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RESPONSE OF *DUNALIELLA SALINA* TEOD. CULTURE TO VARIOUS REGIMES OF FED-BATCH NUTRIENT SUPPLEMENTATION

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There were studied *D. salina* culture growth, β-carotene and total protein accumulation under various fed-batch regimes: supplementation of nitrate and phosphate, nitrate or phosphate solely, and after the switch of nitrate supplied culture to phosphate supplementation and *vice versa*. Pre-cultures, unsupplied with the both or any nutrient, accumulated β-carotene. The sub-cultures supplied in turns with nitrogen and phosphorus, thus surviving one or the other nutrient depletion, regained culture growth ability, lost the ability to accumulate β-carotene and started to accumulate protein. The ability to reserve the available nutrient under another nutrient deficiency was hypothesized as *D. salina* possible ecological strategy, including assimilated carbon storage in triacylglycerides (TAGs) and β-carotene accumulation to prevent TAGs oxidation. The outcomes for industrial *D. salina* culture are discussed.

Key words: *Dunaliella salina, β-carotene, nitrate, phosphate, total protein*

Cultured unicellular microalga *Dunaliella salina* Teod. (*Chlorophyta*) has become a demanded β-carotene-rich food supplement claimed to be health-protective (Buono et al., 2014). Australian manufacturers dominate in this US30 million annually market (Borowitzka, 2013). They harvest the alga from open fertilized saline ponds, where it is grown extensively under natural irradiation. Long sunny season, as well as large pond area (up to 400 ha), favors high yields (Del Campo et al., 2007; Tafreshi, Shariati, 2009).

D. salina inhabits arid or semi-arid regions of the world, where evaporation prevails precipitation and natural hypersaline waters occur (Garcia-Gonzalez et al., 2003). *D. salina* could possibly be cultured in open ponds there or in closed photobioreactors beyond the arid zone. Microalgal culture is better to rely on outdoor natural irradiance, because artificial illumination proved to be economically unfeasible to grow microalgae commercially at the current technical level (Gordon, Polle, 2007). For locations with relatively short summer or with restricted natural resources of saline waters some technically simple measures should be taken to intensify and manage the culture to obtain high yields in shorter time and smaller culture volumes, e.g. as it was implemented in Israel in up to 4000 m^2 raceway ponds, CO_2 supplemented and paddle-wheel stirred (Tafreshi, Shariati, 2009). Adjustment of the medium composition could be one of the options.

Growing β-carotene-rich *D. salina* is complicated, because culture propagation and cellular β-carotene accumulation require the opposite conditions. Optimal conditions (moderate irradiation and salinity, and nutrient enriched medium) are necessary to increase cell number, stress conditions (elevated irradiation and salinity, and nutrient depleted medium) – to enhance β-carotene content

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Experimental design

	Variant											
	I	**II**			**III**			**IV and V**				
	Pre-culture medium, mg/l											
Sub-variant	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**		**9**		**10**	
KNO_3	0	20	40	80	0	0	0	20		40		80	
K_2HPO_4	0	0	0	0	5	10	20	5		10		20	
	Sub-culture medium, mg/l												
Sub-variant	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**8[a]**	**9**	**9[a]**	**10**	**10[a]**
KNO_3	0	0	0	0	20	40	80	0	0	0	0	0	0
K_2HPO_4	0	5	10	20	0	0	0	0	0	0	0	0	0

Comments: a – non-transferred culture, nutrient supplementation discontinued.

(Massyuk, 1973). Traditionally *D. salina* is grown in the batch culture (the culture grows in the medium rich in the nutrients; when nutrient depletion halts culture growth, β-carotene starts accumulating). Or it could be cultured in the two stages (the culture sufficiently pre-grown at the first stage is transferred into the nutrient deficient medium for β-carotene accumulation at the second stage) (Ben-Amotz, 1995).

Nitrate in the medium prevents β-carotene accumulation in *D. salina* cells even under high irradiation and salinity at the transcriptional level (Coesel et al., 2008). Because nutrient acquisition by algae depends on many factors (Giordano, Beardall, 2009), we suppose that fed-batch culture mode, with regular nutrient supplementation on demand, would be more suitable to better control the outdoors *D. salina* culture prone to weather fluctuations. It makes possible to increase, decrease or exclude certain nutrient flexibly depending on weather forecast (cloudiness, precipitation, temperature, wind).

The scientific literature is very scarce about the behavior of *D. salina* in fed-batch culture (Yamaoka et al., 1994) and do not allow to compare and choose the appropriate regimes.

The aim of the research was to study *D. salina* culture growth and β-carotene accumulation under fed-batch supplementation of nitrate and phosphate, nitrate or phosphate solely, and after the switch of nitrate supplied culture to phosphate supplementation and *vice versa*. Total cellular protein was measured as a potential valuable food and feed, by-produced at carotenoid extraction from *D. salina* biomass (Buono et al., 2014).

METHODS

The stock culture of *D. salina*, strain IBSS1, was maintained in the dissolved natural sea salt (specific gravity of the medium 1.15 g/cm^3).

Modified Artari's medium (Vinogradova et al., 2011) was used in the experiments. Osmotic components NaCl (116 g/l) and $MgSO_4 \cdot 7H_2O$ (50 g/l) were added to the medium once, before the inoculation.

The experimental design envisaged 5 different variants with different pre- and sub-culturing regimes and 3 nutrient concentration sub-variants (table):

I. Pre-cultured and sub-cultured at both nutrient supplementation excluded.

II. Pre-cultured at KNO_3 fed-batch supplementation, sub-cultured at K_2HPO_4 fed-batch supplementation.

III. Pre-cultured at K_2HPO_4 fed-batch supplementation, sub-cultured at KNO_3 fed-batch supplementation.

IV. Pre-cultured at both KNO_3 and K_2HPO_4 fed-batch supplementation, transferred for sub-culturing without nutrient supplementation.

V. Pre-cultured at both KNO_3 and K_2HPO_4 fed-batch supplementation, the culture left non-transferred, nutrient supplementation discontinued from the 45th day for another 42 days.

Three initial nutrient concentrations were: KNO_3 – 20, 40, and 80 mg/l, K_2HPO_4 – 5, 10, 20 mg/l (the same ratio maintained - 4:1). KNO_3 and K_2HPO_4 were supplemented first at the inoculation and then every 3-4 days in the half-dose.

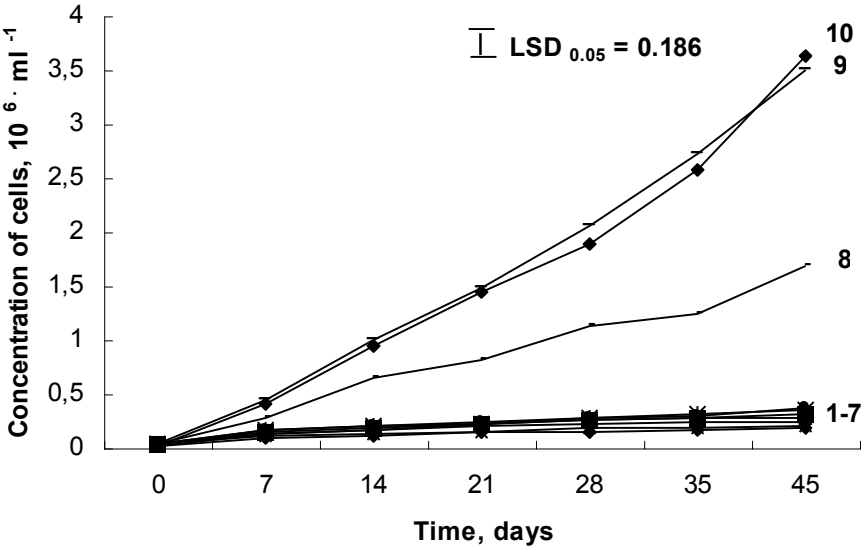

Fig. 1. Growth dynamics of *D. salina* fed-batch culture (see the table for the detailed experimental design).
1 – 0 mg/l KNO_3, 0 mg/l K_2HPO_4; 2-4 – 20, 40 and 80 mg/l KNO_3, respectively, 0 mg/l K_2HPO_4; 5-7 – 0 mg/l KNO_3, 5, 10 and 20 mg/l K_2HPO_4, respectively; 8-10 – 20, 40 and 80 mg/l KNO_3, 5, 10 and 20 mg/l K_2HPO_4 respectively.

Cell suspensions after pre-culturing were centrifuged at 3000 g for 10 min, cells washed with the solution of NaCl (116 g/l) and $MgSO_4 \cdot 7H_2O$ (50 g/l), and inoculated into the media for sub-culturing.

Atmospheric CO_2 dissolved in the medium served the sole carbon source.

Micronutrients were added to the final concentrations: 3.1 mg/l H_3BO_4, 1.94 mg/l $MnSO_4 \cdot 5H_2O$, 0.287 mg/l $ZnSO_4 \cdot 7H_2O$, 0.088 mg/l $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.146 mg/l $Co(NO_3)_2 \cdot 4H_2O$, 0.119 mg/l KBr, 0.083 mg/l KI, 0.048 mg/l $NiSO_4 \cdot 7H_2O$, 0,08 mg/l $CuSO_4 \cdot 5H_2O$.

The other conditions were maintained constant: irradiance – 5 klx from four 32 W "Maxus" lamps, the color temperature 2700 K, 16 hours light, 8 hours dark, 27 °C.

Cultures were inoculated with the initial concentration of the cells *ca*. $15 \cdot 10^3$ per 1 ml, 15 ml of the culture in 25 ml Erlenmeyer flasks.

Cells were counted using Goryaev haemocytometer, or optical density of the culture was measured at 750 nm. Concentration of cells was calculated by the haemocytometer formula or calibration equation for optical density and expressed as 10^6 per 1 ml.

β-carotene was extracted by vigorous shaking culture aliquot (1 ml) with 2 ml of ethyl acetate, the extract absorbance at 440 nm was measured, β-carotene content was calculated using the reference specific extinction $E_{1cm}^{1\%}$=2500 (IARC 1998), and expressed as pg per 1 cell.

For total protein quantification culture aliquots (6 ml) were centrifuged, the pellets washed with Bligh and Dyer (1959) mixture and dissolved in 2 ml of 1n NaOH. The protein was quantified by Lowry (Lowry et al., 1951), calculated using calibration equation by bovine serum albumin, and expressed as pg per 1 cell.

All experiments were carried out in triplicate. The data distributions did not differ from the normal according to Shapiro-Wilk test. To process the data, parametric multifactor analysis of variance was used. The figures present means and least significant differences ($LSD_{0.05}$). The effects discussed are significant at 95% level.

RESULTS

Pre-cultures unsupplied with the both or any nutrient showed insignificant increase of cell concentrations, while the cultures supplied with the both nutrients grew normally (fig. 1). At the lowest nutrient levels (KNO_3 – 20 mg/l, K_2HPO_4 – 5 mg/l) the growth rate conceded to the higher nutrient levels (fig. 1). The culture was irresponsive to KNO_3 increase from 40 to 80 mg/l and K_2HPO_4 increase from 10 to 20 mg/l, showing that the higher nutrient levels were probably supra-optimal under the given experimental conditions (fig. 1).

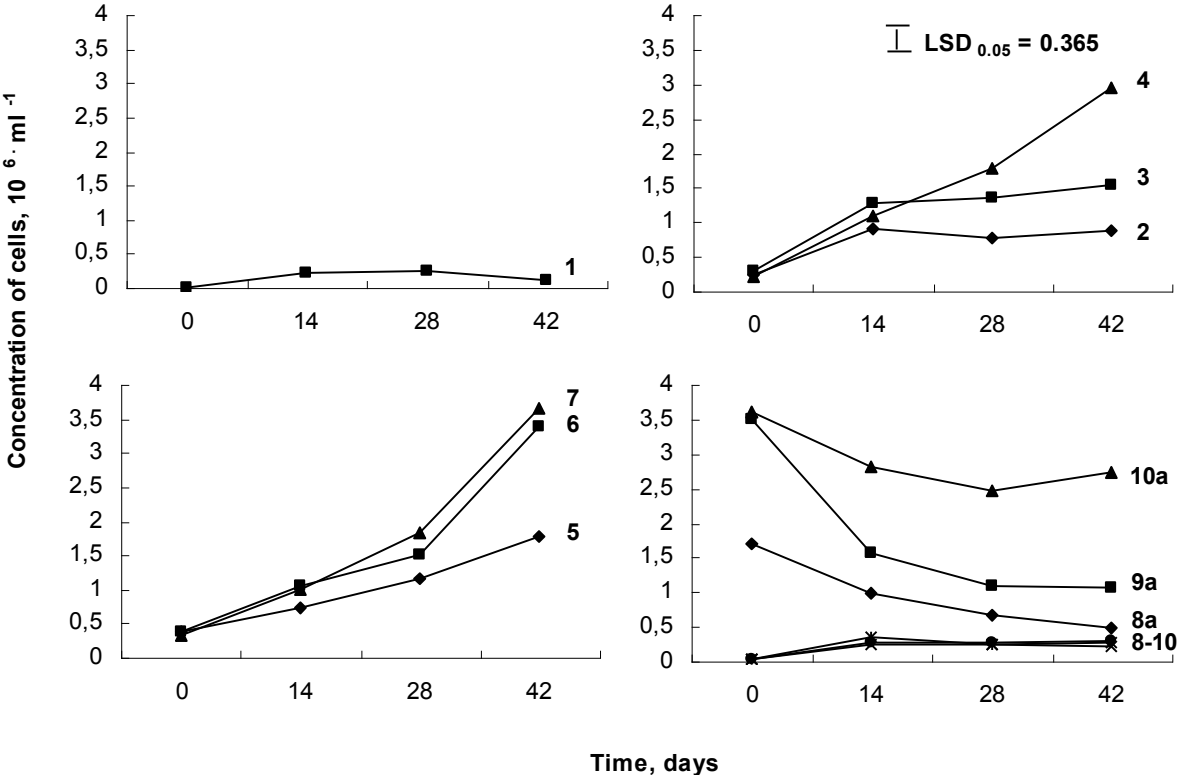

Fig. 2. Growth dynamics of *D. salina* sub-cultures after switching the fed-batch regime.
Here and in figs. 3, 4. 1 – 0 mg/l KNO_3, 0 mg/l K_2HPO_4 to 0 mg/l KNO_3, 0 mg/l K_2HPO_4; 2-4 – from 20, 40 and 80 mg/l KNO_3, respectively, and 0 mg/l K_2HPO_4 to 0 mg/l KNO_3, 5, 10 and 20 mg/l K_2HPO_4, respectively; 5-7 – from 0 mg/l KNO_3, 5, 10 and 20 mg/l K_2HPO_4, respectively, to 20, 40 and 80 mg/l KNO_3, respectively, and 0 mg/l K_2HPO_4; 8-10 – from 20, 40 and 80 mg/l KNO_3 and 5, 10 and 20 mg/l K_2HPO_4, respectively, to 0 mg/l KNO_3, 0 mg/l K_2HPO_4; 8a-10a – 20, 40 and 80 mg/l KNO_3 and 5, 10 and 20 mg/l K_2HPO_4, respectively, supplementation discontinued.

Unsupplied with the both nutrients sub-cultures showed the same growth retardation as the unsupplied pre-cultures, no matter whether the inocula had been taken from: the pre-cultures supplied or unsupplied with the both nutrients (fig. 2). Canceling nutrient supplementation in the cultures pre-fed by the both nutrients resulted in gradual cell concentration decrease (fig. 2).

Surprisingly, the single nutrient supplied cultures regained growth ability, when switched to supplementation with the other nutrient (fig. 2). Growth dynamics in those sub-cultures (fig. 2) were almost similar to the pre-cultures simultaneously supplied with corresponding concentrations of the both nutrients, except the culture growth was slightly retarded after transferring KNO_3 supplied culture to K_2HPO_4 supplied medium (fig. 2).

Pre-cultures unsupplied with the both or any nutrient accumulated more than 20 pg cell^{-1} β-carotene (zero time point fig. 3). When sub-cultured in the fresh media they lost their cellular β-carotene in 14 days (fig. 3). Then, the sub-cultures unsupplied with the both or any nutrient started accumulating β-carotene. To the 42nd day they lined up by β-carotene cellular content in the following order: pre-cultured and sub-cultured without the both nutrients > pre-cultured with the both nutrients supplied, sub-cultured with the both nutrients excluded > pre-cultured with the both nutrients supplied, supplementation discontinued (fig. 3). Within the last two groups the sub-variants lined up by descending nutrient level (fig. 3), i.e. β-carotene accumulation negatively correlated with the concentrations of nutrients, which the cultures had been exposed to during pre-culturing.

The sub-cultures switched from supplementation of the one nutrient to the other did not accumulate β-carotene regardless that the sub-culture medium was depleted in one of the nutrients (fig. 3). In the variants, pre-cultured with KNO_3 and sub-cultured with K_2HPO_4, slight β-carotene accumulation was noticeable in the concentration sub-variants exhibiting growth retardation (fig. 2, 3).

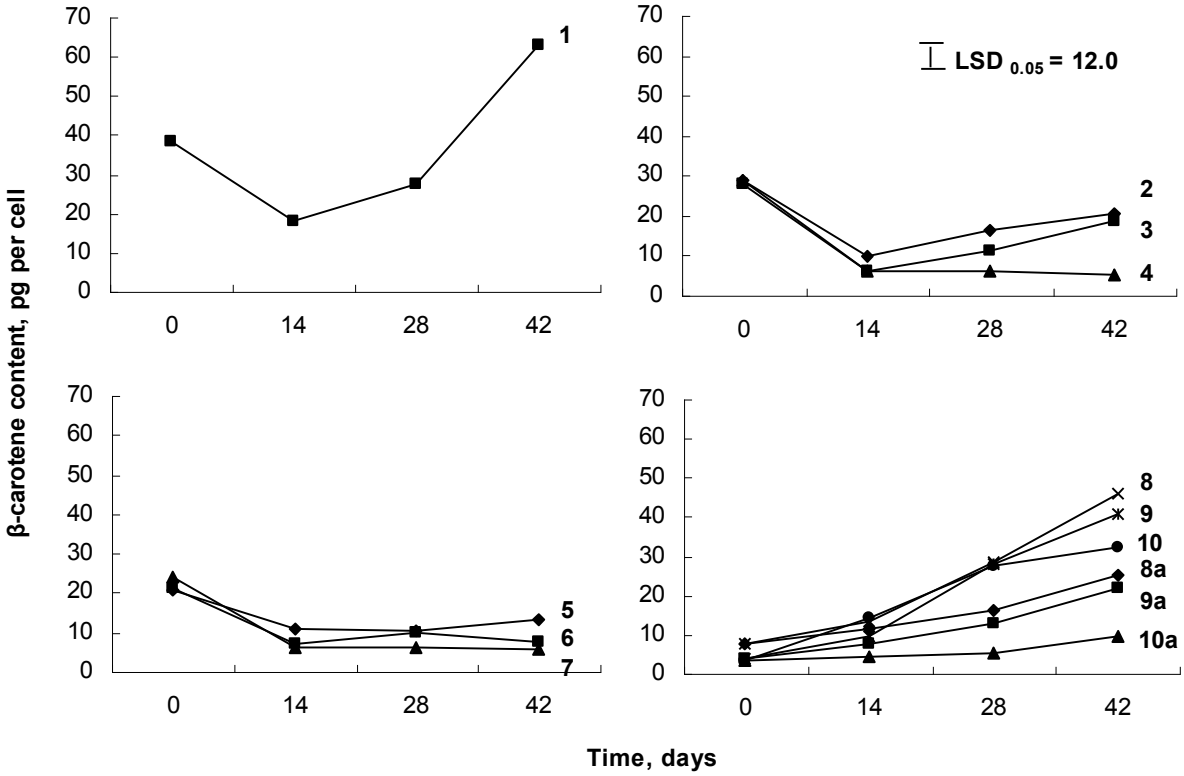

Fig. 3. Cellular β-carotene content in *D. salina* sub-cultures after switching the fed-batch regime. Variants are specified in the caption for fig. 2.

The sub-cultures without the both nutrient supplementation retained the lowest total cellular protein content (fig. 4). The protein level in the cultures after canceling nutrient supplementation was slightly higher (fig. 4). The cultures switched from the one to the other nutrient supplementation demonstrated significant total cellular protein accumulation (fig. 4). Surprisingly, the protein accumulation occurred in the cells placed into the medium without nitrogen source (KNO_3) (fig. 4). In this variant protein accumulation level and durability positively correlated to the nutrients level. Protein accumulation was transient and turned into cellular total protein content decrease (fig. 4), that correlated with culture growth retardation (fig. 2) and β-carotene accumulation (fig. 3).

DISCUSSION

The most prominent finding was that the fed-batch *D. salina* culture, supplied in turns with nitrogen and phosphorus, thus in turns surviving one or the other nutrient depletion, regained culture growth ability and lost the ability to accumulate β-carotene.

The ability to grow in nutrient depleted medium with almost the same rate as in nutrient supplied medium is an indirect proof of the ability to store the available nutrient. One more indirect proof was our earlier finding, that in *D. salina* nitrate and phosphate acquisition occurs independently and proportionally to acquired nutrient concentration even at the other nutrient depletion and culture growth retardation (Komaristaya et al., 2010). This ability is specific to *D. salina*. In many algal species nitrate depletion prevents phosphate acquisition and *vice versa* (Bougaran et al., 2010). *D. salina* has the adaptive advantage under regular periodic supply and limitation of the nutrients, for example, in salt work ponds, where *D. salina* massively develops under periodical supply of fresh portions of seawater fed for NaCl manufacturing.

D. salina is known to store phosphorus in the form of polyphosphates (volutin) (Karni, Avron, 1988). There is no information in the literature on nitrogen reserves in *D. salina* cells. But the increase of nitrite, ammonium and free amino acids intracellular concentrations was observed under sulfur deficiency in *D. salina* (Giordano et al., 2000). Noteworthy, that intracellular phosphates increased too (Giordano et al., 2000).

Some algal species store nitrogen in several forms simultaneously: inorganic ions, amino acids and even proteins (Pueschel, Korb 2001). Our previous data showed total cellular protein increase in

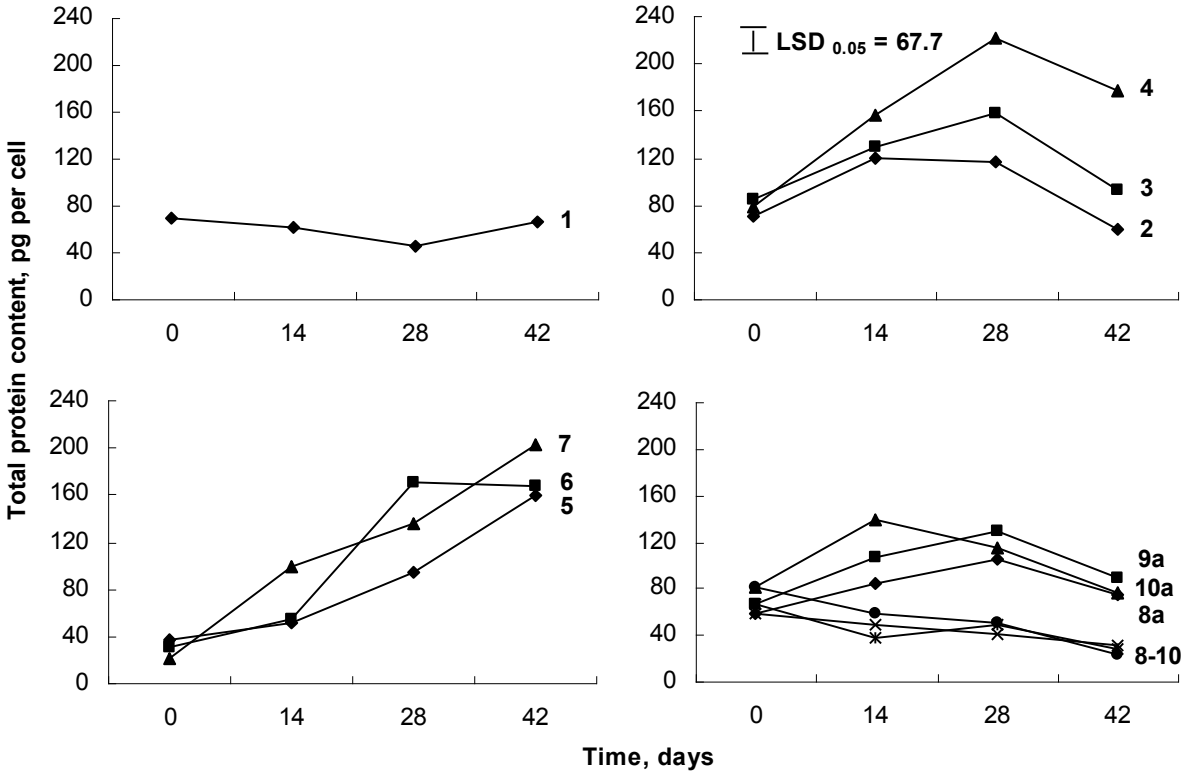

Fig. 4. Total cellular protein content in *D. salina* sub-cultures after switching the fed-batch regime.
Variants are specified in the caption for fig. 2.

D. salina under phosphorus deficiency (Antonenko, Komaristaya, 2010). We had considered proteins as putative nitrogen storage form in *D. salina*, but it could not be the only one. The unexpectable cellular protein increase in nitrogen deprived and phosphorus supplied sub-culture supports this idea. Apparently, the cells, when pre-cultured in nitrate supplied medium, accumulated nitrogen in some small molecules, which started converting into proteins as soon as phosphorus became available. Phosphate pre-supplied culture started accumulating protein as soon as appeared in phosphate deprived and nitrate supplied medium.

The proteomics of *D. salina* stayed out of focus of our research. Earlier we showed that total protein content increase under phosphorus deficiency did not relate to certain enzymatic (catalase) activity changes (Antonenko, Komaristaya, 2010). It means that the accumulated proteins possessed certain specificity. Total cellular protein content in *D. salina* further confirmed its liability to change starting to decrease at the late stages of sub-culturing in nitrate deprived medium. Probably, some specific proteins in *D. salina* cells serve as slowly exchangeable nitrogen reserve in addition to fast exchangeable small molecules reserve. Their composition should be the topic of separate investigation.

Considering possible *D. salina* adaptive strategy to accumulate available nutrients when others are depleted, the question should be asked if the alga is able to accumulate the most important nutrient – carbon, and what is the putative form of carbon storage. Ability to accumulate assimilated carbon could have an adaptive value in hyperhaline habitats: brine heating (up to 38 °C in summer) and its salinity increase due to water evaporation (up to 1.25 g/cm^3 in salterns) cause CO_2 solubility decrease and its deficiency for algae.

This reasoning could lead to another interpretation of already known facts. Triacylglycerides (TAG) synthesis inhibition prevented β-carotene accumulation in *D. salina* (Rabbani et al., 1998). The authors explained that effect as β-carotene synthesis enzymes inhibition by the final product, not being withdrawn into its usual allocation compartment – lipid globules (Rabbani et al., 1998).

From our point of view, lipid globules are very likely to store assimilated carbon in *D. salina*. Algae are known to accumulate TAGs under nutrient deficiency in the forms of lipid globules in cytosol or plastoglobules in chloroplast (Murphy,

2001), but this ability is rare. In the study (Rodolfi et al., 2009) 30 strains belonging to 14 genera of marine and freshwater algae were screened for the ability to store lipids at nitrogen and phosphorus deficiency, but it was revealed for 2 marine species only. We hypothesize that β-carotene in *D. salina* serves to protect storage TAGs from oxidation. It could be further supposed why stresses like high irradiance, suboptimal temperature or increased salinity induce β-carotene accumulation in lipid globules of *D. salina* cells. It is generally known that any stress is accompanied by oxidative stress in photosynthesizing cell.

Good fit of *D. salina* cellular responses to nutrients concentrations shows that the hypothesis of antioxidant protection of assimilated carbon storage could be formulated and verified as a mathematical model based on C:N:P ratio. If confirmed in the future research it would made the biotechnologies of carotenoid synthesis more reliably controlled.

The fact of preventing β-carotene accumulation when intracellular nutrient reserves are claimed for culture growth was new, unobvious and has practical relevance. In the industrial *D. salina* cultures the concentrations of nutrients should be well balanced to avoid limiting culture growth by some nutrients and storing the other, as intracellular nutrients reserves could decrease or even prevent potential β-carotene yield. Because many other environmental factors influence nutrients absorption rate, fed-batch cultivation under continuous control of nutrient absorption is a tool to cope with open culture challenges.

Thus, the following conclusions could be drawn:

- Under single nutrient (nitrogen or phosphorus) deficiency *D. salina* cells accumulate the available nutrient and become able to culture growth under accumulated nutrient deficiency in the medium.

- The nutrient (nitrogen or phosphorus) stored inside *D. salina* cells prevents β-carotene accumulation under that nutrient single deficiency in the medium.

- Under alternating nutrient deficiencies *D. salina* cells accumulate total cellular protein that is likely to serve as slow exchangeable nitrogen reserve.

We hypothesize that *D. salina* possesses an ecological strategy to accumulate available nutrients when population growth is halted by the other nutrient deficiency. Lipid globules are likely to be assimilated carbon reserve, and β-carotene accumulates in them to prevent TAGs oxidation.

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РЕАКЦИЯ КУЛЬТУРЫ *DUNALIELLA SALINA* TEOD. НА РАЗЛИЧНЫЕ РЕЖИМЫ ПОДПИТКИ БИОГЕНАМИ

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Изучали динамику роста культуры *D. salina*, накопления β-каротина и общего белка при различных режимах подпитки: добавлении нитрата и фосфата, только нитрата или фосфата, и после перевода культуры, подпитываемой нитратом, на добавки фосфата, и наоборот. Культуры, которые не подпитывали ни одним или подпитывали только одним биогеном, накапливали β-каротин. Суб-культуры, которые поочередно подпитывались нитратом или фосфатом, и которые поочередно переживали дефицит одного из биогенов, восстанавливали способность к росту культуры, утрачивали способность к накоплению β-каротина и начинали накапливать общий белок. Предполагается, что *D. salina* обладает экологической стратегией, которая заключается в способности накапливать доступный биоген при дефиците другого биогена, включая запасание ассимилированного углерода в виде триацилглицеридов и накопление β-каротина для предотвращения окисления триацилглицеридов. Обсуждаются следствия для промышленной культуры *D. salina*.

Ключевые слова: *Dunaliella salina, β-каротин, нитрат, фосфат, общий белок*

RESPONSE OF DUNALIELLA SALINA TEOD. CULTURE

РЕАКЦІЯ КУЛЬТУРИ *DUNALIELLA SALINA* TEOD. НА РІЗНІ РЕЖИМИ ПІДЖИВЛЕННЯ БІОГЕНАМИ

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Вивчали динаміку росту культури *D. salina*, накопичення β-каротину і загального білка за різних режимів підживлення: за додавання нітрату і фосфату, тільки нітрату або фосфату, і після переведення культури, що підживлювали нітратом, на добавки фосфату, і навпаки. Культури, що не підживлювали жодним або підживлювали лише одним біогеном, накопичували β-каротин. Суб-культури, які по черзі підживлювалися нітратом або фосфатом, і які по черзі зазнавали впливу дефіциту одного з біогенів, відновлювали здатність до росту культури, втрачали здатність до накопичення β-каротину і починали накопичувати загальний білок. Висловлено припущення, що *D. salina* має екологічну стратегією, яка полягає у здатності накопичувати доступний біоген за дефіциту іншого біогена, включаючи запасання асимільованого вуглецю у вигляді триацилгліцеридів і накопичення β-каротину для запобігання окиснення триацилгліцеридів. Обговорюються наслідки для промислової культури *D. salina*.

Ключові слова: *Dunaliella salina, β-каротин, нітрат, фосфат, загальний білок*