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NANOSTRUCTURED FERRIC CITRATE EFFECT ON *Chlorella vulgaris* DEVELOPMENT

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The aim of the research was to study the development of *Chlorella vulgaris* at culturing on the modified Gromov 6 medium with high concentrations of nanostructured ferric citrate and also its effect on photosynthesis pigments accumulation. It was demonstrated that the highest intracellular iron content (15 mg/g of dry mass) in the culture cells was typical with nanostructured ferric citrate content of 30 mg/dm^3 of culture medium, the highest content of chlorophyll *a* — 23 mg/g of dry algae mass, *b* — 7.5 mg/g of dry mass, and carotenoids — 9.2 mg/g of dry mass was observed at nanostructured ferric citrate content of 20 mg/dm^3. The use of nanostructured ferric citrate leaded to an increase in the chlorella biomass yield by 3 times with compared to standard technology. Simultaneously, intracellular iron content in cells increased significantly with the use of nanostructured ferric citrate, which increases their value as a nutritional supplement. In order to increase the biomass yield and intracellular iron content in cells, application of nanostructured ferric citrate is recommended.

Key words: chlorella cells, biomass, intracellular iron, chlorophylls, carotenoids, nanostructured ferric citrate.

The lack of elements participating in biochemical processes in human and animal bodies results in diseases and requires to be introduced additionally. Microalgae is a source of the broad variety of biologically active substances and their consumption increases from year to year [1,2].The enrichment of microalgae cells with elements or compounds in the easily digestible form for humans or animals will make possible to solve the issues of prophylaxis or treatment of diseases caused by deficiency of such compounds. Ferrum is one of these elements.

Iron compounds participate in photosynthesis redox reactions, breathing, carbon exchange, so its deficiency affects the general cell and body metabolism [3]. Enzymes containing heme and non-heme iron are being the active forms of ferrum. The heme structure is like the chlorophyll structure. Moreover, it is known that magnesium ions in this compound are easily replaced with ferrum ions in human and animal bodies [4]. Concurrent content of iron and chlorophyll ions in microalgae cells will benefit to the element flow to the blood and have a positive influence on a body condition.

Culturing conditions, form and content of nutrient medium components influence on cell metabolism and specific product accumulation by microalgae [1, 5, 6]. Consequently, the study of the effect of iron compound concentrations and forms contained in culture medium on its cell content and chlorophyll biosynthesis is an urgent issue.

Standard media for *Chlorella* microalgae cultivation contain iron in the form of salts such as sulphate, chlorideorcitratein concentrations 0.2–2 mg/dm^3. Iron content is increased to 5 mg/dm^3 to intensify growing [4]. Ethylenediaminetetraacetatesodium (Na_2EDTA), a complexing substance, is added to media to avoid ferrum phosphate sedimentation at neutral and alkaline pH values.

The effect of ferrum ion concentration on biomass yield and microalga metabolites

has been studied in works [1,4,6,7, 8].Using $FeSO_4 \cdot 7H_2O$ with ferrum ion concentrations, mg/dm^3, 0.35, 4.89, 9.44 (control specimen) and 13.99, *Chlorella vulgaris* demonstrated the highest biomass growth of 1.03×10^7cell/cm^3 for 21 days of cultivation with minimum ferrum concentration. Moreover, the content of proteins (8.34 mg/g of dry weight, control specimen — 5.15 mg/g) and phenolic compounds (6.34 mg/g, control — 5.80 mg/g) increases with ferrum ion concentration of 0.35 mg/dm^3 [1, 7].

Using ferrum sulphate, the average growth rate of *Chlorella vulgaris* increases by 10.34% and 6.89% at ferrum concentrations 1.48 and 1.97 mg/dm^3, respectively, vs. control specimen(0.986 mg/dm^3), and reaches 0.32 day-1 with concentration 1.48 mg/dm^3. Should ferrum concentration be increased to 2.47 mg/dm^3, biomass growth will be inhibited by 51.72% vs. control specimen. Maximum lipid fraction content (36.4%) is typical for ferrum ion concentration of 1.97 mg/dm^3 [6].

Decrease of $FeCl_3$ concentration to $1.2 \cdot 10^{-5}$ mol/dm^3 results in lipid content elevation up to 56.6% in *Chlorella vulgaris*, which is 3–7 folds more compared to samples cultured on medium with a lesser ferrum concentration. The use of iron chelate complex as a nutrition source does not affect lipid fraction biosynthesis [8].

The quantity of cell ferrum ions is increased with ferrum content growth in culture medium. Fe^{3+} ion content in quantity 25–50 mg/dm^3 and in the form of sulphate or chloride results in medium acidification, and may reach pH = 3.8 which has a negative effect on microalgae development [4]. Consequently, it will be required to introduce a chelating agent to culture medium to increase cell ferrum ion content.

The addition of a complexing substance at ferrum ion concentration of 0.25–3 mg/dm^3 increases the growth rate of microalgae biomass. If ferrum concentration exceeds 5 mg/dm^3, the introduction of a chelating agent will not influence on *Chlorella vulgaris* development [4].

Should ferric citrate be used, a higher biomass yield will be typical for a larger iron (III) ions concentration (4.8×10^{-5} mol/l), while lipid yield is higher at the decrease of ion concentration twice. The biomass yield increase at elevation of ferrum ion concentration in the medium is conditioned by the fact that ferrum is a cofactor of the photosystems I and II and impacts on cell light absorption, photochemical energy conversion, electron transfer and carbon fixation [9].

The study of a nanostructured ferric citrate effect on its accumulation by the organism (its contentin blood)had been conducted on mice[10]. It was found out that introduction of ferrum ions in composition of nanostructured citrate, the same as with ferrum sulphate, leads to the elevated ferrum ion content in a studied object.

The concentration of ferrum ions influences on fixation of other microelements in *Chlorella vulgaris* [11]. The ferrum ion content in quantity 25–50 mg/dm^3 in the form of chloride or sulphate reduces content of phosphorus, zinc, copper, magnesium, mangan in *Chlorella* cells and increases chlorophyll content [4].

Based on the foregoing, it is possible to state that an effect of high nanostructured ferric citrate concentrations on microalgae cells may result in the change of biosynthetic process direction of cells due to which its content may be heightened in microalgae cells and chlorophyll biosynthesis — structural analogues of heme. Therefore, the study of the effect of different nanostructured ferric citrate concentrations on ferrum ion accumulation and the change of *Chlorella vulgaris* cell metabolism are being a pressing issue.

The objective of this work is to define a rational ferrum ion concentration for *Chlorella vulgaris* culturing with a high content of ferrum ions and chlorophyll.

The following tasks have been solved to achieve a set objective:

– to establish an effect of different forms and concentrations of ferrum ions in culture medium on its assimilation and development of *Chlorella vulgaris* cells;

– to clarify a dependence on nanostructured ferric citrate concentration of photosynthetic pigment biosynthesis by microalgae cells.

Materials and Methods

The study has been carried out with microalgae *Chlorella vulgaris* ACKU531–06 from the collection of the Taras Shevchenko National University of Kyiv (Ukraine).

The microalgae were cultivated in photoreactors of volume 1.2 dm^3 with the airlift stirring system. The mixing was carried out with bubbling airusing the compressor Resunair-pump AC — 9601. Bubbling speed is 0.003 m^3/h. CO_2 as a carbon source was added to bubbling air through the reducer system. CO_2 volume introduced to the photobioreactor had been varied depending on microalgae

concentration. Culture temperature is 30 and 18 °C.

The Gromov 6 medium with a variable concentration of sulphate and nanostructured ferric citrate had been used as a basic nutrient medium. The medium was autoclaved for 1 hour at temperature 120 °C and pressure 250 kPa.

Nanostructured ferric citrate was provided by the Ukrainian State Science and Research Institute of Nanobiotechnologies and Resource Efficiency.

Natural lighting along with artificial lighting was used concurrently as a source of light energy to intensify the development of microalga culture. Lighting with LEDs had been carried out in regime: light — 4 hours and darkness — 4 hours. The ratio of red (620–770 nm), green (510–550 nm) and blue (440–480 nm) wavelength ranges is — 2:1:1. The use of such LED combination allows to provide chlorophylls *a, b* and carotenoids of photosystems I and II with energy, which has a positive effect on biomass growth [12].

Vacuum filtrationon the vacuum filtering station PVF–35(47)/1 VN (Russia) was used to separate biomass from culture medium.

Methods for defining growth of biomass, ferrum, and content of photosystem pigments in Chlorella vulgaris cells

The change in cell size and morphology has been observed using the microscope TM XSP–139TP (Ulab, China).

Cell concentration and growth were defined using the standard microscopic method and Goryaev's chamber [13] by the formula:

$$X = (a \cdot 4000 \cdot y)/b, \text{ cell/mcl}, \quad (1)$$

where X — quantity of cells defined in suspension, in 1 mm^3; a — total cells in suspension calculated in certain volume of the chamber; b — quantity of counted small squares; y — suspension dilution. The count is carried out with low power of the microscope (lens 8×, eyepiece 10×).

Spectral analysis was applied to determine the biomass growth rate using the spectrophotometer ULAB 102 at wavelength 460 nm.

Specific rate of microalga cell growth μ during the day was calculated according to the standard method [13].

The concentration of chlorophyll *a* and *b* and carotenoids was established with Ricci method [14].Ethanol 96% was used for extraction due to hydrophobic pigment properties. The obtained solution density was determined using the spectrophotometer Ulab 102 at wavelength 664 nm and 649 nm. Chlorophyll *a* and *b* content was calculated by the formulas:

$$Ca = 13.36A664 - 5.19\ A_{649}, \quad (2)$$

$$Cb = 27.43A649 - 8.12\ A_{664}, \quad (3)$$

where Ca — chlorophyll *a* content (mg/cm^3); Cb — chlorophyll *b* content (mg/cm^3); A_{649} – light absorption at 649 nm; A_{664} – light absorption at 664 nm.

Carotenoid content was calculated by the formula:

$$C\ x + c = \\ = (1000A_{470} - 2.13Ca - 97.63Cb)/209, \quad (4)$$

where C x+c — carotenoid content (mg/cm^3); Cb — chlorophyll *b* content (mg/cm^3); Ca — chlorophyll *a* content (mg/cm^3); A_{470} – light absorption at 470 nm.

Chlorella vulgaris cell isolation from culture medium has been carried out by filtering off suspension with the vacuum pump PVF–35(47)/1 VN (Russia). The sediment was dried to an absolute dry weight in the drying oven 2B–151 at temperature105 °C. Biomass content was defined by the formula:

$$C_B = \frac{(m_k - m_n)\cdot 1000}{V}, \text{ g/dm}^3, \quad (5)$$

where, m_k — filter weight with biomass after drying; m_n — filter weight; 1000 — conversion to volume of 1 dm^3; V — aliquot from which it was received.

Residual iron content in culture liquid has been determined by standard method using sulfosalicylic acid [15].

Results and Dicussion

Table 1 illustrates the biomass growth for 7 days of culturing with the use of ferrum ion sulphate and nanostructured citrate in different concentrations as a source and which correspond to the standard Gromov 6 medium, and in concentration that is being optimal according to the published data [4].Culture temperatureis 30±1 °C.

As control medium with low content of $FeSO_4 \cdot 7H_2O$ was used, as it usually is a source of Fe^{2+} ions in most of standard cultural mediums.

It may be stated based on the obtained results that a source of ferrum ions influences

Table 1. ***Chlorella vulgaris*** **biomass growth at culturing with different iron content and sources**

No.	Ferrum ion sources	Fe^{2+} concentration, mg/dm^3	Biomass growth, g/dm^3
Control	$FeSO_4 \cdot 7H_2O$	2± 0.1	0.50± 0.025
1	$FeSO_4 \cdot 7H_2O$	5± 0.25 *	0.60 ± 0.03 *
2	$FeC_6H_5O_7$	2± 0.1	0.72 ± 0.04 *
3	$FeC_6H_5O_7$	5± 0.25 *	1.24 ± 0.06 *

Note. Here and after: $M \pm m$, $n = 5$; * $P < 0.05$ with compared to control.

on *Chlorella vulgaris* development, for example, if the concentration is increased. Ferrum ions in the form of nanostructured complex salt with citrate like ferrum sulphate result in increase of biomass growth rate. The increase of ferric citrate concentration in the medium in 2.5 folds enhances biomass accumulation in 1.7 folds and exceeds biomass growth rate twice if sulphate is used under such conditions.

The ferrum ion medium content has been increased in 2.5, 5 and 8 folds relatively to standard concentration in control sample to define a rational concentration of nanostructured ferric citrate for culturing *Chlorella vulgaris* with a high ferrum ion content and its effect on photosynthesis pigment biosynthesis. The culturing has place at temperature 18 ± 1 °C. Fig. 1. illustrates dynamics of changes of optical microalgae suspension density while cultured depending on nanostructured ferric citrate concentration. Should ferrum ion concentration be increased 8 folds (15 mg/dm^3) vs. Control medium (1.87 mg/dm^3) suspension density will increase 4 folds. Specific growth rate under such conditions makes up 0.137 day-1 and 0.103 day-1, respectively.

It should be mentioned that the elevated ferric citrate concentration impacts of morpho-physiological cell state. If ferrum ions have a low content, the cells will have smaller size (1–2 μm) and form colonies. If ferrum ion concentration is 15 mg/dm^3, the cells will be of intense green colour and have size of 4–5 μm, and they do not form colonies.

Figure 2 illustrates biomass growth depending on the medium nanostructured ferric citrate concentration at temperature 18 ± 1 °C. As it is seen from Fig. 2, if medium ferric citrate concentrations are maintained at the level from 20 to 30 mg/dm^3, the culture biomass growth will be slowed down as for concentration of 15 mg/dm^3.

The efficiency of ferrum absorption by *Chlorella vulgaris* cells for 7culture days depending on nanostructured citrate metal complex concentration in the medium is given in Table 2.

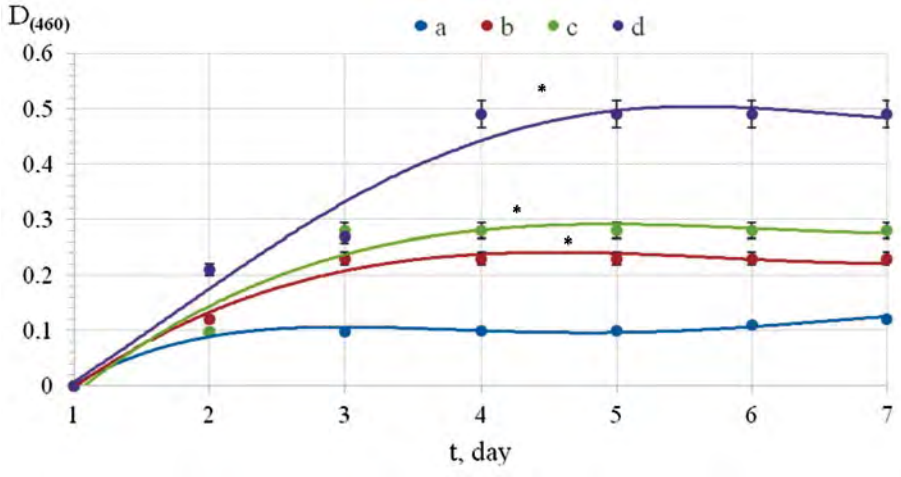

Fig. 1. **Change of optical microalgae suspension density, $D_{(460)}$, when cultured (t) depending on ferric citrate content, mg/dm^3:** *a* (Control sample) — 1.87; *b* — 5; *c* — 10; *d* — 15

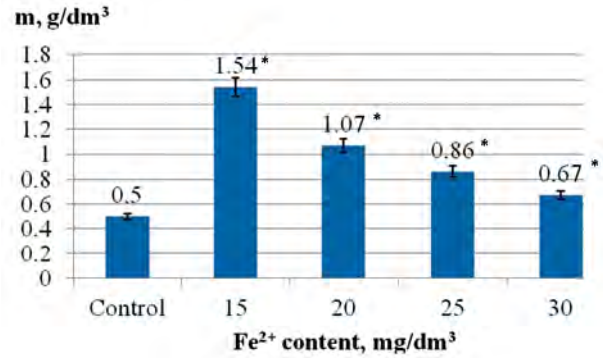

***Fig. 2. Chlorella vulgaris* biomass yield (m) depending on ferrum ion content in culture medium, mg/dm^3**

It may be affirmed proceeding from the above results that the efficiency of ferrum ion biosorption elevates with the increased concentration of nanostructured citrate ferrum complex in nutrient medium, but the biomass growth is reduced. Consequently, ferrum ion adsorption is enhanced almost twice at the content of nanostructured citrate with ferrum ion concentration of 20 mg/dm^3 relatively to ferrum ion content of 15 mg/dm^3, and the biomass growth is decreased by 30.5%.

Fig. 3 demonstrates a specific content of photosynthesis pigments (chlorophylls a and *b*, carotenoids)in *Chlorella vulgaris* cells depending on nanostructured citrate ferrum complex concentration in culture medium.

It is seen on Fig. 3 that chlorophyll and carotenoid contents are elevated with the increased medium iron concentration up to 20–25 mg/dm^3. In comparison to standard medium that is used as control sample under same experiment conditions, with $FeSO_4$ as Fe ions source, content of Chlorophyll *a* was 2.4 mg/g, *b* 0.6 mg/g and Carotenoids 1.1 mg/g, which is lower than when using Fe citrate.

The highest specific chlorophyll content is typical for iron concentration of 20 mg/dm^3 that in equivalent to adsorbed ferrum amount makes up 1.55 mg/mg Fe^{2+}, for concentration of 15 mg/dm^3 — 1.47 mg/mg Fe^{2+}, and 25 mg/dm^3 — 1.23 mg/mg Fe^{2+}.The similar trend is observed for carotenoid content. The content of 20 mg/dm^3, if nanostructured ferric citrate is used, is being a rational concentration for culturing in order to obtain *Chlorella vulgaris* cells with a high content of ferrum ions and chlorophyll.

The effect of high nanostructured ferric citrate contents on Chlorella vulgaris cell metabolism

Unlike the studies [4] stating that the use of chelate complexes of ferrum ions with concentration in the medium more than 5 mg/dm3does not affect the biomass growth, the results obtained for nanostructured ferric citrate demonstrate a dependence of *Chlorella vulgaris* biomass growth of nanostructured ferric citrate concentration. At the same time, the biomass growth has a dome-shaped dependence with maximum growth with ferrum ion content of 15 mg/dm^3. Subsequent elevation of medium ferrum ion content in such a form leads to the reduction of biomass growth. Such dependence might be explained by the reduced flow of other elements to cells, in particular, of mangan and phosphorus, and which affects chlorophyll synthesis and the run of photosynthetic processes of the dark stage.

The deceleration in microalgae biomass growth due to nanostructured ferric citrate content from 20 to 30 mg/dm^3 can be explained by the fact that with the exceeded medium ferrum content above certain threshold, the efficiency of energy conversion via photosynthetic route is lowered and the culture may undergo a toxic effect due to ferrum excess [16].

***Table 2.* Efficiency of *Chlorella vulgaris* ferrum biosorption depending on nanostructured ferric citrate concentration**

№	Ferrum ion source	Starting Fe^{2+} concentration, mg/dm^3	Residual Fe^{2+} concentration, mg/dm^3	Biomass Fe^{2+} content, Fe^{2+} mg/g
Control	$FeSO_4$	2	0.4±0.01	0.2 ±0.01
1	$FeC_6H_5O_7$	1.87	0.34±0.01*	0.42 ±0.02*
2	$FeC_6H_5O_7$	15	0.12±0.01*	8.05±0.4*
3	$FeC_6H_5O_7$	20	0.16±0.01*	15.45±0.7*
4	$FeC_6H_5O_7$	25	1.27±0.06*	22.99±1.1*
5	$FeC_6H_5O_7$	30	1.52±0.07*	35.42 ±1.7*

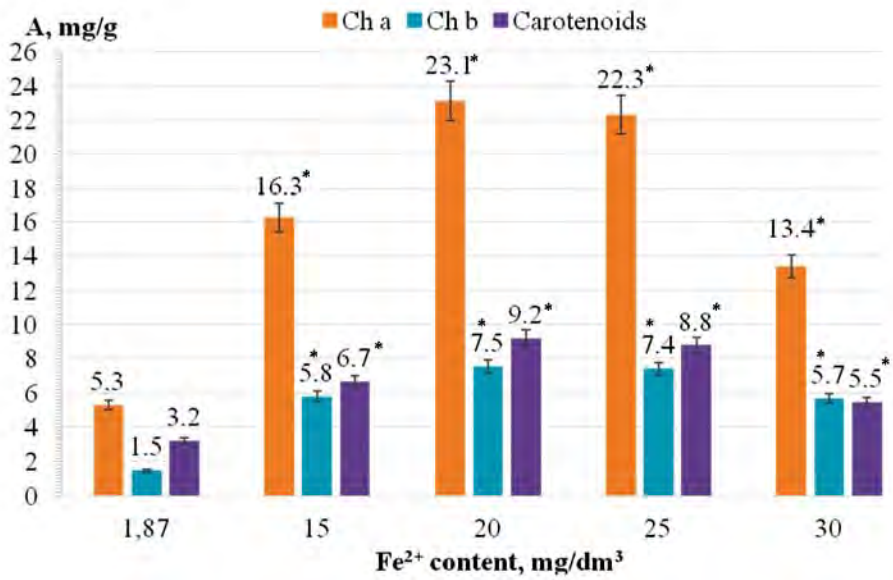

Fig. 3. **Specific content of chlorophyll *a, b* and carotenoids (A, mg/g of algae dry mass) in *Chlorella vulgaris* cells depending on nutrient medium ferrum ion content, mg/dm^3**

Such dependency can be also explained by a lowered flow of other elements to cells, in particular, of mangan and phosphorus, and which affects chlorophyll synthesis and the run of the dark photosynthesis stage processes that lead to the biomass growth reduction.

The flow of ferrum ions to cells may be carried out via two routes [17–19]. First of all, a cell surface may retain metal ions in the form of complex citrate salt due to compounds containing carboxyl, hydroxyl, sulphate, phosphate or amine groups, the latter one — due to bonding of citrate oxygen — amine hydrogen or of ferrum ions — unseparated electron pair of nitrogen [19–22].This process depends on pH [19, 22, 23], algae genus [24, 25], and biomass concentration [22]. However, this process does not depend on cell energy consumption. The part of ferrum ions with high medium concentration remains adsorbed on cell surface. Another path is absorption and accumulation of ferrum ions inside of the cell. This is a slow process, which includes active transport of metal ions via a cell membrane. At the same time, transport of ferrum ions in the form of citrate may be carried out both in the form of ions and complex salt. The process of ion transfer to cell is sensible to low temperatures, inhibitors as well as to the lack of energy source [18].

The fact that the elevation of initial ferrum ion content results in its increase in cell biomass while it growth is lowered confirms that if nanostructured ferric citrate is used, there will be two paths for its utilization that are typical for *Chlorella vulgaris*– sorption and flow to cells. Moreover, the use of nanostructured citrate increases its specific contents. the use of other iron sources — chloride, sulphate and common citrate, where its maximum content reaches 3 mg/g [4]. The use of nanostructured ferric citrate also reduces the biomass growth at concentration of 20 mg/dm^3, which confirms its flow to cells and inhibition of their development. If ethylenediaminetetraacetate complexes with ferrum ions in the form of complex salt are used, the concentration that does not affect cell development will make up 50 mg/dm^3. It means that it will be better to use nanostructured citrate to elevate cell Fe^{2+} content.

Should such salt be used, pH will not change during the growing. In our case, pH varied within 8.1–8.5.Plasm permeability for ferrum ions is enhanced under such conditions [4].

A pigment content dependence of medium ferrum ionconcentration has a maximum at 20 mg/dm^3. The increase in this adsorption level lowers both biomass growth and pigment content, which is explained by the change in membrane permeability for other elements and by the reduction of the flow to cells.

Therefore, ferrum ions in the form of nanostructured citrate complex penetrate easier to a cell and contribute to the enhancement of biomass growth. The elevated concentration of nanostructured ferric citrate heightens cell ferrum ion content and photosynthesis pigment content in several times if compared with the use of other ferrum forms.

Thus, it was demonstrated that introduction of ferrum ions in the form of nanostructured citrate complex to culture medium enhances biomass growth twice at concentration of 5 mg/dm^3, if compared with sulphate. Maximum biomass growth of *Chlorella vulgaris* is typical with ferrum ion concentration of 15 mg/dm^3, which increases biomass growth three times, if compared with standard culture conditions.

It was defined that a high content of medium nanostructured ferric citrate improves the ion adsorption level by cells. Moreover, specific ferrum content in biomass is higher in several times, if compared with the use of other sources of ferrum ions.

It was proved that 20 mg/dm^3 nanostructured citrate content in culture medium is being a rational ferrum ion concentration for enhanced accumulation of ferrum ions in *Chlorella vulgaris* biomass and for photosynthesis pigment biosynthesis.

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ВПЛИВ НАНОСТРУКТУРОВАНОГО ЦИТРАТУ ЗАЛІЗА НА РОЗВИТОК *Chlorella vulgaris*

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Метою роботи було дослідити розвиток культури *Chlorella vulgaris* під час культивування на модифікованому середовищі Громова № 6 за високих концентрацій наноструктурованого цитрату заліза, зокрема вплив останнього на накопичення пігментів фотосинтезу та внутрішньоклітинного заліза у клітинах водорості. Показано, що максимальний вміст заліза у клітинах культури, що дорівнює 15 мг/дм^3 сухої маси, спостерігається за вмісту у середовищі культивування наноструктурованого цитрату заліза в концентрації 30,0 мг/дм^3, хлорофілу *a* — 23,0 мг/г, *b* — 7,5 мг/г та каротиноїдів — 9,2 мг/г сухої маси — за його концентрації 20,0 мг/дм^3. Застосування наноструктурованого цитрату заліза призводить до збільшення приросту біомаси у 3,0 рази порівняно з культивуванням за стандартною технологією. Вміст внутрішньоклітинного заліза істотно зростає у разі використання у середовищі культивування наноструктурованого цитрату заліза, що підвищує якість цих клітин як харчової добавки. Для збільшення виходу біомаси клітин хлорели доцільно застосовувати в середовищі культивування наноструктурований цитрат заліза.

Ключові слова: клітини хлорели, біомаса, внутрішньоклітинне залізо, хлорофіли, каротиноїди, наноструктурований цитрат заліза.

ВЛИЯНИЕ НАНОСТРУКТУРИРОВАННОГО ЦИТРАТА ЖЕЛЕЗА НА РАЗВИТИЕ *Chlorella vulgaris*

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Целью работы было изучить развитие культуры *Chlorella vulgaris* при культивировании на модифицированной среде Громова № 6 при высоких концентрациях наноструктурированного цитрата железа, в частности влияние последнего на накопление пигментов фотосинтеза и внутриклеточного железа в клетках водоросли. Показано, что наибольшее содержание внутриклеточного железа в клетках культуры, равное 15 мг/дм^3, наблюдается при содержании в среде культивирования наноструктурированного цитрата железа в концентрации 30,0 мг/дм^3, хлорофилла *a* — 23,0 мг/г, *b* — 7,5 мг/г и каротиноидов — 9,2 мг/г сухой массы — при его концентрации 20,0 мг/дм^3. Применение наноструктурированного цитрата железа приводит к увеличению прироста биомассы в 3,0 раза по сравнению с культивированием по стандартной технологии. Содержание внутриклеточного железа значительно возрастает при использовании наноструктурированного цитрата железа, что повышает качество этих клеток как пищевой добавки. Для увеличения выхода биомассы клеток хлореллы целесообразно применять в среде культивирования наноструктурированный цитрат железа.

Ключевые слова: клетки хлореллы, биомасса, внутриклеточное железо, хлорофиллы, каротиноиды, наноструктурированный цитрат железа.