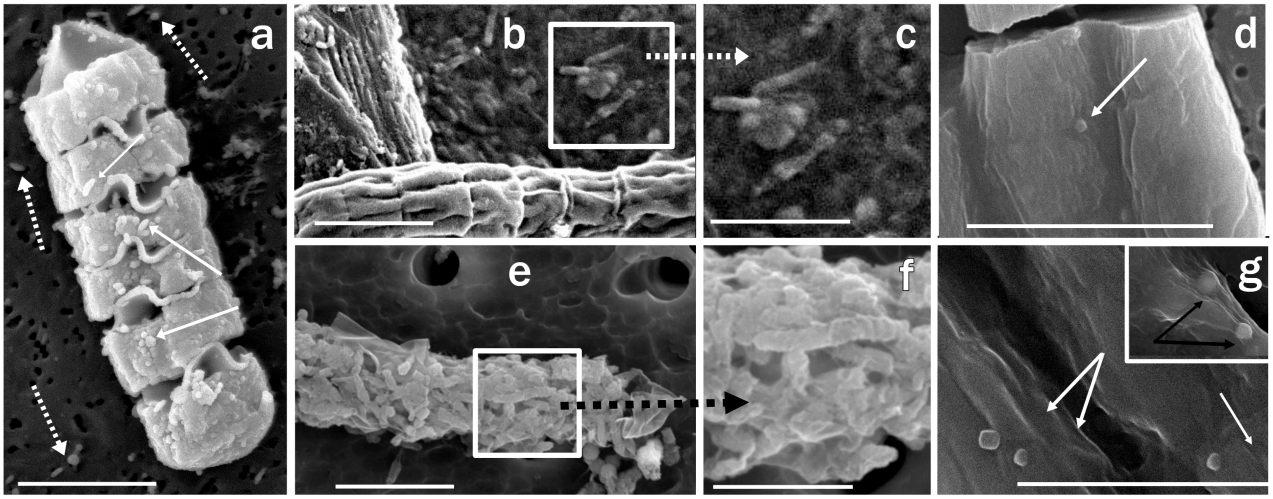

Fig. 3. *Limnospira platensis* trichomes in reactivated samples after 2 and 19 years of storage under anhydrobiosis condition (according to scanning electron microscopy data): a, *L. platensis* trichomes and associated bacteria after 2 years of storage; b, deformed *L. platensis* trichomes and associated bacteria after 19 years of storage; c, *L. platensis*–associated bacteria in a culture suspension of samples after 19 years of storage; d, bacterial cells on the surface of the Cyanobacteria mucilaginous sheath; e, destroyed *L. platensis* trichomes in a culture suspension of samples after 19 years of storage; f, bacterial cells on destroyed trichomes; g, bacterial cells on the surface and in the Cyanobacteria mucilaginous sheath. Scale bars are 5 μm

Dynamics of C-phycocyanin content in *Limnospira platensis* samples at different storage periods. All samples available (1, 2, 12, and 19 years, the present study; and 4 and 17 years, described previously [Kharchuk et al., 2022]) exhibit a decline in C-PC concentration as the storage period increases. The highest C-PC content in dehydrated *L. platensis* cells was recorded after 1 year of storage: the mean value was (5.88 ± 0.68) %. A statistically significant drop (unpaired *t*-test, $p < 0.05$) was registered between 1 and 2 years of storage, and also between 1 and 4 years: down to (2.64 ± 0.3) % and (2 ± 0.4) %, respectively. After 12 years of storage, there was a further decrease in C-PC concentration by 1.1–2 times (compared to that in the previous period of study), but it was statistically nonsignificant (unpaired *t*-test, $p > 0.05$). The lowest C-PC content was observed after 19 years of storage: (0.8 ± 0.02) % (Fig. 4).

Interestingly, the longer the storage period, the lower the variability of C-PC concentration. Apparently, due to the effect of this factor, along with temperature and RM, there is a drop in C-PC content, and the pigment concentration itself can be considered as an indicator of viability of *L. platensis* cells. Our data provide evidence for the fact that conditions for dehydration and subsequent storage of cyanobacteria, commonly used for commercial production of biomass, differ from conditions for optimal preservation of *L. platensis* in the state of anhydrobiosis to ensure subsequent reactivation of viable C-PC-rich cells. These findings can be applied in selecting conditions for preserving cultures of microalgae and cyanoprokaryotes in the state of anhydrobiosis.

Conclusion. The highest C-phycocyanin (C-PC) content in anhydrobiotic cells of *Limnospira platensis* was registered in samples dehydrated at +30 °C: 5.6 and 3.4% for those stored for 2 and 12 years, respectively. A shift in dehydration temperature to +50 and +60 °C seemed to trigger primary destruction of cyanobacterial trichomes. The pigment concentration declined for both periods of study: down to 1.9% for 2 years of storage and 0.54% for 12 years.

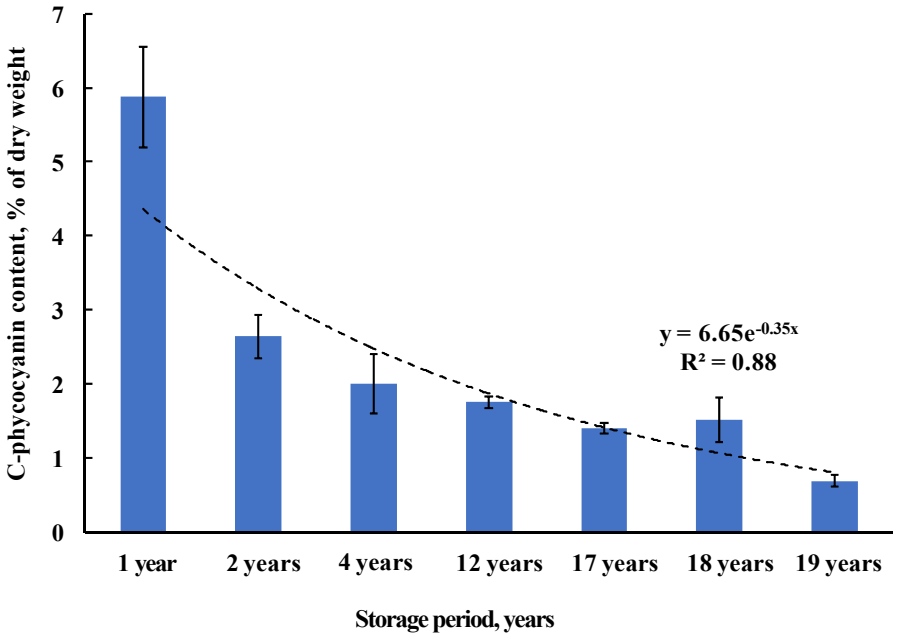

Fig. 4. C-phycocyanin content in dehydrated *Limnospira platensis* samples at different storage periods (averaged data obtained at dehydration temperatures of +30…+60 °C and residual moisture of 6.6–15.2%). The dotted line shows the trend. Data for years 4 and 17 is from [Kharchuk et al., 2022]

A range of residual moisture was wider than the known optimum for *L. platensis*: the values varied within 6.9–15.4%. No correlation was determined between residual moisture of preserved cultures and dehydration temperature. However, most often samples with high residual moisture (12–15.2%) were recorded at high dehydration temperatures (23% of the total sample). Lower residual moisture (6.6–8.54%) was registered in 47.4 % of samples stored for a long time (more than 12 years). Apparently, residual moisture levels below and above optimal ones triggered irreversible processes in cyanobacterial cells, and also secondary destruction of trichomes; moreover, C-PC content dropped to the minimum. After long-term storage, suspension of reactivated cyanobacteria exhibited active development of microflora and dominance of disintegrated trichomes.

A trend was noted for the data array, regardless of dehydration temperature and residual moisture: there was a decline in C-PC concentration with increasing storage period of samples. For 2–4 and 4–12 years, a decrease was statistically significant (unpaired *t*-test, $p < 0.05$); then, it was non-significant (unpaired *t*-test, $p > 0.05$). It is likely that the long storage period, along with dehydration temperature and residual moisture, contributed to the degradation and complete destruction of C-PC in samples. Its content can be considered an indicator of viability of *L. platensis* cells.

Conditions, commonly used for commercial production of cyanobacterial biomass for further production of dietary supplements, medicines, food additives, and other biotechnological products, differ from conditions required for microalgae and cyanoprokaryotes to enter the state of anhydrobiosis with subsequent reactivation of their viable cells with high C-PC concentration. The following parameters are optimal for cyanobacteria to enter the state of anhydrobiosis: dehydration at +30 °C and residual moisture of 9–11% during storage for no more than two years. Obtained results may be useful for the proper preservation of algal cultures in collections.

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ФАКТОРЫ, ВЛИЯЮЩИЕ НА КОНЦЕНТРАЦИЮ С-ФИКОЦИАНИНА В КЛЕТКАХ *LIMNOSPIRA PLATENSIS* (GOMONT) K. R. S. SANTOS & HENTSCHKE (*SPIRULINA*) ПРИ РАЗЛИЧНЫХ СРОКАХ ХРАНЕНИЯ В ОБЕЗВОЖЕННОМ СОСТОЯНИИ

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Limnospira platensis (Gomont) K. R. S. Santos & Hentschke, 1973 (*Spirulina*) обладает высокой пищевой ценностью: в её состав входит до 70 % белка, а также каротиноиды, витамины группы B, витамин E и другие питательные вещества и минералы. Оптимальное хранение цианобактерий достигается высушиванием, и в таком виде биомасса долгое время не теряет своих полезных свойств. Кроме того, клетки сохраняют жизнеспособность, переходя в состояние ангидробиоза, что является крайне важным для сохранения биоразнообразия цианопрокариот и микроводорослей в хранилищах. Среди биохимических компонентов *L. platensis* особый интерес вызывает пигмент С-фикоцианин (С-фц), который обладает антиоксидантными, иммуномодулирующими и онкопротекторными свойствами и входит в состав фотосинтезирующих пигментных комплексов цианобактерий. Целью данной работы было определить содержание С-фц в образцах *L. platensis*, сохраняемых в состоянии ангидробиоза от 1 до 19 лет, и выявить основные факторы, влияющие на снижение концентрации пигмента. Использованы стандартные (биохимический и оптический) методы исследования биомассы *L. platensis*, а также микроскопический — для контроля сопутствующей микрофлоры. Максимальное количество С-фц получено при температуре +30 °C (5,6 и 3,4 % для 2-го и 12-го года хранения соответственно). Дегидратация при более высоких температурах, +50 и +60 °C, приводила к снижению содержания пигмента (1,9 и 0,54 % для 2-го и 12-го года хранения соответственно). Чёткой зависимости между

уровнем концентрации С-фц и остаточной влажностью не обнаружено, однако в 23 % образцов при высоких значениях температуры дегидратации выявлена повышенная остаточная влажность (12–16 %). Хранение в течение 19 лет, в отличие от хранения в течение 2 лет, характеризовалось доминированием разрушенных трихомов и массовым развитием сопутствующих бактерий. Обнаружено снижение концентрации С-фц и уменьшение вариабельности показателя при увеличении сроков хранения образцов. По-видимому, длительность хранения, наряду с температурой и остаточной влажностью, оказывала влияние на концентрацию пигмента. Вероятно, содержание С-фц может рассматриваться как индикатор жизнеспособности клеток *L. platensis*. При переводе цианобактерий в состояние ангидробиоза оптимальны дегидратация при температуре +30 °С и хранение образцов при остаточной влажности 9–11 % (срок — не более 2 лет). Результаты могут быть полезны для сохранения биоразнообразия и получения биомассы, богатой С-фц, в ангидробиозных коллекциях водорослей и цианобактерий.

Ключевые слова: цианобактерии, ангидробиоз, микроводоросли, дегидратация, остаточная влажность, температура, сроки хранения