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MANUFACTURING FEATURES OF DUNALIELLA SALINA AR-1 AND THE INFLUENCE OF SOLES ON THE APPLICATION OF β-CAROTINS

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Abstract: The new Aral strain Dunaliella salina AR-1 has been isolated and its development cycle has been studied. The appearance, structure and reproduction of green microalgae Dunaliella salina is described. It is shown that its main type of reproduction is reproduction through the formation of palmels. Maximum accumulation of biomass was observed at the concentration of NaCL 2.5-3.0 M and pH= 6.5-7.0. The composition of β-carotenes in the biomass of green and brown form of Dunaliella salina AR-1 has been studied, as well as the effect of nitrogen and phosphorus salts content on this process.

Keywords: microalgae, Dunaliella salina, β-carotenes, stresses, hepersalted water bodies.

Introduction

Due to the favorable climate (5 - 6 months of warm sunny days per year), in addition to crop production, Uzbekistan can successfully develop another branch of agriculture - industrial cultivation of microalgae in open reservoirs. Moreover, the Aral Sea region is suitable for industrial cultivation of microalgae Dunaliella salina. Commercial products of such cultivation are the dried biomass of this microalgae, rich in vitamins (especially provitamin A - β-carotene), valuable omega-3 fatty acids and glycerine. From this biomass you can get orange food dye E-160a(ii) - natural beta-carotene, multivitamin preparations for people and animal feed additives.

Single cell green algae of the genus Dunaliella are the most salt tolerant among eukaryotic photosynthesizing organisms, which are able to survive in extremely different salines (0.05-5 M NaCl). As the salinity of the environment increases in Dunaliella cells, the pigment apparatus, respiratory and photosynthetic metabolism, as well as nucleic metabolism, are rebuilt. According to recent data, when the NaCl concentration increases from 0.5 M to 3.0 M, 76 salinduced proteins are detected in Dunaliella salina cells [1]. By methods of two-dimensional gel electrophoresis and mass spectrometry (MS) 80% of these proteins were fully identified, their localization and function were determined. These include enzymes of central metabolic pathways such as photosynthesis, energy production, protein synthesis and degradation, biosynthesis of amino acids, chaperones, antioxidants, and others. Previously, some authors have shown that an increase in NaCl concentration causes a change in activity of many enzymes in D. salina cells. These data suggest that D. salina has many metabolic pathways that are well conjugated, labile and closely related to the functioning of the photosynthetic apparatus [2].

Despite numerous studies, the factors and mechanisms of induction of supersynthesis of β-carotene in Dunaliella salina cells, as well as its functions, have not been finally clarified [3]. It is known that the maximum intensity of carotenesis is achieved by simultaneous action of the following factors: increased salt content, temperature and illumination; lack or absence of nitrogen and phosphorus; presence of carbon sources. The value of these factors for

carotinogenesis induction is different [4].

Methods and materials

Extraction was carried out from natural water in the amount of 0.1 ml by sowing into a liquid mineral medium OPS (basic nutrient mixture), capable of supporting the growth of hyperheaded dunalyl species for several months.

The basis for selection and determination of microbial abundance is obtaining of accumulation cultures by means of creation of elective conditions. When creating the elective conditions take into account the peculiarities of physiology and metabolism of microorganisms: their requirements to food sources, the attitude to the acidity of the medium, aeration, temperature and other factors. Pure culture was obtained from accumulating cultures. The limit dilution method (MNR) was used to obtain a clean crop. For this purpose, 15 tubes with 9 ml of medium were taken. In each tube of the first row 1 ml of preliminary diluted initial sample was added. The contents were thoroughly stirred and 1 ml from each tube of the first row were transferred to the tubes of the second row. In the same way the content was transferred from the second row tubes to the third row tubes. As a result, in each row dilutions of 1:1000; 1:10000; 1:100000 are obtained, the tubes of each row contain 10, 1, 01 mm3 of the original sample. Tube rack with tubes placed on the window for a few months before the green environment. Thus, in some tubes of the third row received a clean culture.

The purity of the culture was tested by sowing for a dense nutrient medium OPS in Petri dishes. In cultivation of 1:100000, the growth of individual colonies was found on cups, according to morphological and cultural properties of Dunaliella salina culture [5].

Common carotenoids and chlorophylls were determined in acetone extracts by spectrophotometric method on specific absorption at 440, 480, 630, 644, 645, 662, 663 and 750 nm [6].

Results and their discussion

We analyzed 17 samples from hypergaline reservoirs of different regions of Uzbekistan - (Republic of Karakalpakstan, Namangan, Khorezm, Ferghana and Bukhara regions), from which 4 strains of Dunaliella salina were obtained (Table 1).

Table 1: **Sources of microalgae emissions**

№	Variants	ContentNaCl g/l	Location	samples
1	Sample No. 1 (pH - 5.0)	250	Republic of Karakalpakstan, Kungrad District, lake - top layer	*Dunaliella salina*
	Sample No. 2 (pH - 5.0)		Republic of Karakalpakstan, Kungrad district, lake - bottom layer	
	Sample No.3 (pH - 5.0)		Republic of Karakalpakstan, Kungrad District, standing water (upper layer)	
	Sample No. 4 (pH - 5.0)		Republic of Karakalpakstan, Kungrad District, standing water (bottom layer)	
	Sample No. 5 (pH - 5.0)		Republic of Karakalpakstan, Kungrad District, Aral Sea -1	Saving culture of microalgae is obtained
	Sample No. 6 (pH - 5.0)		Lake Ustyurt -1	
2	Sample No. 1 (pH - 5.0)	50	Namangan region Mingbulak rayon, water body (upper layer)	Saving culture of microalgae is obtained
	Sample No. 2 (pH - 5.0)		water body	

	Sample No. 3 (pH - 5.0)		water body	
	Sample No. 4 (pH - 5.0)		water body (bottom layer)	
	Sample No. 5 (pH - 5.0)		Namangan oblast, Chust district, Yangiyer village, water reservoir (bottom layer)	
3	Sample No. 1 (pH - 5.0)	200	Khorezm region, Urgench city, lake (upper layer)	*Dunaliella minuta*
	Sample No. 2 (pH - 5.0)		Khorezm region, town Urgench Lake (lower layer)	
4	Sample No. 1 (pH - 5.0)	30	Ferghana region, Yazyavan district, Yuldashabad village, water body (middle layer)	Algae crops obtained
	Sample No. 2 (pH - 5.0)		Ferghana region, Yazyavan district, Yuldashabad village, water body (middle layer)	
5	Sample No. 1 (pH - 5.0)	50	Bukhara region, lakes (middle layer)	
	Sample No. 2 (pH - 5.0)		Bukhara region, lakes (middle layer)	
	Overall	**5 areas, 17 samples**		

It is shown that during cultivation of microalgae dunalella in solution of four salts (g/l): NaCL-116, MgSO4x.7. H2O-5,0., KNO3-2,5., K2HPO4-0,2., H2O-1 liter, at mixing, bubbling by air, illumination 5000-10000 lk (lux) and T0= 24-280C, the culture on the 5th day goes to the stationary phase at the density of culture about 1 g/l (2 million cells in ml).

At repeated reseeding of microalgae growing on OPS media (basic nutrient mixture), only one crop (Dunaliella salina R-1) was resistant to NaCL. It is shown that this crop is capable of growth and development at all salt concentrations. Maximum accumulation of biomass was observed at NaCL concentrations of 2.5-3.0 M and pH= 6.5-7.0 (Table 2).

Table 2 : **Effect of NaCl concentration on biomass accumulation Dunaliella salina AR-1**

Day	**Molarity**				
	1.5	**2.0**	**2.5**	**3.0**	**3.5**
3	$3.1x10^5$	$1.6x10^5$	$1.1x10^6$	$1.4x10^6$	$1.3x10^5$
5	$5.8x10^5$	$7.2x10^5$	$4.8x10^6$	$4.6x10^6$	$3.4x10^5$
7	$8.5x10^5$	$8.2x10^5$	$7.2x10^6$	$9.2x10^5$	$5.7x10^5$
9	$1.2x10^6$	$5.1x10^5$	$4.6x10^7$	$5.3x10^7$	$7.1x10^5$
11	$8.5x10^5$	$9.0x10^5$	$8.9x10^6$	$9.1x10^6$	$9.2x10^4$

Representatives of the genus Dunaliella are characterized by a fairly diverse form of cells: oval, ellipsoidal, ovoid, pear-shaped, sometimes spherical, cylindrical or spindle-shaped; radial or bilaterally symmetrical, rarely dorsified or slightly asymmetrical. Cell sizes are very diverse. Lengths may vary from 5 µm to 29 µm, width from 4 µm to 20 µm; cell volume from 70 to 4500 cubic microns. (fig.1).

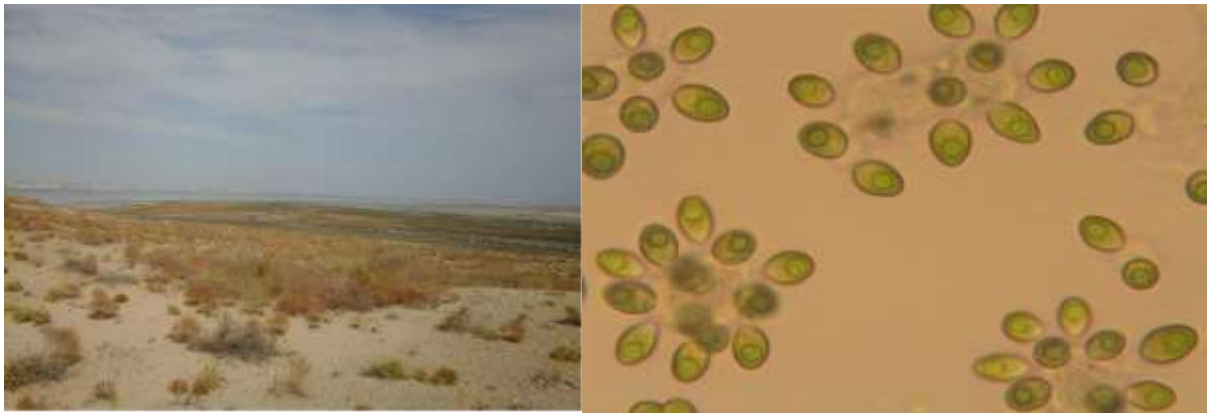


Fig. 1. Place of extraction - Republic of Karakalpakstan, Kungrad region, lake - upper layer and morphology of Dunaliella salina AR-1 cells.

Cells of Dunaliella salina AR-1 strain have a broad-oval shape, with an extended back and slightly narrowed front end, length 10-15 microns, width 7-10 microns, radially symmetrical green chromotophorus, cells with two elastic, homodynamic harnesses, approximately equal lengths, movable. The edge is smooth, the consistency is soft. On a dense agarized environment forms smooth colonies. On the liquid medium growth in the form of green flake-shaped sediment is observed. Strict autotrophs. Optimal temperature for growth is 26-280C. Temperature 45-46 º C causes cell death. It grows on media with addition of NaCl in concentration 1.5-4.5%.

Dunaliella salina AR-1 strain, isolated by us from hyper-saline water bodies of the Aral Sea region, has some peculiarities in reproduction. When cultivating it in Arthari medium with 2M NaCl and total salinity of about 140 g/l, we rarely observed cell division in a mobile state. Basically, mobile green cells, growing up to 10 - 15 microns, are covered with mucus and form palmels, which sink to the bottom and, attached to the walls of the cultural vessel, move like amoeba. In this amoeba-like form, they reproduce by division or budding. Then amoeba-like forms are destroyed and small (1-2 microns) monads of dunalella emerge from them. They actively photosynthesize, grow and, reaching the size of 10-15 microns, again turn into palmella and further into amoebic-like forms.

This process can be observed especially clearly in the synchronous culture of dunalella, which was obtained by centrifuging cells at different stages of development at 2000 g 30 min. At the same time, only individuals of Dunalella sized 1-2 microns remain in the supernatant. After adding to the supernatant a concentrate of biogenic elements (KNO3, K2PO4), up to the final concentrations, as in the Arthurian medium, lighting 5 - 10 kLk and air bubbling, all cells for 5 days simultaneously grow to the size of 10-15 microns. The dynamics of biomass increase of this synchronous culture is presented in Fig. 2.

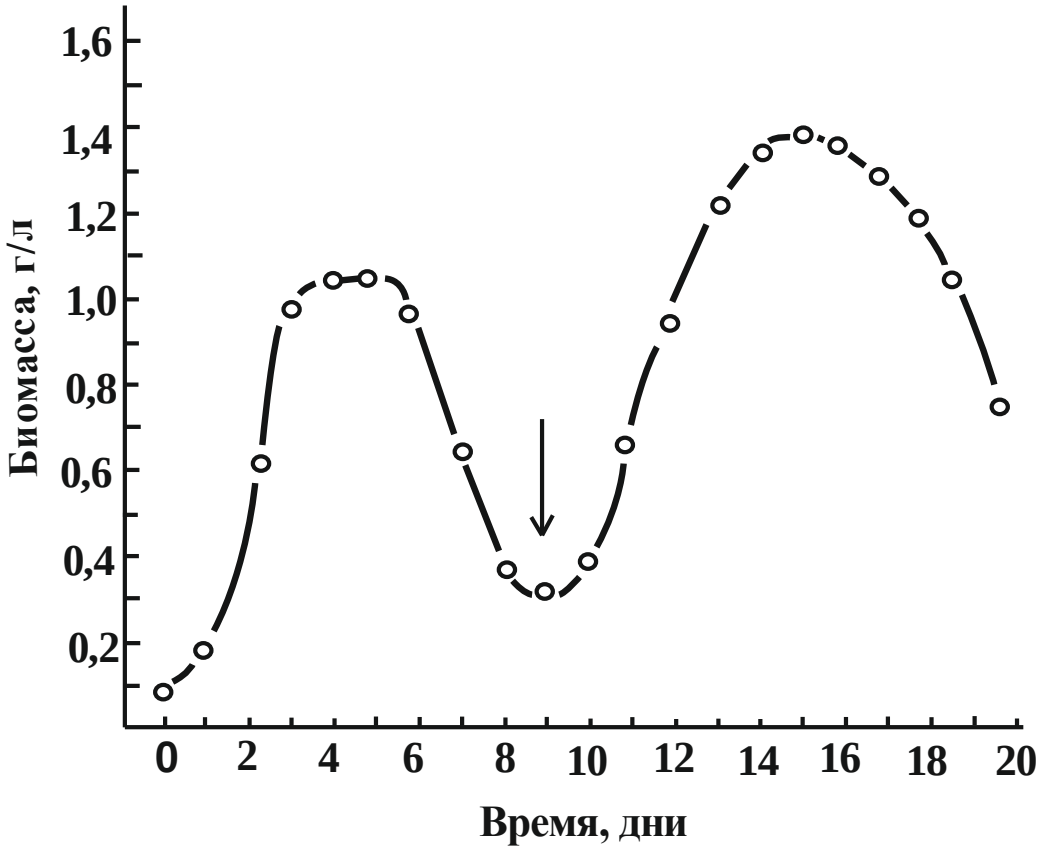


Fig. 2. Dynamics of Dunaliella salina biomass change in synchronous culture in 10 l laboratory bioreactor with illumination of 6000 - 8000 lux (108 - 144 µMols-1m-2) and air bubbling (5-6 l/m). The arrow shows the addition of the consumed biogenic elements concentrate (KNO3, K2HPO4) to the initial Arthari medium concentrations.

On day 6, the cells begin to round off, secrete mucus and turn into palmella, which due to the secretion of mucus in one direction and flowing there grainy content performs amoeboid movements. These palmellae are lowered to the bottom and attached to the walls of the vessel. On day 8, almost all cells turn into amoeba-like palmels with fine graining. The concentration of free floating cells in the medium decreases (Fig. 3,4).

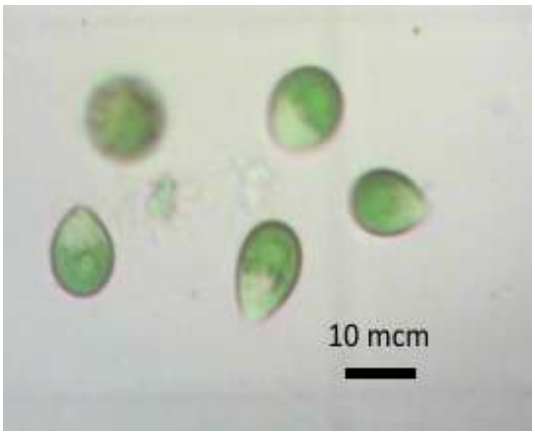
Further on the 9th day amoeba-like palmellae begin to collapse and on Wednesday many small individuals go out, and on the 10th day of amoeba-like palmellae at the bottom there are only transparent shells, and in the environment float dunalella size of 1 - 2 microns. When added to the medium concentrate of consumed biogenic elements (KNO3, K2HPO4) to the initial concentrations of the medium Arthari, the cycle of development of dunalella repeats (Fig.3, 4).

All this development cycle of dunalella occurs at salt concentration about 140 g/l (2M NaCl + 0.2MMgSO4) and illumination 6000 - 8000 lux (108 - 144 µMols-1m-2).

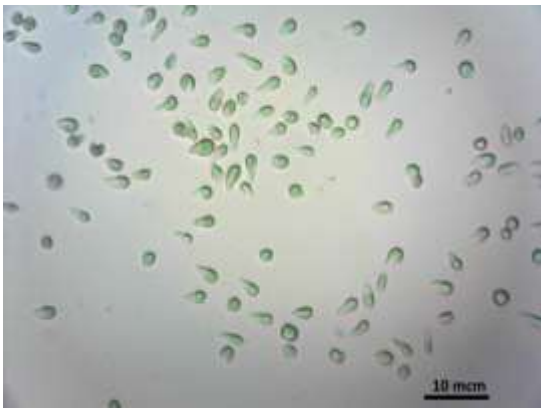
In this cycle of dunalella development, the stage of 1-2 µm specimens is convenient to use as an inoculant to produce green biomass.

In some cases, in amoeba-like palmellae, along with small dunalellae, large individuals of about 10 microns in size are preserved. It is possible that large individuals are the result of division of the mother, and small individuals are the result of separation from her (fig.4).

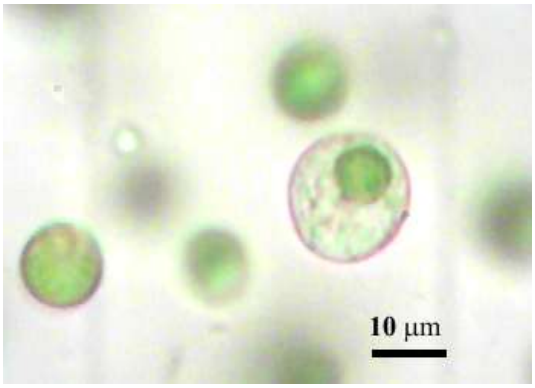
Such formation of palmellae was described for D.salina, isolated from saltworks near the coastal town of Visahapatnam (India) after the rainy season, when



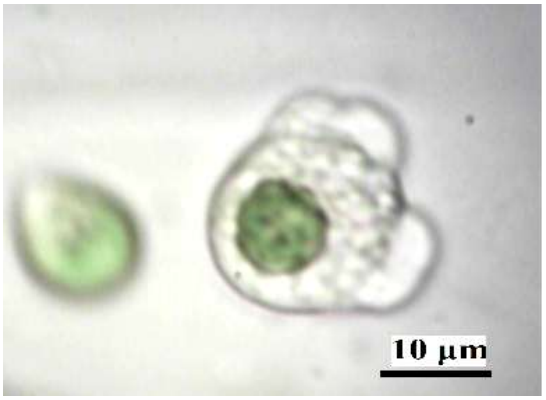
1 day



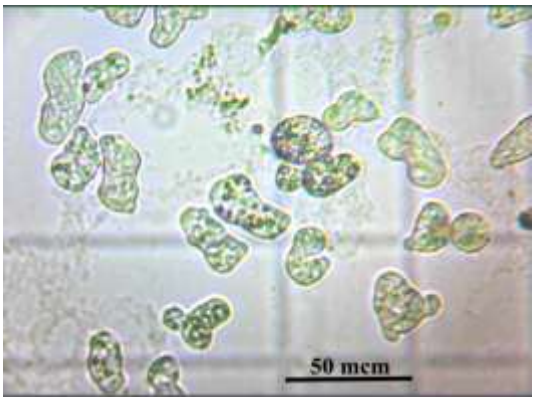
5 day



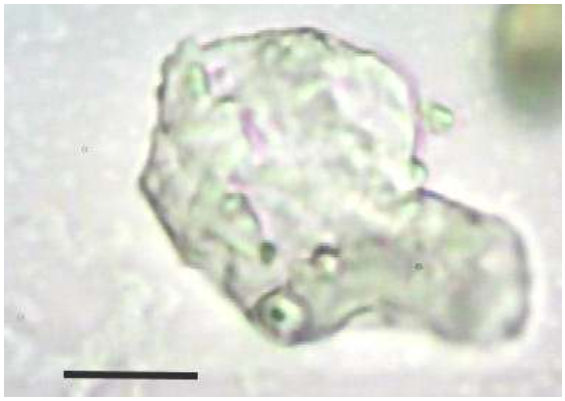
6 day



6 day



8 day

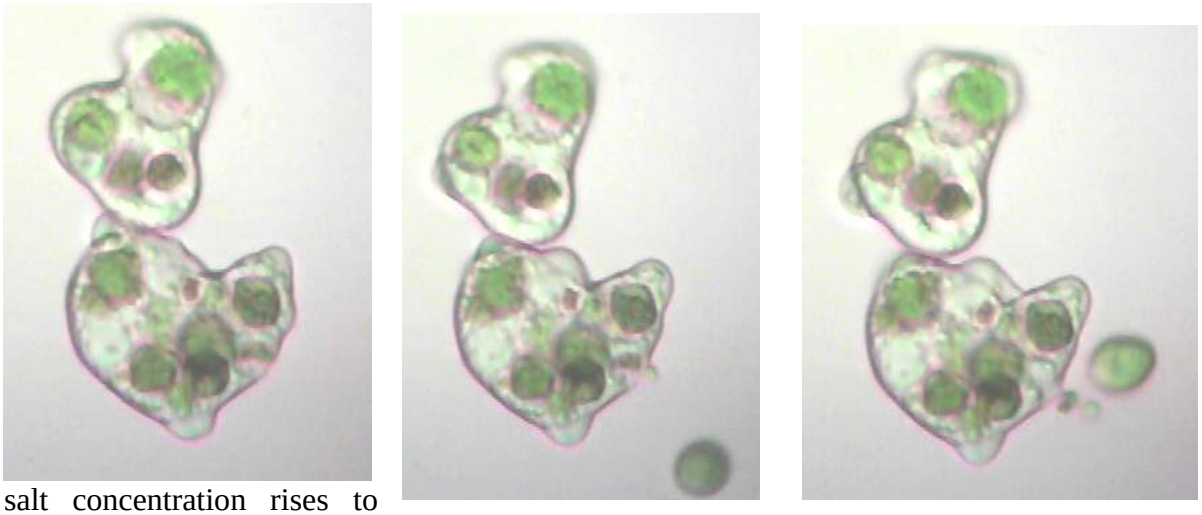


10 day

Fig. 3. Pictures of synchronous culture cells in the process of their development

The salt concentration decreases sharply [7].

It is possible that preferential reproduction through palmella of Dunaliella salina AR-1 strain is a reaction to stress conditions in the Aral lakes, which are characterized by low salt concentrations (below 50 g/l) in winter and spring, and in summer the salt concentration rises to a saturation and many lakes dry up. Even in the main Western Aral Sea in September the



salt concentration rises to 180 g/l.

Рис. 4. Процесс выхода из амёбоподобной пальмеллы маленькой особи

In addition, in summer shallow water temperature can rise above 40 ° C, and at night fall to 15°C.

If the culture at the stage of adult cells stop adding nutrients and water to maintain salt concentrations of 140 g / l, as well as increase the illumination above 20 kLk, cells begin to accumulate carotene and yellow, and at a concentration of salts above 250 g / l, as a result of evaporation of water, begin to turn into yellow orange cysts (Fig. 5.).

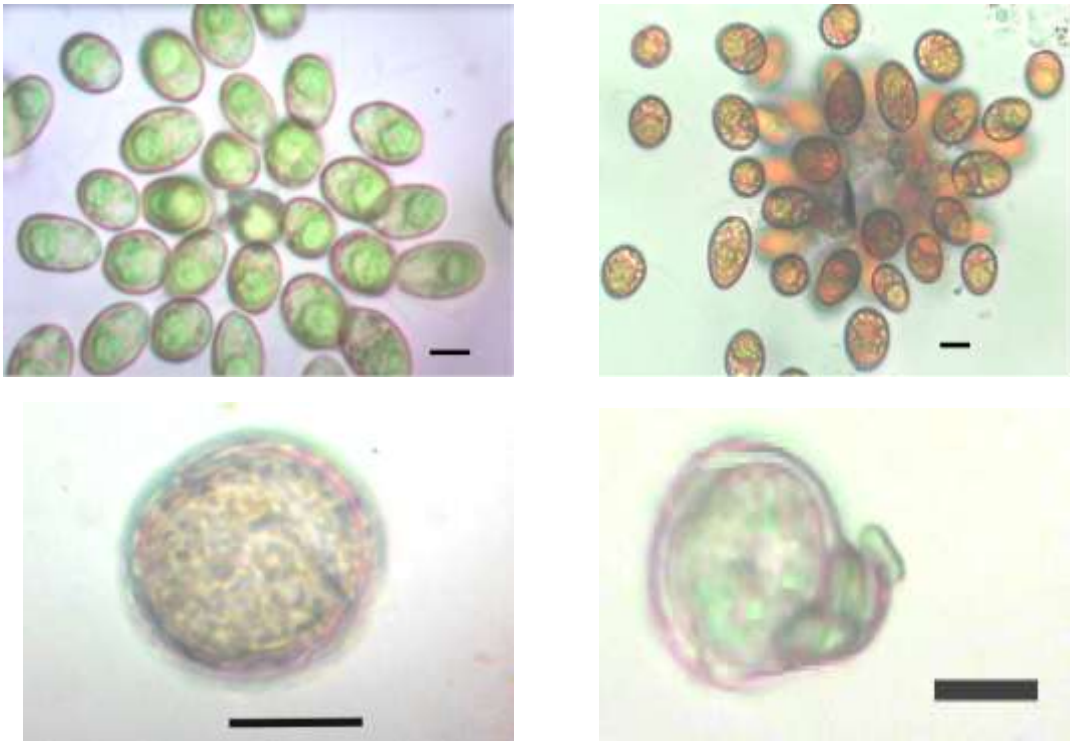


Fig.5. Different stages of Dunaliella salina AR-1 cell development

Biomass of yellow cells and cysts is the target product of dunalella cultivation. Green cells, yellow cells, cyst and cyst from which young cells emerge.

In some rare cases, we have observed a transverse division of mobile cells, which is

described in the literature as the main breeding for microalgae of the genus Dunaliella and even kidney breeding, which is not yet described for Dunaliella (Fig.6).

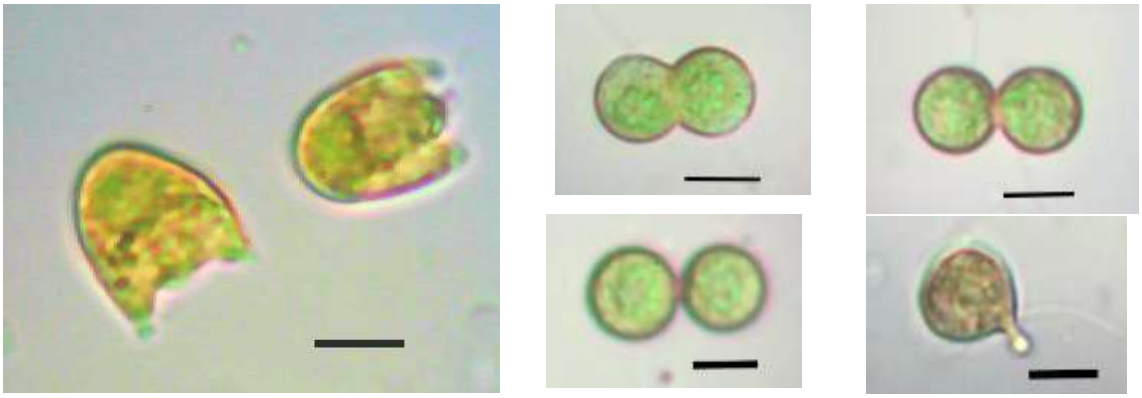


Fig.6. Cell division and patching Dunaliella salina AR-1

Microalgae were cultivated in Artari environment: NaCl (116 g/l), Mg2SO4 2H2O (50 g/l), KNO3 (2.5 g/l), K2NRO4 (0.2 g/l), NaHCO3 (1). 0 g/l) + trace amounts of trace elements (H3BO3, MnCl2, ZnSO4, MoO3, NH4VO3, FeSO4, Ca(NO3)2) pH 8.0, at illumination 5000 - 10000 lux (90 - 180 µMol (photons) s-1m-2). Under such conditions, if we add expendable amounts of biogenic elements (KNO3 and K2NRO4), D. salinaAR-1 develops in green form. If you increase the illumination above 30000 lux (540 µMols-1m-2), do not add consumed nutrients and do not add evaporating water, the concentration of salts in the culture medium will increase and at a concentration of about 250 g / l D. Salinaar passes into a yellow orange form due to the accumulation of large amounts of carotenoids. It is the yellow-orange form that is the purpose of industrial cultivation of D. salina. Biomass was collected by separation on milk separator and lyophilically dried.

General carotenoids were determined in acetone extracts by spectrophotometric method on specific absorption at 440, 480, 630, 644, 645, 662, 663 and 750 nm (Table 3).

Table 3: Composition of common carotenoids in green and yellow form D.salina AR-1

Pigments	General carotenoids
Green form, mg/g % of dry biomass	1.6 – 2.2 0.16 – 0.22
Yellow form, mg/g % of dry biomass	16.3-23.6 1.63-2.3

As can be seen from the results, the maximum accumulation of carotenoids was 2.3% of the dry biomass weight, while industrial stacks contain up to 10% of carotenoids from dry mass. It is known that by regulating the content of nitrogen and phosphorus in the medium, cultivation under conditions favorable for cell division of salinity, illumination and temperature can influence the accumulation of β-carotene in D.salina cells. We have tested different concentrations of nitrogen and phosphorus, which are much lower than the growth optimum for D.salina, and are 0.5-1 g/l NaNO3 and 0.02-0.25 g/l K2HPO4 [8]. Nitrogen was applied in the form of KNO3 depending on the variant of experience (without application - nitrogen exclusion, 20, 40 and 80 mg/l), phosphorus - in the form of KH2PO4 (without application - phosphorus exclusion, 4, 9, as well as the concentration of 45 mg/l relating to the

optimal range).

In all variants of the experiment, during the first 6 days of cultivation, the content of β-carotene in cells sharply decreased, the cells lost orange color (Fig. 7). This period corresponded to the most intensive growth of cultures, which is also confirmed by literature data [9]. Growth of cultures in the variants of experience with the exception of biogens continued until the exhaustion of nitrogen and phosphorus in the medium. In the process of culture growth in these variants, the accumulation of β-carotene and restoration of cellular orange color began on the 24th day (see Fig. 7). On media with phosphorus exclusion and suboptimal nitrogen concentrations, cultures accumulated β-carotene and grew twice as intensively as on all media with nitrogen exclusion. The maintenance of suboptimal concentrations of both biogens (20-80 mg/l KNO3 and 4- 9 mg/l KH2PO4) in the medium significantly stimulated crop growth, but suppressed carotenogenesis, and cells remained green.

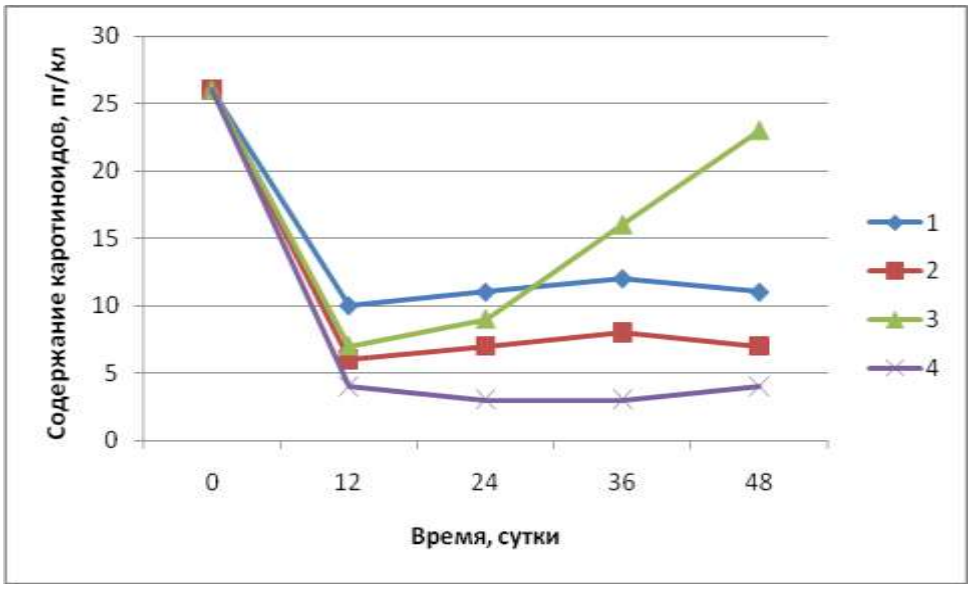


Fig. 7. Dynamics of β-carotene content in Dunaliella salina cells in media:
1 - without KNO3 and KH2PO4 application; 2 - without KNO3, 4-9 mg/l KH2PO4 application;
3 - without application of KNO3, 45 mg/l KH2PO4; 4 - 20-80 mg/l KNO3, without application of KH2PO4;
5 - 20-80 mg/l KNO3, 4-45 mg/l KH2PO4, without KH2PO4 application.

The results obtained show that carotinogenesis is induced in Dunaliella salina only when the nitrogen concentration in the medium decreases during cultivation to zero, which is confirmed by data from a number of researchers [10,11]. The induction of synthesis of high amounts of carotenoids, also called carotinogenesis, is a characteristic response of unicellular algae to the action of stressors of various nature (high salinity, UV radiation, intense lighting, extreme temperatures). Therefore, carotinogenesis, along with other responses to stressors, is an adaptive response that ensures the survival of microalgae of the genus Dunaliella in extreme habitat conditions.

Conclusion

Thus, the photoautotrophic microalgae Dunaliella salina is characterized by living in highly concentrated salt water bodies (withstands environmental salinity of 2.5 to 500 g/l) with a content of sodium chloride of about 10-11% (maintains viability even at 20-

25% NaCl content). At salt concentration of 140 g/l and illumination higher than 20 kLq, carotene starts to accumulate in D.salina AR-1 microalgae cells, and at salt concentration higher than 250 g/l, as a result of water evaporation, cells turn into yellow orange cysts. The biomass of yellow cells and cysts is the target product of dunalella cultivation. The results also show that carotinogenesis is induced in Dunaliella salina only when the nitrogen concentration in the medium decreases during cultivation. It should be noted that preferring a neutral-alkaline reaction of the medium, D.salina reacts negatively to sharp changes in it and sudden changes in osmotic pressure, and dilution of the concentrated medium can lead to the destruction and death of cells of the culture. Further investigation of the influence of individual factors and their combinations on the carotinogenesis process will allow to find out the mechanisms of carotinogenesis induction and β-carotene function in Dunaliella salina AR-1.

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