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## NUTRIENT STORED INSIDE *DUNALIELLA SALINA* CELLS PREVENTS β-CAROTENE ACCUMULATION UNDER ITS MEDIUM DEFICIENCY

Annotation. At alternating cultivation cycles under deficiencies of nitrogen or phosphorus *D. salina* culture grew limitedly at the first cultivation, then intense after transferring the culture. Cellular β-carotene accumulated during the first cultivation, and then almost did not. *D. salina* cells are able to accumulate the available nutrient at the other nutrient deficiency and to spend the intracellular nutrient reserve for culture growth when the other nutrient re-supplied to the medium.

The microalga *Dunaliella salina* Teod. is cultivated to manufacture natural β-carotene. Detailed history of research and the current state of the cultivation technology can be found in the reviews (Oren, 2005; Lamers et al., 2008; Ramos et al., 2011). The technology encounters a problem: "The optima of culture growth and β-carotene accumulation do not coincide" (Massjuk 1973), *i.e.* single cultivation cycle can result in either much biomass with low β-carotene content or little biomass with high β-carotene content. To resolve this contradiction, the two-stage cultivation was proposed: during the first stage, the conditions are aimed at biomass growth, during the second - at β-carotene accumulation (Massjuk, 1973). At that, ready for harvesting biomass is yet missing at the first stage of cultivation cycle.

*D. salina* is successfully cultivated at the industrial scale in Australia (Curtain, 2000) and Israel (Ben-Amotz, 2004). Ukraine possesses natural resources – salt lakes and salterns – to grow this alga too (Massjuk, 1973). *D. salina* biomass and so called mixed carotenoids product (which consists mostly of β-carotene) are of great demand in the world market. Thus, further improvement of *D. salina* cultivation technology remains of current interest.

It was found that, while the culture grew normally in the presence of both nitrogen and phosphorus, deficiency of any of these nutrients independently induced β-carotene accumulation in *D. salina* cells (Komaristaya et al., 2010). What if one can reach a compromise between culture growth and β-carotene accumulation under alternating nitrogen and phosphorus deficiencies? That would allow constantly producing the biomass with high β-carotene content starting from the first cultivation cycle, depriving and re-supplying nitrogen or phosphorus every time at transferring the culture.

The objective of this research was to study the dynamics of culture growth and β-carotene accumulation in *D. salina* when culturing under conditions of alternating nitrogen and phosphorus

deficiencies.

We did not reach the compromise between culture growth and β-carotene accumulation in *D. salina* culture under alternating nitrogen and phosphorus deficiencies. The culture still either grew intense or accumulated β-carotene. Pre-incubated with nitrogen or phosphorus only, the culture acquired the ability to grow under this nutrient deficiency (*i.e.* the cells accumulated the available nutrient) and lost the ability to accumulate β-carotene under this nutrient deficiency in the medium.

The ability to store nutrients was itself quite expectable for algal cells. *D. salina* is known to store phosphorus in the form of polyphosphates (volutin) (Karni and Avron, 1988). New and unobvious was that intracellular nitrogen or phosphorus reserve prevented the induction of β-carotene accumulation by nitrogen or phosphorus deficiency in the medium. It could be the reason of inability to prove that nitrogen or phosphorus deficiencies induce β-carotene accumulation in some researches (Ben-Amotz et al., 1982).

Despite cultivation mode with alternating deficiencies appeared practically inapplicable, it showed an important physiological specificity of *D. salina*. In some other algal species single nutrient deficiency inhibited absorption of the other nutrient (Bougaran et al., 2010). Earlier we found that *D. salina* acquired independently nitrogen and phosphorus: nitrogen concentration in the medium did not influence phosphorus acquisition, and *vice versa* (Komaristaya et al., 2010). The results of this study proved that the cells accumulated the available nutrient when culture growth was limited by the other nutrient, and spent the accumulated nutrient when limiting nutrient became available. This ability must give to the species 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.

We had considered proteins as putative nitrogen storage form in *D. salina*, because their intracellular content can vary greatly (Antonenko, Komaristaya, 2010). Some algal species store nitrogen in the forms of inorganic ions, amino acids and even proteins (Pueschel, Korb, 2001). Our previous data showed that *D. salina* accumulated proteins at phosphorus deficiency (Antonenko, Komaristaya 2010). From the present study, this species accumulated nitrogen in the other forms as well, more likely in some small molecules. The cellular protein increase in nitrogen deprived sub-culture supports this idea. Apparently, for protein synthesis phosphorus was necessary, which appeared sufficient in the second sub-culture, or protein synthesis just lagged behind nitrogen accumulation, which occurred in some precursor molecules. Reclaiming nitrogen for culture growth in the second sub-culture lagged too. Maybe, protein is a slowly exchangeable form of nitrogen storage in *D. salina*.

The other proof of low molecular weight nitrogen reserve forms in *D. salina* is intracellular

concentrations increase of nitrite, ammonium and free amino acids under sulfur deficiency (Giordano et al., 2000). Noteworthy, that under sulfur limitation intracellular phosphates increased too (Giordano et al., 2000).

Intracellular reserve nitrogen and phosphorus quantification remained beyond the focus of the current research targeted to testing alternating deficiencies cultivation mode on *D. salina*. The ability of culture to grow under certain nutrient deficiency served as the test function to prove the nutrient intracellular accumulation, and biological tests are known to be even more sensitive than the methods of analytical chemistry that makes our results robust.

The proteomics of alternating nutrient deficiency stayed out our focus too. Earlier we showed that total protein content increase under phosphorus deficiency was not accompanied by certain enzymatic (catalase) activity increase (Antonenko, Komaristaya, 2010). It means that the proteins accumulated possessed certain specificity. Their composition could be the topic of separate investigation.

Taking into account possible adaptive ability of *D. salina* to accumulate all available nutrients when some nutrient is deficient, the question should be asked if the alga accumulates the most important nutrient – carbon, and what is the putative form of carbon storage. The ability to accumulate assimilated carbon could have an adaptive value in hyperhaline habitats too: 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.

β-carotene accumulation in *D. salina* followed the same pattern as accumulation of the other nutrients. It occurred at culture growth inhibition by the deficiency of nitrogen or phosphorus (Komaristaya et al., 2010), and sulfur (Giordano et al., 2000); and cellular β-carotene content decreased when all the nutrients are available and the culture grew intense.

All these allow another interpretation of some already known facts. Triacylglycerides synthesis inhibition prevented β-carotene accumulation in *D. salina* (Rabbani et al., 1998). The authors explained that effect from the point of view of β-carotene synthesis enzymes regulation by final product withdrawal and allocation into specialized cellular compartments – lipid globules (Rabbani et al., 1998).

We are far from the idea that so complex, active and multifunctional substance as β-carotene serves in *D. salina* cells as assimilated carbon reserve. Triacylglycerides are more likely to store assimilated carbon. Algae are known to accumulate triacylglycerides at the nutrient deficiency in the forms of lipid globules in cytosol or plastoglobules in chloroplast (Murphy, 2001), but most species are exceptions from that. 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.

It could be 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. In *D. salina* lipid globules β-carotene could protect storage lipids from oxidation.

This hypothesis could be extrapolated to the other industrial producents of carotenoids: like the microalga *Haematococcus pluvialis* Flotow em. Wille, which accumulates astaxanthin, and the mold *Blakeslea trispora* Taxter, which accumulates β-carotene. The analysis of published data supports our hypothesis. Both in *H. pluvialis* (Boussiba, 2000) and *B. trispora* (Menschel et al., 2005) intense biomass growth disagrees with carotenoid accumulation. In *H. pluvialis* nitrogen deficiency and carbon excess stimulate astaxanthin accumulation (Kang et al., 2005). In *B. trispora* phosphorus deficiency (Menschel et al., 2005) and specific carbon nutrition, that leads to triacylglycerides deposition (Ciegler et al., 1959), facilitate β-carotene accumulation.

Probably, all these species are able to store the nutrients that are available, including assimilated carbon, providing culture growth is inhibited by one or several nutrients deficiencies. And carotenoids protect assimilated carbon lipid deposits from oxidation.

This hypothesis is the further detailed elaboration of earlier expressed antioxidant hypothesis of secondary carotenoids function (Grunewald et al., 2000) and has not been proposed before. The other hypotheses include: photoprotection (Bidigare et al., 1993), synthesis of regulatory apocarotenoids similar to vitamin A, abscisic acid, and trisporic acids (Olson, 1993), synthesis of cell walls of resting life cycle stages – zygotes and cysts (Komaristaya, Gorbulin, 2006). Possible multifunctionality of secondary carotenoids should not be excluded.

Good fit of *D. salina* cellular responses to nutrients concentrations in the present study 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 not only contribute to our knowledge on the species able to accumulate carotenoids but also made the biotechnologies of carotenoid synthesis more reliably controlled.

From the practical point of view, classical two-stage mode of culturing *D. salina* remains the most effective. Our study adds to the classical mode the precaution: during the first stage of cultivation the concentrations of nitrogen and phosphorus should be well balanced and not overabundant to avoid limiting culture growth by some nutrients and storing the other, because intracellular nutrients reserves would decrease or even prevent potential β-carotene yield at the second cultivation stage. As many other environmental factors could influence nutrients absorption rate, fed-batch cultivation mode under continuous control of nutrient absorption could be useful in that respect.