ISSN: 2224-5286 (Print)
ISSN: 2518-1491 (Online)

ACADEMIC SCIENTIFIC JOURNAL OF CHEMISTRY

№4
2025

ISSN 2518-1491 (Online),
ISSN 2224-5286 (Print)

CENTRAL ASIAN ACADEMIC
RESEARCH CENTER

ACADEMIC SCIENTIFIC JOURNAL OF CHEMISTRY

4 (465)

October – December 2025

PUBLISHED SINCE JANUARY
1947 PUBLISHED 4 TIMES A YEAR

ALMATY, NAS RK

ACADEMIC SCIENTIFIC JOURNAL OF CHEMISTRY
ISSN 2518-1491 (Online),
ISSN 2224-5286 (Print)
Owner: «Central Asian Academic Research Center» LLP (Almaty).
The certificate of registration of a periodical printed publication in the Committee of information of the Ministry of Information and Social Development of the Republic of Kazakhstan **№ KZ23VPY00121156,**
issued 05.06.2025
Thematic scope: *organic chemistry, inorganic chemistry, catalysis, electrochemistry and corrosion, pharmaceutical chemistry and technology.*
Periodicity: 4 times a year.
http://chemistry-technology.kz/index.php/en/arhiv

Editorial address: JSC «D.V. Sokolsky institute of fuel, catalysis and electrochemistry», 142, Kunayev str., of. 310, Almaty, 050100, tel. 291-62-80, fax 291-57-22, e-mail: orgcat@nursat.kz

«ACADEMIC SCIENTIFIC JOURNAL OF CHEMISTRY»
ISSN 2518-1491 (Online),
ISSN 2224-5286 (Print)
Меншіктенуші: «Орталық Азия академиялық ғылыми орталығы» ЖШС (Алматы қ.).
Қазақстан Республикасының Ақпарат және қоғамдық даму министрлігінің Ақпарат комитетінде 05.06.2025 ж. берілген **№ KZ23VPY00121156** мерзімдік басылым тіркеуіне қойылу туралы куәлік.
Тақырыптық бағыты: *органикалық химия, бейорганикалық химия, катализ, электрохимия және коррозия, фармацевтикалық химия және технологиялар.*
Мерзімділігі: жылына 4 рет.
http://chemistry-technology.kz/index.php/en/arhiv

Редакцияның мекенжайы: 050100, Алматы қ., Қонаев к-сі, 142, «Д.В. Сокольский атындағы отын, катализ және электрохимия институты» АҚ, каб. 310, тел. 291-62-80, факс 291-57-22, e-mail:orgcat@nursat.kz

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«ACADEMIC SCIENTIFIC JOURNAL OF CHEMISTRY».
ISSN 2518-1491 (Online),
ISSN 2224-5286 (Print)

Собственник: Республиканское общественное объединение ТОО «Центрально-азиатский академический научный центр» (г. Алматы).

Свидетельство о постановке на учет периодического печатного издания в Комитете информации Министерства информации и общественного развития Республики Казахстан **№ KZ23VPY00121156,** выданное 05.06.2025 г.

Тематическая направленность: *органическая химия, неорганическая химия, катализ, электрохимия и коррозия, фармацевтическая химия и технологии.*

Периодичность: 4 раз в год.

http://chemistry-technology.kz/index.php/en/arhiv

Адрес редакции: 050100, г. Алматы, ул. Кунаева, 142, АО «Институт топлива, катализа и электрохимии им. Д.В. Сокольского», каб. 310, тел. 291-62-80, факс 291-57-22, e-mail:orgcat@nursat.kz

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ACADEMIC SCIENTIFIC JOURNAL OF CHEMISTRY
ISSN 2224–5286
Volume 4.
Number 465 (2025), 197–211

https://doi.org/10.32014/2025.2518-1491.331

IRSTI 31.27.19
UDC 636.085.16:582.263:66.0

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EFFICIENCY OF MODIFIED MEDIUM FOR PIGMENT AND ANTIOXIDANT BIOSYNTHESIS IN *CHLORELLA VULGARIS*

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Abstract. The relevance of this study lies in developing a method to increase biologically active substances (BAS) in *Chlorella vulgaris* biomass by chemically modifying the Bogdanov medium. To enhance the growth and metabolic characteristics of Chlorella vulgaris, the availability of nitrogen, iron and other trace elements that regulate photosynthetic activity and biosynthesis of BAS is necessary. This article examines the effect of adding urea and iron nanoparticles on the increase in the cell concentration of *Chlorella vulgaris* and the content of pigments and antioxidant substances. The study applies spectrophotometric methods to measure optical density and chlorophylls, carotenoids, flavonoids, and polyphenolic compounds. The results of the study show that the use of the modified medium resulted in a 22% increase in the biomass and optical density of the suspension. Chlorophylls *a* and *b* levels increased to 11.1 and 5.2 mg/mL, respectively, indicating improved photosynthetic activity resulting from the integration of nitrogen and iron into metabolic pathways. Carotenoid concentrations were 3.3 mg/mL, while flavonoids and polyphenols concentrations - 0.2%, indicating enhanced cellular antioxidant activity. The conducted studies demonstrate the effectiveness of chemical modification of Bogdanov's medium for stimulating the biosynthesis of pigments and antioxidant compounds in *Chlorella vulgaris*. The practical significance of this study lies in the potential use of Chlorella vulgaris for the development of effective feed additives and biostimulants that enhance animal productivity, soil fertility, and seed germination.

Keywords: *Chlorella vulgaris,* chemical modification, Bogdanov nutrient medium, chlorophylls, carotenoids, flavonoids, polyphenols

Funding: *This research was funded by the Science Committee of the Ministry of Science and Higher Education of the Republic of Kazakhstan (Grant No.* AP*23488216).*

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CHLORELLA VULGARIS ҚҰРАМЫНДАҒЫ ПИГМЕНТТЕР МЕН АНТИОКСИДАНТТАРДЫҢ БИОСИНТЕЗІ ҮШІН МОДИФИКАЦИЯЛАНҒАН ҚОРЕКТІК ОРТАНЫҢ ТИІМДІЛІГІ

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Аннотация. Бұл зерттеудің өзектілігі хлорелла үшін қолданылатын Богданов өсіру ортасын химиялық модификациялау арқылы *Chlorella vulgaris* биомассасындағы биологиялық белсенді заттардың (ББЗ) мөлшерін мақсатты түрде арттыру әдісін әзірлеумен байланысты. *Chlorella vulgaris*-тің өсуі мен метаболитикалық сипаттамаларын жақсарту үшін фотосинтетикалық белсенділікті және ББЗ биосинтезін реттейтін азот, темір және басқа да микроэлементтердің болуы қажет. Бұл мақалада несепнәр мен темір нанобөлшектерін өсіру ортасына қосудың *Chlorella vulgaris* микробалдырларының жасушалық концентрациясының артуына және пигменттер мен антиоксиданттық заттардың мөлшеріне әсері қарастырылады. Зерттеуде микробалдырдың оптикалық тығыздығы мен хлорофиллдердің, каротиноидтардың, флавоноидтардың және полифенолды қосылыстардың құрамын бағалау үшін спектрофотометриялық талдау әдісі қолданылады. Зерттеу нәтижелері модификацияланған ортаны пайдалану биомасса мен суспензияның оптикалық тығыздығының 22%-ға артуына әкелгенін көрсетеді. Хлорофилл a және *b* деңгейлері сәйкесінше 11,1 және 5,2 мг/мл дейін өсті, бұл азот пен темірдің жасушалық метаболизмге енуіне байланысты фотосинтездің күшеюін көрсетеді. Каротиноидтардың концентрациясы 3,3 мг/мл тең болды, ал флавоноидтар мен полифенолдардың концентрациясы 0,2%-ті құрады, бұл жасушалардың антиоксиданттық белсенділігінің артқанын көрсетеді, бұл қарқынды фотосинтез кезінде микробалдырды тотығу стрессінен тиімдірек қорғауды қамтамасыз етеді. Жүргізілген зерттеулер *Chlorella vulgaris*-тегі пигменттер мен антиоксидантты қосылыстардың биосинтезін ынталандыру үшін Богданов өсіру ортасын химиялық модификациялаудың тиімділігін көрсетеді және бұл тәсілді құнды метаболиттердің жоғары деңгейімен биомасса алу үшін перспективалы етеді. Бұл зерттеудің практикалық маңыздылығы жануарлардың өнімділігін, топырақтың құнарлығын және тұқымның өнуін жақсартатын тиімді жем қоспалары мен биостимуляторларды әзірлеу үшін *Chlorella vulgaris*-ті пайдалану мүмкіндігінде жатыр.

Түйін сөздер**:** *Chlorella vulgaris,* химиялық модификация, Богданов өсіру ортасы, хлорофилдер, каротиноидтар, флавоноидтар, полифенолдар

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ЭФФЕКТИВНОСТЬ МОДИФИЦИРОВАННОЙ СРЕДЫ ДЛЯ БИОСИНТЕЗА ПИГМЕНТОВ И АНТИОКСИДАНТОВ В CHLORELLA VULGARIS

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Аннотация. Актуальность исследования связана с разработкой способа целенаправленного увеличения содержания биологически активных веществ (БАВ) в биомассе Chlorella vulgaris путем химической модификации питательной среды Богданова для культивирования хлореллы. Для усиления ростовых и метаболических характеристик Chlorella vulgaris необходимы доступность азота, железа и микроэлементов, регулирующих фотосинтетическую активность и биосинтез БАВ. В статье рассматривается влияние добавления мочевины и наночастиц железа на увеличение концентрации клеток микроводоросли Chlorella vulgaris, а также содержания пигментов и антиоксидантных веществ. В работе используется спектрофотометрический метод анализа, позволяющий оценивать оптическую плотность культуры, содержание хлорофиллов, каротиноидов, флавоноидов и полифенольных соединений. Результаты показывают, что применение модифицированной среды привело к увеличению биомассы и оптической плотности суспензии на 22 %. Содержание хлорофиллов a и b увеличилось до 11,1 и 5,2 мг/мл соответственно, отражая усиление фотосинтеза за счет включения азота и железа в метаболизм клетки. Концентрации каротиноидов составили 3,3 мг/мл, а флавоноидов и полифенолов — 0,2 %, что указывает на увеличение антиоксидантной активности и более эффективную защиту клеток от окислительного стресса при интенсивном фотосинтезе. Проведенные исследования демонстрируют эффективность химической модификации среды Богданова для стимуляции биосинтеза пигментов и антиоксидантов в Chlorella vulgaris, что делает данный подход перспективным для получения биомассы с повышенным содержанием ценных метаболитов. Практическая значимость работы состоит в возможности использования Chlorella vulgaris при создании эффективных кормовых добавок и биостимуляторов, повышающих продуктивность животных, плодородие почв и всхожесть семян.

Ключевые слова: *Chlorella vulgaris,* химическая модификация, питательная среда Богданова, хлорофиллы, каротиноиды, флавоноиды, полифенолы

Introduction. The rapid development of biochemical research into natural compounds is focusing attention on the search for effective sources of biologically active substances (BAS). The single-celled microalga *Chlorella vulgaris* is characterized by rapid growth, high adaptability to environmental changes, and the ability to synthesize a wide range of BAS (Kelebek et al., 2025; Rinawati et al., 2020).

The biosynthesis of BAS is a complex of interconnected biochemical reactions involving primary photosynthetic metabolism and secondary metabolism, which produces pigments, phenolic compounds, and antioxidants. During the first stage of photosynthesis, carbohydrates are synthesized in *Chlorella vulgaris* cells. These serve as the primary source of the carbon skeleton for the subsequent formation of BAS through the assimilation of nitrogen, phosphorus, and sulfur. Secondary metabolites are formed from the products of primary metabolism through sequential chemical reactions - condensation, oxidation, reduction, and cyclization. These chemical transformations result in the formation of chlorophylls, carotenoids, flavonoids, and phenolic compounds, which perform protective and antioxidant functions, preventing photo oxidative damage to cellular structures (Zúñiga et al., 2016). According to research (Daliry et al., 2017; El-fayoumy et al., 2020), the chemical composition of microalgae depends on cultivation conditions, which regulate the biochemical metabolic reactions in *Chlorella vulgaris* cells. The most important parameters influencing the biosynthetic activity of microalgae are the concentration of mineral salts in the nutrient medium, illumination, temperature, and pH of the medium. Changing the physical parameters and the content of macro- and microelements allows for targeted control of metabolism and stimulation of the synthesis of individual groups of BAS. For example, nitrogen starvation promotes the accumulation of not only lipids, but also carotenoids and phenolic compounds, which is due to an adaptive and protective response to metabolic stress. At the same time, the addition of Mg^{2+}, Fe^{2+}, and Fe^{3+} salts activate an increase in chlorophylls content (Fang, et al., 2024; Cichonski, 2022).

One of the accessible and rapid methods for assessing the intensity of biosynthetic processes in *Chlorella vulgaris* cells based on the content of pigments and phenolic compounds is spectrophotometric analysis (Rinawati et al., 2020). Based on a literature review, secondary metabolites characteristics were compiled for conducting spectral analysis (Table 1).

Table 1. Characteristics of the main biologically active substances of microalgae *Chlorella vulgaris*

Biologically active substance	Functions	Maximum absorption spectra, nm	Extraction agent	Sources
Chlorophyll *a*	Photosynthesis, photoprotection, antioxidant activity	662	acetone	Rinawati et al., 2020
Chlorophyll *b*		645	acetone	Rinawati et al., 2020
Carotenoids		470	acetone	Rinawati 2020
Flavonoids	Antimicrobial and antioxidant activity, anti-inflammatory effect	420	ethanol	Mitishev et al., 2023
Polyphenols		745	methanol	Fomenko et al., 2023

Chlorophyll’s photosynthetic activity is explained by its chemical structure, which consists of a porphyrin ring with a magnesium atom and a hydrophobic side chain. Conjugated double bonds in the porphyrin ring absorb photons in the red region of the spectrum, enabling electron transport. During photochemical degradation, the electronic structure of the porphyrin complex is disrupted by the removal of magnesium from the molecule, resulting in the formation of brown pheophytin (Figure 1). This leads to a change in the absorption spectrum and a loss of the characteristic green color:

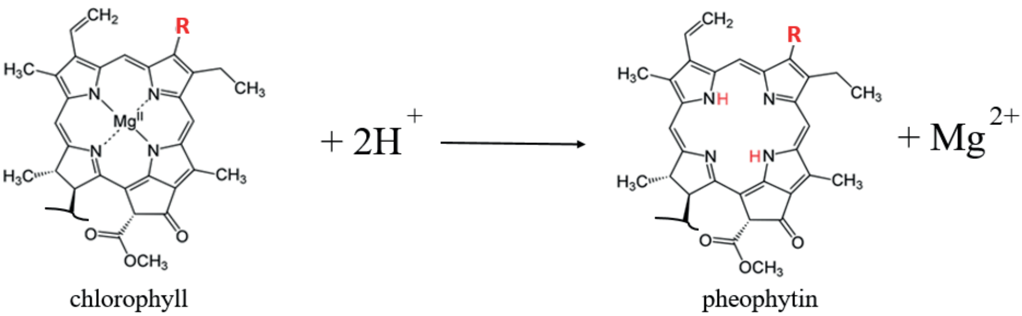

Figure 1 – Conversion of chlorophylls to pheophytin as a result of protonation and removal of Mg^{2+} from the porphyrin ring (R for chlorophyll *a* is $-CH_3$, chlorophyll *b* is -CHO)

In their study Nilova and co-authors (Nilova et al., 2021) attribute the photosynthetic and antioxidant activity of carotenoids to the presence of a long chain of conjugated double bonds. This structure allows the molecule to efficiently absorb light in the blue region of the spectrum, transfer energy to chlorophylls, and quench excited states of chlorophylls, preventing the formation of reactive oxygen species. Conjugated bonds also provide carotenoids with a high ability to neutralize free radicals. During photochemical degradation, cleavage of double bonds leads to a change in the absorption spectrum and a decrease in the antioxidant activity of carotenoids, accompanied by a loss of their characteristic yellow-orange color.

As noted by Fomenko and co-authors (Fomenko et al., 2023), the antioxidant properties of phenolic compounds (polyphenols and flavonoids) are due to the presence of aromatic rings with hydroxyl groups capable of donor-acceptor reactions. Due to their ability to donate hydrogen atoms, transfer electrons, and chelate transition metal ions, polyphenols suppress free-radical oxidation chain reactions. Light-induced degradation leads to the formation of quinones, simple phenols, and low-molecular-weight organic acids, accompanied by a decrease in color intensity and antioxidant activity. This also weakens the absorption spectrum in the UV region and leads to a loss of biological activity. Despite differences in structure, all of the listed secondary metabolites are susceptible to photo degradation when exposed to light, accompanied by disruption of their π-systems, changes in absorption spectra, and a decrease in biological activity. This must be taken into account during their isolation and spectrophotometric analysis of *Chlorella vulgaris*. Therefore, to preserve the chemical and physiological properties of the biologically active substances and obtain accurate spectrophotometric data, extraction is performed in the dark or under dim, diffused light.

According to patents (Grabarnik et al., 2018; Bogdanov, 2018), modifying N.I. Bogdanov's nutrient medium with microelements and organic matter optimizes the supply of mineral ions to enhance the photosynthetic and metabolic activity of cells. Despite the presence of a significant number of studies (Wong et al., 2017; El-fayoumy et al., 2020) devoted to the study of individual groups of BAS in microalgae, a comprehensive analysis of the influence of the mineral composition of Bogdanov's medium on the biosynthesis of pigments and phenolic compounds remains insufficiently studied. Therefore, analytical monitoring of BAS synthesis in *Chlorella vulgaris* biomass cultivated on a chemically modified nutrient medium is becoming increasingly important.

The aim of the study was to evaluate the influence of the composition of the control and modified Bogdanov nutrient media on the accumulation of BAS (chlorophylls, carotenoids, polyphenols and flavonoids) in *Chlorella vulgaris* cells using spectrophotometric analysis.

Materials and methods. The object of the study was a commercial strain of *Chlorella vulgaris* IFR C-111 (Figure 2), acquired from the laboratory of the "Scientific and Technological Water Center" LLP (Petropavlovsk, Republic of Kazakhstan). Micrographs of the cells were obtained using an optical microscope. (Micros, Austria).

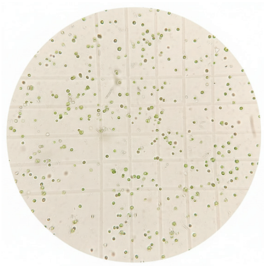

Figure 2 – Microscopic image of *Chlorella vulgaris* IFR C-111 cells

The cultivation procedure was carried out in laboratory conditions in a light-tight grow box (Figure 3), which includes: 1) high-pressure sodium lamp (HPS); 2) air circulation fan; 3) exhaust fan; 4) ventilation holes; 5) thermostat; 6) timer; 7) conical flasks with microalgae suspension; 8) gas outlet tubes; 9) laboratory shaker; 10) carbon dioxide cylinder.

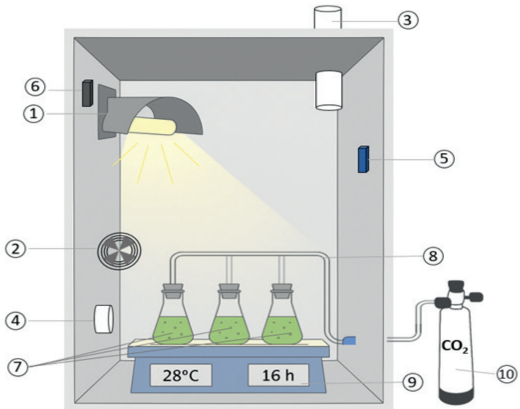

Figure 3 – Light-tight grow box for growing microalgae

Chlorella vulgaris was grown in 250 mL flasks, each with a working volume of 160 mL of medium and 40 mL of inoculum. The photoperiod was 16 hours of light and 8 hours of darkness. The temperature of the culture medium was maintained at 28^0C±2 ^{0}C, and the pH was adjusted by bubbling an air mixture containing 1.5% carbon dioxide until a value of 7.0±0.2 was reached. The cultivation duration was 14 days. The microalgae suspension was continuously stirred on a shaker to prevent settling of the microalgae cells. Bogdanov's nutrient medium was used as a control. A modified medium was prepared by adding urea (3 mg/L) and iron nanoparticles stabilized with chitosan. The synthesis of iron nanoparticles is described in our work (Orazzhanova et al., 2024).

Microalgae cell growth was assessed spectrophotometrically (UV/VIS, Specord 210 plus, Germany) at 683 nm, and cell counts were performed daily in a Goryaev chamber for 14 days.

After cultivation, the biomass was separated by centrifugation at 3000 rpm, washed twice with deionized water, and decanted. The *Chlorella vulgaris* paste was then freeze-dried (Scientz-12) at -59.1^{0}C until dry biomass was obtained (Figure 4).

Figure 4 – Biomass of *Chlorella vulgaris* before (left) and after freeze-drying (right)

The main BAS (chlorophylls, carotenoids, flavonoids, and polyphenols) were isolated from the dry biomass of *Chlorella vulgaris* using sequential extraction with various solvents (Table 2). Sample preparation included homogenization in a porcelain mortar to a powder state and extraction under various conditions depending on the chemical nature of the compounds (Figure 5). The extracts were incubated for 30 min at room temperature, and the liquid phase was separated by centrifugation at 3000 rpm for 10 minutes. The precipitate was re-treated with a fresh portion of solvent, the extracts were combined, and filtered before spectrophotometric analysis (Butova et al., 2018).

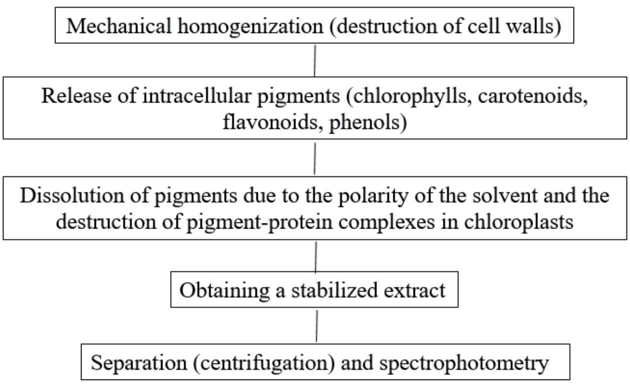

Figure 5 – Flow chart of biologically active substances extraction with organic solvents

To isolate lipophilic pigments, 90% acetone was used, ensuring efficient dissolution of chlorophylls *a* and *b*, and carotenoids. Spectrophotometric measurements were performed at wavelengths of 662 nm (chlorophyll *a*), 645 nm (chlorophyll *b*), and 470 nm for carotenoids (Wong et al., 2017) using formulas (1-4).

$$\text{chlorophyll } a\ (C_a) = 11.24\ A_{662} - 2.04\ A_{645} \quad (1)$$

$$\text{chlorophyll } b\ (C_b) = 20.13\ A_{645} - 4.19\ A_{662} \quad (2)$$

$$\text{total chlorophylls} = \text{chlorophyll } a + \text{chlorophyll } b \quad (3)$$

$$\text{total carotenoids} = \frac{1000\ A_{470} - 1.90\ C_a - 63.14\ C_b}{214} \quad (4)$$

Flavonoids extraction was performed with 60% ethanol, as flavonoid compounds exhibit moderate polarity and readily dissolve in aqueous alcohol. Quantitative determination of flavonoids was performed using a complexation reaction with $AlCl_3$. 1 mL of 2% $AlCl_3$ solution was added to 1 mL of the ethanol extract, the mixture was incubated for 30 min at room temperature, and the optical density was measured in the range 390-420 nm relative to the control. Complex formation between flavonoids and aluminum ions proceeds according to the following scheme:

$$Flav - OH + Al^{3+} \rightarrow [Flav - O - Al]^{2+} + H^+$$

The content of total flavonoids, recalculated for rutin and absolutely dry matter (X, %), was determined using the formula 5 (Mitishev et al., 2023):

$$X = \frac{A \times m_0 \times 100 \times 50 \times 2 \times 25 \times 100}{A_0 \times m \times 2 \times 50 \times 25 \times (100 - W)} \quad (5)$$

Polyphenolic compounds were isolated using 70% methanol, which ensures a high degree of phenolic group extraction due to its high polarity. Polyphenols content was determined using the Folin-Ciocalteu method in accordance with GOST R ISO 14502-1-2010. The 0.5 mL of methanol extract, 2.5 mL of Folin-Ciocalteu reagent, and 2 mL of 7.5% Na_2CO_3 solution were added to the reaction mixture. The mixture was incubated for 30 min at 25 ^{0}C in the dark, after which the optical density was measured at 765 nm. The concentration of phenolic compounds was calculated using a gallic acid calibration curve.

All experiments were performed in biological and analytical triplicate. We used the descriptive statistics method, the results are presented as the mean ± standard deviation.

Results. Cultivation of *Chlorella vulgaris* in Bogdanov's nutrient medium (control) and its modified form reveals differences in culture growth intensity depending on the composition of the nutrient medium. Enhanced growth was observed with the modified nutrient medium compared to the control. Analysis of optical density dynamics (683 nm) in the control medium revealed a slow increase from 0.653 to 1.389 by day 14. Meanwhile, the optical density of the *Chlorella vulgaris* suspension reached 1.691 on a medium modified with urea and iron nanoparticles. Differences in culture growth rates depending on the composition of the nutrient medium were also observed in the dynamics of *Chlorella vulgaris* cell counts. In the control Bogdanov's medium, the increase in cell concentration was gradual and remained the lowest among the studied variants throughout the entire cultivation period (1.16×10^7 cells/mL). The addition of urea and iron nanoparticles resulted in enhanced growth, cells entered the exponential phase earlier, and maximum concentrations reached 1.4×10^7 cells/mL, indicating improved nitrogen availability and intensified cell division processes (Figure 6).

This growth dynamic is consistent with literature and our data indicating the ability of iron nanoparticles to increase the bioavailability of micronutrients and enhance the metabolic and photosynthetic activity of microalgae (Plutakhin et al., 2017; Orazzhanova et al., 2024).

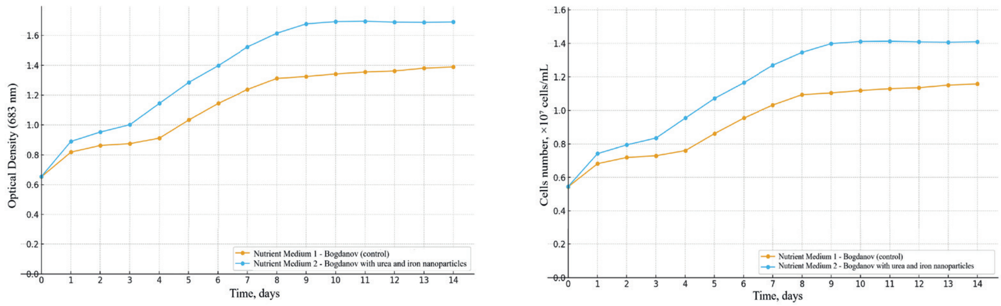

Figure 6 – The effect of chemical modification of Bogdanov's nutrient medium on the growth dynamics of *Chlorella vulgaris* cells

The obtained results of spectrophotometric analysis of BAS showed that modification of Bogdanov medium leads to an increase in the content of all BAS in *Chlorella vulgaris* cells (Figures 7-9). In the control medium, the concentrations of chlorophylls *a* and *b* were 29.3 and 25.6 mg/mL, respectively, while the addition of urea and iron nanoparticles increased the amount of chlorophyll *a* to 31.7 mg/mL, chlorophyll *b* to 44.8 mg/mL, and total chlorophylls reached 76.5 mg/mL. The content of carotenoids increased from 1.6 mg/mL (control) to 3.4 mg/mL upon the introduction of urea with iron nanoparticles. The concentration of flavonoids in the control medium was 0.059%, while in the modified medium it increased to 0.073%, which corresponds to an increase of 23.7%. Similar changes were noted for polyphenols, with their content increasing from 0.11% in the control to 0.20% in the modified medium. When using a medium containing urea and iron nanoparticles, the concentration of polyphenols increased by 81.8%.

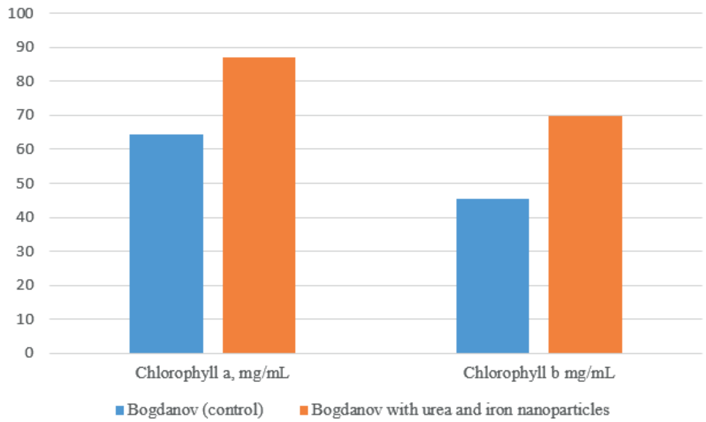

Figure 7 – Chlorophylls content (*a, b*) in *Chlorella vulgaris* cultivated in control and modified media

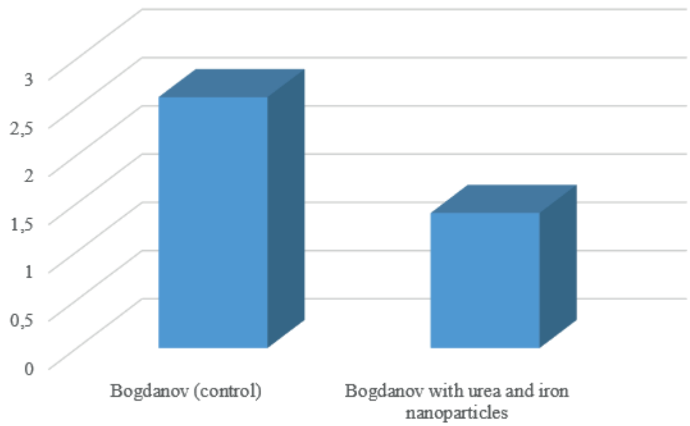

Figure 8 – Carotenoids content in *Chlorella vulgaris* cultivated in control and modified media

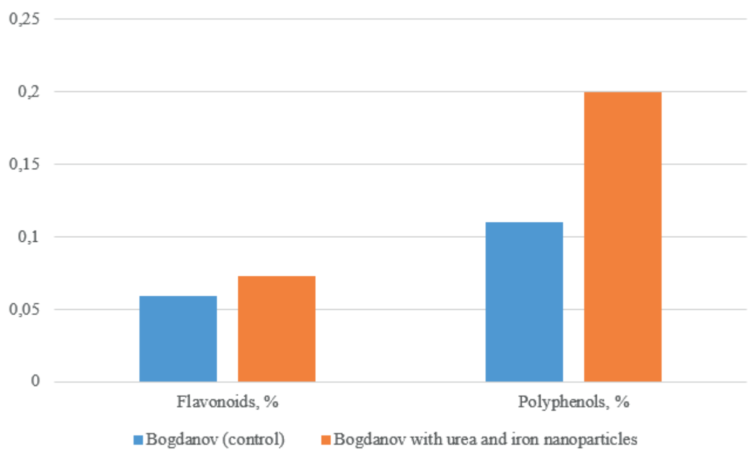

Figure 9 – Content of flavonoids and carotenoids in *Chlorella vulgaris* cultivated in control and modified media

Discussions. The addition of urea and iron nanoparticles to Bogdanov's nutrient medium provides nitrogen and iron nutrition, enhancing the biosynthesis of secondary metabolites (chlorophylls, carotenoids, flavonoids, and polyphenols). According to research (Kim et al., 2016; Yaakob et al., 2021), nitrogen forms in the form of nitrate and urea accelerate microalgae growth, as the end product NH_4^+ is formed during metabolism during the reduction of nitrate and urea. However, these nitrogen sources are incorporated into microalgae metabolism differently. For example, in the biochemical process, nitrates are first reduced to nitrites by iron-containing enzymes, and then nitrites are reduced to ammonium ions:

$$NO_3^- + 2e^- + 2H^+ \rightarrow NO_2^- + H_2O$$

$$NO_2^- + 6e^- + 8H^+ \rightarrow NH_4^+ + 2H_2O$$

Both reactions require large amounts of NADPH and ATP, as well as the active participation of iron-containing enzymes. The presence of iron nanoparticles in the nutrient medium increases the availability of trace elements necessary for the functioning of iron-containing enzymes in the redox reactions of phototrophic metabolism in microalgae. Therefore, when culturing with a nitrate nitrogen source, biomass growth and the synthesis of metabolites (pigments and antioxidant compounds) are slower due to the energy expenditure on nitrate reduction (Moore et al., 2025).

Unlike nitrates, urea is incorporated into nitrogen metabolism more quickly and without the need for additional energy expenditure, as it is directly hydrolyzed to ammonium ions by the enzyme urease:

$$CO(NH_2)_2 + 3H_2O \rightarrow 2NH_4^+ + HCO_3^- + OH^-$$

The resulting ammonium ion is involved in the synthesis of protein amino acids, which are main precursors for the synthesis of secondary metabolites. Urea, as a source of readily available nitrogen, enhances this biosynthesis, thereby increasing photosynthetic and antioxidant activity (Kim et al., 2016).

The results of our study show that chemical modification of Bogdanov's nutrient medium by adding urea and iron nanoparticles leads to increased metabolic efficiency in *Chlorella vulgaris*. The increased content of chlorophylls *a* and *b* in the modified medium reflects the activation of the enzymatic formation of porphyrin rings. The simultaneous increase in the concentration of carotenoids, flavonoids, and polyphenols enhances photo protective mechanisms, which may be a compensatory response to the increased intensity of photochemical processes.

Conclusion. The modified Bogdanov medium supplemented with urea and iron nanoparticles promotes the bioproductivity of *Chlorella vulgaris* and increases the biosynthesis of pigments and antioxidants, resulting in changes in the cellular biochemical composition. Cultivation in the modified medium promoted microalgal growth during the exponential phase, accompanied by a 22% increase in culture optical density relative to the control. The concentrations of chlorophylls *a* and *b* increased from 8.3 to 11.1 mg/mL and from 3.9 to 5.2 mg/mL, respectively, reflecting enhanced photosynthetic performance. The concentration of carotenoids increased from 2.1 mg/mL (control) to 3.3 mg/mL, which is associated with enhanced photoprotective functions. An increase was also observed the changes in antioxidant compounds were also observed: flavonoids content increased from 0.059% (control) to 0.073%, and polyphenolic compounds from 0.11% (control) to 0.2%, indicating an increased ability of cells to neutralize free radicals. Flavonoids and polyphenols content increased by 23.7% and 81.8%, respectively, ensuring the antioxidant activity of the biomass.

Thus, the obtained *Chlorella vulgaris* biomass with an increased content of biologically active substances (pigments, polyphenols and antioxidants) can be considered as a promising feed additive and biofertilizer for increasing the productivity of animals and birds, soil fertility and the viability of agricultural crop seeds.

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ISSN 2518-1491 (Online), ISSN 2224-5286 (Print)

Ответственный редактор *А. Ботанқызы*
Редакторы: *Д.С. Аленов, Т. Апендиев*
Верстка на компьютере *Г.Д. Жадырановой*

Подписано в печать 23.12.2025.
Формат $60x88^{1}/_{8}$. Бумага офсетная. Печать – ризограф.
13,5 п.л. Заказ 4.

«Central Asian Academic Research Center» LLP
Алматы, Қонаев к-сі, 142