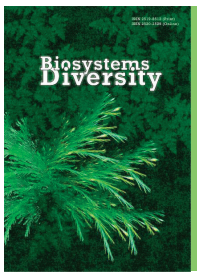

Biosystems Diversity

ISSN 2519-8513 (Print)
ISSN 2520-2529 (Online)
Biosyst. Divers., 2019,
27(3), 244–249
doi: 10.15421/011934

Halotolerant strain of *Chlorococcum oleofaciens* from the Lake Elton Biosphere Reserve

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Article info

Received 24.07.2019
Received in revised form 21.08.2019
Accepted 22.08.2019

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Ignatenko, M. E., Yatsenko-Stepanova, T. N., & Khlopko, Y. A. (2019). Halotolerant strain of Chlorococcum oleofaciens from the Lake Elton Biosphere Reserve. Biosystems Diversity, 27(3), 244–249. doi:10.15421/011934

Chlorococcum oleofaciens is one of the most studied representatives of the *Chlorococcum* genus, both on the ultrastructural and molecular levels. This alga is very interesting due to its ability to hypersynthetize saturated and unsaturated fatty acids and the possibility of using it as a promising object for biofuel production. This research is devoted to the study of the halotolerant strain of *Ch. oleofaciens* Ch-1 extracted from the water of the Khara River (Lake Elton Biosphere Reserve, Russia, a UNESCO World Heritage site), mineralization of 14‰. The strain *Ch. oleofaciens* Ch-1 was studied at the morphological level (light microscopy), as well as using molecular genetics methods (18S rDNA). The objectives of the study included establishing the range of halotolerance of the allocated strain of *Ch. oleofaciens* as a whole, revealing borders of level of mineralization that are optimum for algae growth, and also tracing features of its morphology and cycle of development in the conditions of various salinity. In the course of the studies performed it was established that the extracted strain of *Ch. oleofaciens* Ch-1 differed from the typical one by greater variability of some morphological features and had a wide ecological valence: the range of its halotolerance was 0–60‰. The maximum values of quantitative development of *Ch. oleofaciens* Ch-1 were registered at mineralization of 0–14‰. It is shown that with increasing salinity in the development cycle of the strain, the duration of the adaptation phase increases, the exponential phase decreases, small celled forms are replaced by large celled forms and reproduction features are noted. The obtained results can be used for selection of optimal conditions for cultivation of the halotolerant strain of *Ch. oleofaciens* for biotechnological purposes.

Keywords: *Chlorococcum* morphology; halotolerance; 18S rDNA.

Introduction

Chlorococcum oleofaciens Trainor & Bold (=*Chlorococcum croceum* P. A. Archibald & Bold, *Ch. sphacosum* P. A. Archibald & Bold, *Ch. granulosum* P. A. Archibald) is one of the most studied representatives of the *Chlorococcum* genus, both on the ultrastructural and molecular levels (Kawasaki et al., 2015; Maltsev & Konovalenko, 2017). This alga is very interesting due to its ability to hypersynthetize saturated and unsaturated fatty acids and the possibility of its use as a promising object for biofuel production (Abomohra et al., 2012; Del Río et al., 2015, 2017). *Ch. oleofaciens* is widespread throughout the globe and is a typical representative of soil flora according to numerous research data (Andreyeva, 1998; Guiry & Guiry, 2019).

In 2014, during the study of water samples from the brackish Khara River (Lake Elton Biosphere Reserve, Russia, a UNESCO World Heritage site), we found and allocated an algae strain identified as *Ch. oleofaciens*. At present, the example of individual representatives of the *Chlorococcum* genus has shown that environmental salinity can be considered a factor regulating the production of algae biomass and the way they accumulate intracellular metabolites (fatty acids) (Kirrolia et al., 2012; Beevi & Sukumaran, 2015; Feng et al., 2016). Proceeding from the aforesaid, we identified the need to establish the range of halotolerance of the allocated strain of *Ch. oleofaciens* as a whole, to reveal borders of the level of its mineralization that are optimum for algae growth, and also to trace features of its morphology and cycle of development in the conditions of various salinity.

Materials and methods

Allocation and cultivation of algae. The object of the study was *Chlorococcum oleofaciens* Trainor & Bold Ch-1 strain of green microalgae extracted from the water of the Khara River (selection point "Chyortov Most (Devil's Bridge)" (49º14'14.6"N 46º38'51.1"E), mineralization of 14‰).

The Khara River is one of the seven tributaries of Europe's largest hyperhaline lake, Lake Elton (Lake Elton Biosphere Reserve, Russia, a UNESCO World Heritage site). The river has a flat watercourse of about 40 km long, with a slow flow (0.2–0.4 m/s) and a depth from 0.1–0.8 m (Burkova, 2012). The predominance of saline sediments, solonchaks and saline soils in the catchment basin area determines an increased level of water salinity (Kanapatskiy et al., 2018), with maximum salinity recorded in the lake/river mixing zone. The rivers belong to the sodium-potassium chloride class according to the ratio of the main water ions. According to the Venetian classification system (1958) of natural waters by mineralization level, the waters of the Khara River can be characterized as brackish (0.5–30.0‰).

Algae were separated into a pure culture using micropipettes with a Nikon Eclipse Ts2 inverted microscope.The algologically pure culture was maintained on Knop's medium (g/L: $Ca(NO_3)_2$ – 0.25; $MgSO_4 \times 7H_2O$ – 0.06; KH_2PO_4 – 0.06; KCl – 0.08; Fe_2Cl_6 – 1 drop of 1% solution) with addition of sodium chloride in the amount of 14 g/L. The cultivation was carried out under conditions of artificial insolation with a photoperiod of 16:8 hours (day : night) at +23 – +24 ºC.

Purification, amplification and DNA sequencing. DNA purification, preparation of amplicons, and further sequencing of nucleotide sequences were carried out on the basis of Syntol CJSC (Russia).

Molecular phylogenetic analysis. To perform the molecular phylogenetic analysis, a sample of sequences of the 18S rDNA gene with a length of at least 1650 pairs of nucleotides from GenBank (NCBI) corresponding to green algae of the order Chlamydomonadales was made based on the literature data (Temraleeva et al., 2017; Maltsev & Konovalenko, 2017; Mikhailyuk et al., 2018; Temraleeva & Moslalenko, 2019). Cyanoprokaryota such as *Geitlerinema splendidum* 014 JQ712602.1 (scientific name: *Geitlerinema splendidum* (Greville ex Gomont) Anagnosti-

dis) and *Geitlerinema amphibium* I013-0021 KY550458.1 (scientific name: *Anagnostidinema amphibium* (C. Agardh ex Gomont) Strunecký, Bohunická, J. R. Johansen & J. Komárek) were used as an outgroup. Nucleotide sequence alignment was performed in the Unipro UGENE shell program by ClustalW algorithm. The model of nucleotide substitutions was constructed using the program jModelTest 2 v2.1.10. The GTR model was the optimal one. Phylogenetic relationships were reconstructed using the maximum likelihood method (ML) in the Unipro UGENE shell analysis using the program PhyML. Statistical support for the tree topology was assessed using bootstrap analysis (1000 repetitions).

Determination of the halotolerance range. The range of halotolerance of *Ch. oleofaciens* Ch-1was estimated by growing a culture on media with different NaCl concentrations: 0‰, 10‰, 20‰, 30‰, 40‰, 50‰, 60‰, 70‰, 80‰, 90‰, 100‰ (Knop's medium was used as a basis; NaCl concentration of 14‰ was used as the control). For this purpose, 2 mL of algae suspension was inoculated in 30 mL of medium with different levels of mineralization. The experiment was conducted in triplicate. The number of algae (N, cells/L) was estimated by the method of direct counting in the Nageotte Counting Chamber (Assistent, Germany) of 0.01 cm^3 with the use of the light microscope "Axiostar plus" (Carl Zeiss, Germany) after 0, 3, 5, 7, 10, 14, 18, 21 and 25 days of the experiment with the subsequent recalculation by the formula:

$$N = k \times n \times (A/a),$$

k – the coefficient showing how many times the volume of the counting chamber is less than 1 cm^3; n – the number of organisms detected on the lines viewed (squares); A – the number of lines (squares) in the counting chamber; a – number of lines (squares) on which the algae were counted.

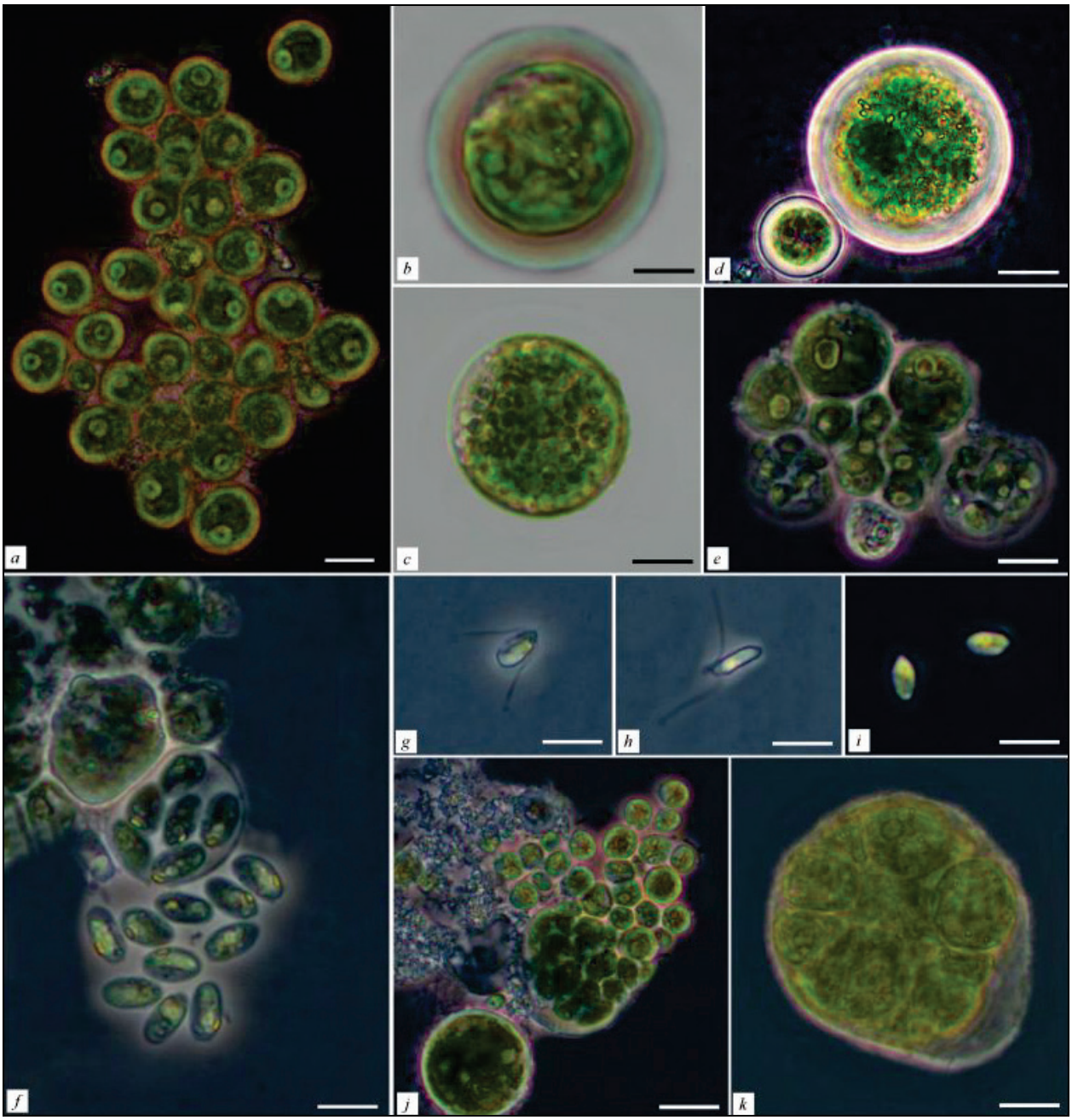

Fig. 1. *Chlorococcum oleofaciens* strain Ch-1: *a* – vegetative cells: pyrenoid with continuous starch sheath (salinity of 14‰); *b* – vegetative cell with thick cell wall at 50‰ salinity; *c* – vegetative cell with thin cell wall at 50‰ salinity; *d* – mature vegetative cells with thick cell wall at 14‰ salinity (4,5-month-old cultures); *e* – cluster of cells of several generations (salinity of 20‰); *f* – zoospores release from zoosporangium: zoospores ellipsoidal to cylindrical with wall-adjacent chloroplast which lies in about half of the cell cavity and anterior ellipsoidal stigma (salinity of 14‰); *g* – zoospore: two flagella longer than the cell (salinity of 14‰); *h* – zoospore flattened on one side (salinity of 14‰); *i* – zoospores after the stop (salinity of 14‰); *j* – aplanospores: release from aplanosporangium (salinity of 14‰); *k* – an aplanosporangium with aplanospores (salinity of 50‰); scale bars – 10 μm; magnification of ×630 (*a*) or ×1000 (*b*, *c*, *d*, *e*, *f*, *g*, *h*, *i*, *j*, *k*)

The impact of salinity was estimated by the rate of change in cell number (specific growth rate); for this purpose, the rate of cell division (r, divisions/day) was calculated by the formula:

$$r = (\ln N_2 - \ln N_1)/\ln 2\Delta t,$$

N_1 and N_2 – the number of cells in the field of vision at the moments t_1 and t_2, Δt – the time interval between t_1 and t_2 (Davidovich et al., 2016).

Changes in cell size under the influence of the mineralization gradient were determined with the help of a microline on the Axioskop microscope (Carl Zeiss, Germany) at magnification of ×40, with the measurement of at least 100 algae cells at each salinity level.

Microphotographs of algae were made in the Center of Collective Use of Scientific Equipment "Microorganism persistence" of the Institute of Cellular and Intracellular Symbiosis of the Ural branch of the Russian Academy of Sciences using the "Axioskop" microscope (Carl Zeiss, Germany) with the help of an "Axiocam" digital camera (Carl Zeiss, Germany) at magnification of ×630, ×1000 and a phase-contrast microscopy.

Results

Morphological characteristics of the strain Chlorococcum oleofaciens Ch-1. The *Chlorococcum oleofaciens* Ch-1 strain extracted from the brackish Khara River with a salinity of 14‰ had the following characteristics: vegetative cells are usually spherical (Fig. 1a); the thickness of the cell wall in a young culture does not exceed 1 μm; the cell wall thickens as the culture ages, reaching 4.6–6.5 μm (4.5 months of cultivation) (Fig. 1d). There is one wall-adjacent cupped chloroplast, with a narrow hole. There is one pyrenoid, the starch sheath of the pyrenoid is continuous (Fig. 1a). Reproduces by zoospores or aplanospores. 16 or more zoospores are formed in zoosporangium (Fig. 1f). Ellipsoidal or cylindrical zoospores are 4.6–9.2 μm long and 2.3–6.9 μm wide, sometimes flattened on one side, with two flagella that are longer than the size of the zoospore's body. The chloroplast in a zoospore is wall-adjacent, lies in about half of the cell cavity, with anterior ellipsoidal stigma (Fig. 1g, h, i). 4–64 aplanospores of 2.3 to 9.2 μm in size are formed in aplanosporangium (Fig. 1j).

Phylogenetic analysis based on the 18S rDNA gene. Phylogenetic analysis of the *Ch. oleofaciens* Ch-1 strain based on fragments of the 18S rDNA gene was performed using species of the *Chlorococcum* genus, as well as other closely related genera of the Chlamydomonadales order (*Macrochloris*, *Neospongiococcum*), and representatives of Cyanoprokaryota of the order Oscillatoriales were included in the sample as an outgroup. Reconstruction of phylogenetic relationships by the method of maximum likelihood revealed that the halotolerant water isolate of *Ch. oleofaciens* Ch-1 that we extracted is grouped with other strains of *Ch. oleofaciens*, including the typical strain *Ch. oleofaciens* SAG 213-11 (Fig. 2).

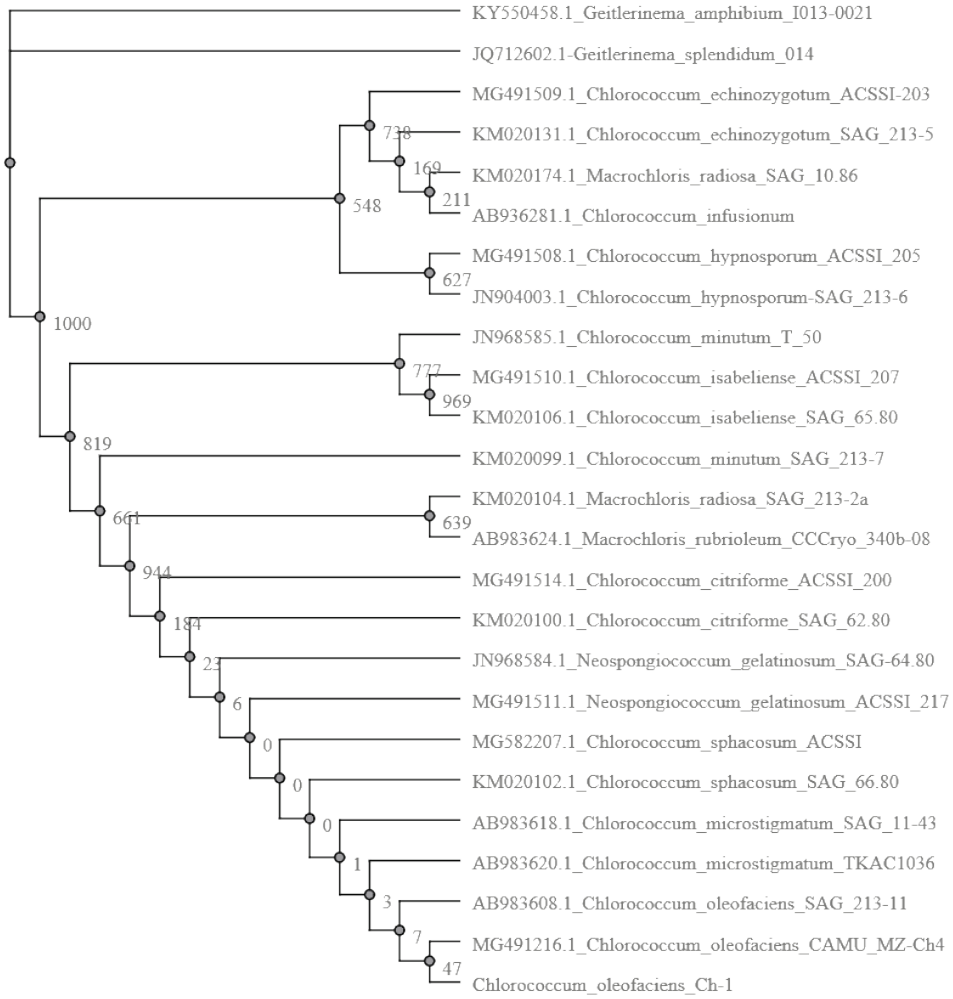

Fig. 2. Phylogenetic tree of green microalgae of the family Chlorococcaceae constructed using the maximum likelihood (ML) method, using the 18S rDNA: model of nucleotide substitution – GTR

It should also be noted that Temraleeva & Moslalenko (2019) indicate the need for taxonomic revision and combine *Neospongiococcum gelatinosum* (P. A. Archibald & Bold) Ettl & Gärtner, *Chlorococcum citriforme* P. A. Archibald & Bold, *Ch. microstigmatum* P. A. Archibald & Bold, *Ch. sphacosum* P. A. Archibald & Bold and *Ch. oleofaciens* into a single species based on 18S rDNA distances, changes in ITS2 conserved regions, differences in the secondary structure of the spacer and in the conservative motif of helix III.

Range of halotolerance of Chlorococcum oleofaciens Ch-1. Halotolerance of *Ch. oleofaciens* Ch-1 was estimated by growing a culture on media with different NaCl concentrations: 0‰, 10‰, 20‰, 30‰, 40‰, 50‰, 60‰, 70‰, 80‰, 90‰, 100‰. A 14‰ mineralization corres-

ponding to the natural mineralization of the water body from which the strain was extracted was chosen as the control one in the experiment. The adaptation period was absent in the culture development cycle at this salinity level. The exponential phase lasted for 18 days, but the rate of strain division varied, reaching 0.56 ± 0.07 divisions/day by the seventh day and gradually decreasing to 0.17 ± 0.02 divisions/day by day 18 of the experiment. Later, there was no increase in the culture number, which indicated that the culture had reached the stationary phase (Fig. 3).

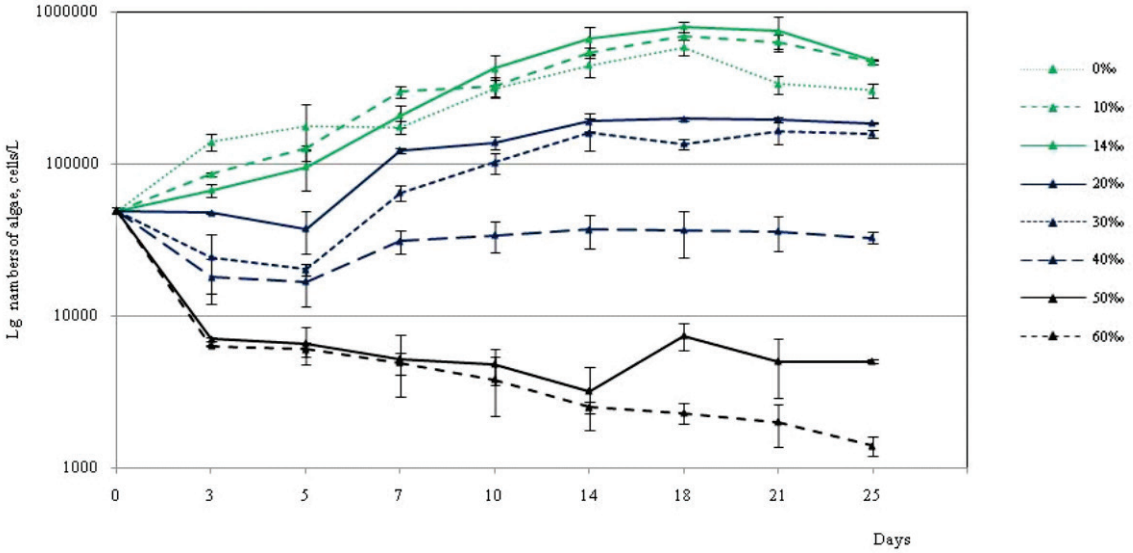

Fig. 3. Population dynamics of *Chlorococcum oleofaciens* Ch-1 in the mineralization gradient (x ± SE, n = 3)

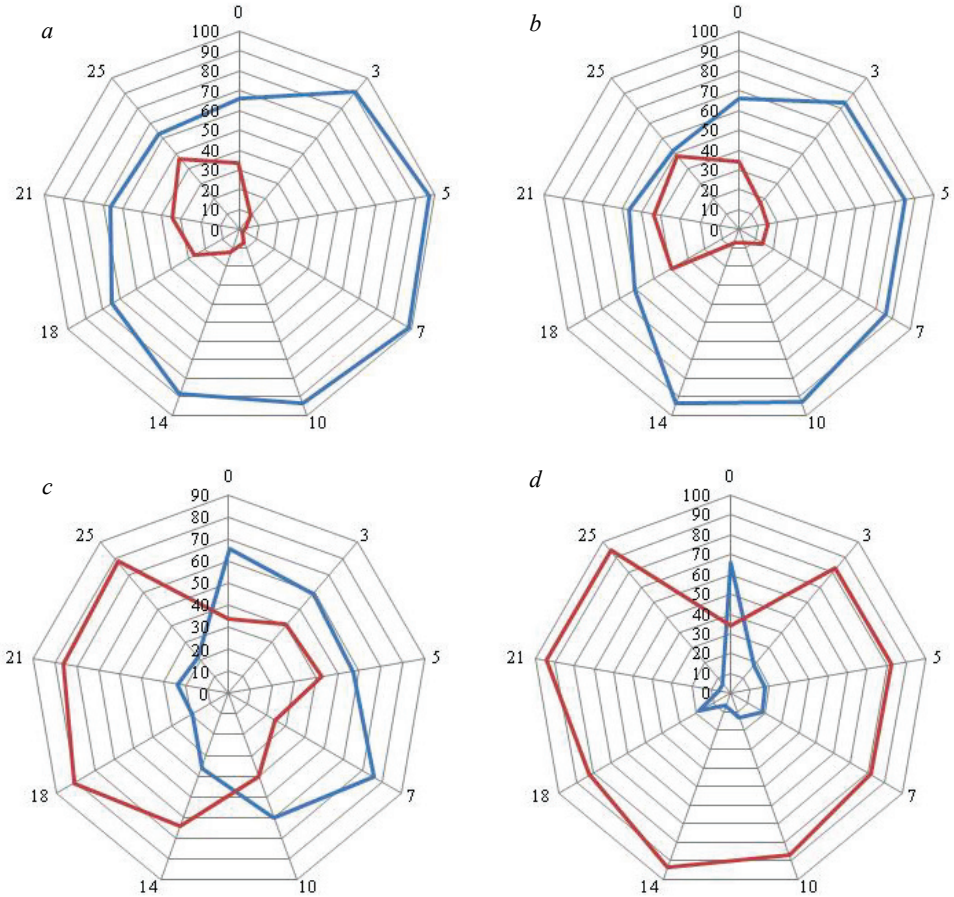

Fig. 4. Dynamics of the ratio of *Chlorococcum oleofaciens* Ch-1 cell size in the mineralization gradient:
a – 0‰, *b* – 14‰, *c* – 20‰, *d* – 50‰, blue line – small celled forms (up to 10 µm), red line – large celled forms (over 10 µm), n = 3

The size of *Ch. oleofaciens* Ch-1 cells at this level of salinity varied 2.3–36.8 µm. Throughout the exponential growth phase, small cellular forms (up to 10 µm) prevailed in the culture, which is connected with intensive cell multiplication of the strain during this period. From day 18 of the experiment, after the crop had reached the stationary phase, the ratio of small celled to large celled (over 10 µm) forms shifted towards the latter (Fig. 4b). Culture reproduction was carried out by zoospores and aplanospores (mobile zoospores, along with aplanospores, were recorded in the culture only during the first five days of the experiment, and the aplanospores were recorded during the rest of the experiment). A similar trend in

the dynamics of the number, growth rate, strain size and reproduction method of *Ch. oleofaciens* Ch-1 was observed during the vegetation period under the conditions of mineralization of 0‰ and 10‰ (Fig. 3, 4a).

During cultivation of *Ch. oleofaciens* Ch-1 on 20‰, 30‰ and 40‰ NaCl media, the culture underwent a 5-day adaptation stage, during which the number of algae decreased 1.3, 2.4 and 2.9 times, respectively, compared to the initial level. The adaptation period was followed by the growth phase of the population, the duration of which also depended on the level of salinity of the environment: up to 10 days with salinity of 20‰ and 30‰ and 5 days with salinity of 40‰. In addition, the specific growth rate of the strain at 40‰ salinity was two times lower than that of *Ch. oleofaciens* Ch-1 cultivated at 20‰ and 30‰ salinity. Cultures reached the stationary phase during vegetation in the media with NaCl concentration of 20‰ and 30‰ from the 14th day of the experiment, at 40‰ – from the 10th day (Fig. 3). Depending on the salinity level, differences in reproduction methods were also observed. Reproduction of the strain of zoospores and aplanospores was recorded only at 20‰ mineralization, where mobile zoospores were detected during the first five days of the experiment as they were in the control. In the media with NaCl concentration of 30‰ and 40‰, the process of zoospore formation was not recorded, and the reproduction of the culture was carried out by aplanospores. And with the increase in the level of mineralization, the size of aplanospores increased: the range of variation was 2.3–9.2 μm at 20‰; 4.6–9.2 μm at 30‰ and 6.9–9.2 μm at 40‰. In addition, under salinity conditions of 20‰, 30‰, and 40‰, the nondisjunction of aplanospores after the rupture of the aplanosporangium shell was often recorded. While remaining grouped, cells continued to grow and reproduce, which led to the formation of clusters of cells of several generations at once (Fig. 1e). The size of the strain was also directly related to the level of mineralization, and large celled forms (more than 10 μm) prevailed over small celled forms by the end of the experiment in all its variants (20‰, 30‰, 40‰) (Fig. 4c).

Algae cultivation with a salinity of 50‰ was accompanied by a long period of adaptation (up to 14 days), during which the number of algae decreased by a factor of 15.3 compared to the initial level. This was followed by a short exponential phase (from 14 to 18 days of cultivation), during which an insignificant increase in the number of *Ch. oleofaciens* Ch-1 was observed (Fig. 3). Throughout the experiment, large cells (up to 50.6 μm) with a thick cell wall prevailed in the culture (Fig. 1b, c, 4d). In these conditions, the strain was multiplied only by the formation of aplanospores. The size of aplanospores varied in the range of 9.2–18.4 μm, which was significantly larger than the size of the aplanospores detected at lower concentrations of NaCl (Fig. 1k). The formed aplanospores remained inside the aplanosporangium for a long time and remained grouped in the case of a rupture of the shell of the latter. The peculiarity of spore formation in this salinity is the number of aplanospores in the sporangium, which did not exceed 16, while at a lower mineralization the number of aplanospores could reach 64.

At 60‰ salinity,the number of *Ch. oleofaciens* Ch-1 decreased significantly throughout the experiment, and at the same time, morphological deformation of cells was noted – the protoplast shrank, many cells were adhered by symbiotic bacteria. However, culture has maintained its vitality (Fig. 3). Salinity over 60‰ (70‰, 80‰, 90‰, 100‰) caused the complete death of *Ch. oleofaciens* Ch-1 by the 3rd day of the experiment.

Discussion

Algae of the *Chlorococcum* genus have a wide geographical distribution and are found in a wide variety of habitats: from the hot springs of Central Asia to soils and water bodies of Antarctica (Andreyeva, 1998; Novis et at., 2015; Guiry & Guiry, 2019). The vast majority of species are typical of soil flora, some of them are found in diverse freshwater bodies, and only two species are marine (*Chlorococcum endozoicum* Collins, *Ch. submarinum* Ålvik) (Guiry & Guiry, 2019). However, the literature provides data on the detection of other species of the *Chlorococcum* genus in conditions of increased salinity. For example, *Chlorococcum infusionum* (Schrank) Menegh is described as the dominant species in the algocenosis of saline soils in the area of Lake Shara-Nur, Russia (Lopatovskaya et al., 2017). *Ch. oleofaciens* was found in the clayey outcrops and shell sand of Cape Kazantip, Black Sea (an area with a migratory shoreline subject to salinization) (Mikhailyuk et al., 2018).

The *Ch. oleofaciens* Ch-1 strain that we studied was extracted from the water of the brackish Khara River (Lake Elton Biosphere Reserve, Russia, a UNESCO Natural Heritage site) at a salinity of 14‰. The morphology of vegetative cells and zoospores of the halotolerant isolate corresponded to the first description of *Ch. oleofaciens* by Trainor & Bold (1953), as well as to the descriptions given in later sources (Archibald & Bold, 1970; Andreyeva, 1998). The only exceptions were the zoospore sizes (4.6–9.2 μm in length and 2.3–6.9 μm in width) and the length of flagella exceeding the cell size. Mismatches between the size of zoospores and the typical diagnosis are also noted by other authors (Andreyeva, 1998; Kawasaki et al., 2015), suggesting strain differences (Kawasaki et al., 2015). Zoospores with flagella exceeding the cell length were recorded by Maltsesev & Konovalenko (2017) in the *Ch. oleofaciens* MZ-Ch4 strain isolated from the forest leaf litter of the Samara Forest, Dnipropetrovsk region, Ukraine.

In the course of the experiment it was shown that the *Ch. oleofaciens* Ch-1 strain differed in a wide range of halotolerance – from 0‰ to 60‰. The maximum values of quantitative development of *Ch. oleofaciens* Ch-1 were registered at mineralization of 0–14‰. The results obtained are consistent with other researchers' data indicating the coincidence of the optimal algae growth zone in the experiment with the salinity values recorded in natural habitats (Finenko & Lanskaya, 1971; Markina & Aizdaicher, 2010; Davidovich et al., 2016).

In spite of the wide range of salt tolerance, some peculiarities were observed in the culture development cycle depending on the salinity level. Thus, under conditions of increasing mineralization, the exponential phase of development was reduced (up to 18 days at 0–14‰ salinity, up to 10 days at 20–30‰ salinity, up to 6 days at 40‰ salinity and less than 5 days at 50‰ salinity), which, according to Rai et al. (2013), is a reflection of the stressful effects of high concentrations of sodium chloride. Similar changes (reduction of the duration of the exponential phase under conditions of increased salinity) were recorded by Singh et al. (2018) in the experiment with *Chlorococcum humicola* (Nägeli) Rabenhorst and *Chlorella vulgaris* Beyerinck when growing them on media with NaCl concentration from 25 to 1000 mM. Decrease in the rate of algae growth at high concentrations of NaCl in the environment is primarily associated with deterioration of cell membrane permeability, change in osmotic potential, intracellular pH, development of ionic imbalance, including accumulation of Na^+ and Cl^- ions by the cell, and loss of such important microelements as Mg, Si, S, Ca, Mn, Fe, and Zn (Demidchik et al., 2014; Singh et al., 2018). It has also been shown in a number of studies that high concentrations of sodium chloride lead to degradation of chloroplast membranes, which leads to a decrease in photosynthesis and, as a consequence, limitation of algae growth (Zhila et al., 2011; Kirrolia et al., 2012).

In addition to reducing the rate of strain growth in conditions of increasing salinity, we also recorded morphological changes. Thus, according to the literature, the diameter of the *Ch. oleofaciens* cell is 10–15 μm and it increases with the age of the culture up to 46 μm, also the cell wall thickens with the aging of the culture (Andreyeva, 1998). However, at 50‰ mineralization during the experiment, cells with the size of 32.2 μm were detected as early as 7 days, and the cell wall thickness varied in the range of 2.3–4.6 μm; by 21 days, cells with the diameter of 46.0 μm were registered; by 25 days of experiment, the maximum recorded cell size was 50.6 μm (cell wall thickness 6.5 μm). Vegetation of the strain on the media with concentration of 30‰ and 40‰ was accompanied by similar changes, but they were less pronounced – the cells with thickened cell wall and diameter exceeding 30 μm were found in the culture only after 10 days, but in contrast to the previous experience (50‰) their size by the end of the experiment did not exceed 46.0 μm. In the course of algae cultivation on the medium with 20‰ NaCl, large-size cells (over 30 μm) were detected on 18th day of the experiment. The dimensional characteristics of the *Ch. oleofaciens* Ch-1 strain grown under conditions of low salinity (0–14‰) throughout the experiment (25 days) corresponded to the typical diagnosis, cells reaching 46 μm in diameter were registered in cultures at the age of 4.0–

4.5 months. Other authors have also noted an increase in cell size in response to the stress of sodium chloride, attributing it to inhibition of the fission process, changes in the fatty acid composition of algae, and accumulation of various osmolytes in the cell (Klochkova et al., 2006; Zhila et al., 2011; Kirrolia et al., 2012; Singh et al., 2018).

Conclusions

In the course of the studies performed it was established that the extracted strain of *Ch. oleofaciens* Ch-1 differed from the typical one by the greater variability of some morphological features and had a wide ecological valence: the range of its halotolerance was 0–60‰. At the same time, the cultural growth curves can be conditionally combined into 3 groups:

a) 0‰, 10‰, 14‰ – optimum zone: in these conditions the maximum indices of culture growth were revealed, there were cells of typical colouring and morphology in experimental samples, intensive reproduction of algae and predominance of small cell forms during the whole period of the experiment were noted;

b) 20‰, 30‰, 40‰ – the *Ch. oleofaciens* Ch-1 development cycle included a clearly defined adaptation phase and a short exponential phase. The culture underwent a number of morphological changes, such as an increase in the cell size, thickening of the cell wall, nondisjunction of aplanospores after the rupture of the aplanosporangia shell; the ratio of small and large cell forms towards the end of the experiment shifted towards the latter;

c) 50‰ and 60‰ limit of the tolerance zone of *Ch. oleofaciens* Ch-1. The period of adaptation was characterized by a significant duration. Morphological changes similar to those described above occurred at earlier stages of the development cycle. Algae remained viable throughout the experiment, but there was no increase in the number of algae, and 3–25 days the crop consisted of large-celled forms by 80–98%.

The obtained results can be used for selection of optimal conditions for cultivation of the halotolerant strain of *Ch. oleofaciens* Ch-1 for biotechnological purposes.

The authors are grateful to A. O. Plotnikov PhD, MD., Head of the Center of Collective Use of Scientific Equipment "Microorganism persistence" of the Institute of Cellular and Intracellular Symbiosis of the Ural branch of the Russian Academy of Sciences, for his assistance at all stages of the study.

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