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OPTIMIZATION OF *Chlorella vulgaris* Beij. CULTIVATION IN A BIOREACTOR OF CONTINUOUS ACTION

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The aim of the research was the development and testing of bioreactor for intensive cultivation of algae *Chlorella vulgaris* Beij. with fully controlled conditions within the operating parameters according to the selected evaluation criteria of the cultivation process. To check the functional efficiency of the designed photobioreactor the growth of *Chlorella vulgaris* Beij. (*Chlorophyta*) in Fitzgerald's medium modified by Zender and Gorham № 11 under the artificial illumination with daylight electric lamps (intensity of 2 500 Lx) for 16 hours a day at 22–25 °C was studed. It was found that at stabilization of culture conditions the maximum value of culture density was observed at the 18th day of cultivation. At this moment, the amount of cells reached 269.2 ± 3.0·10^9 cells/l, while cells amount in stationary phase was within 110.1 ± 4.9·10^9 cells/l. This made possible the continuous chlorella cultivation with an average productivity in stationary mode of about 110 ± 4 mg/l of dry mass with protein content about 60 mg, carbohydrates about 35 mg and lipids about 12 mg of dry mass /l. Sunlight and activators of biosynthesis of organic substances of individual classes allow to change the ratio of proteins, carbohydrates and lipids that is prospective for further research.

Key words: chlorella, photobioreactor, continuous cultivation.

In today's market of raw biomaterials, there is the need to obtain relatively inexpensive and high quality biomass of photosynthetic microalgae, including chlorella, spirulina and dunaliella containing a number of organic compounds used in food, pharmaceutical, cosmetic industry as substitutes for synthetic preservatives, and for biofuel production that is on the front burner [1, 2]. Recently, microalgae are widely used as new sources of bioactive compounds in the prevention and treatment of disease through antioxidant, antimicrobial, antiviral, anticancer, anti-inflammatory and anti-allergic activity [3]. In this sense, *Chlorella vulgaris* is traditionally valuable, which includes components necessary for normal metabolism of an animal body, which allows the use of algae as a highly effective complex bioadditive [4]. Chlorella metabolites exhibit antitoxic [5] and anti-sclerotic effects [6]. We have shown that by including in its composition of exogenous trace elements, this algae can form biologically active complexes with potentially pharmacological action [7, 8].

Methods of microalgae cultivation include their adequate illumination, providing carbon dioxide and other nutrients [9, 10]. Since carbon dioxide is the main and sometimes the only source of carbon, chlorella can intensely develop only at its sufficient quantity. Small industrial machinery and laboratory cultivators usually use bottled carbon dioxide, which is in a mixture with air at 2–5% of carbonic acid content or in pure form. Necessary condition of cultivation is also maintaining the temperature and pH value of the culture medium. Depending on the temperature optimum, chlorella strains are divided into thermophilic (35–37 °C), mesophilic (25–27 °C) and cryophilic (10–15 °C). The pH value during cultivation should be maintained in the range of 5.5–6.5.

Currently, the number of cultivators is designed for intensive cultivation of microalgae considering biological features

of cultures [11–14]. The most perfect of these cultivation methods is flow-through cultivation of algae at which cells yield taking away, adding of fresh culture medium and stabilization of culture optical density are performed automatically. The main advantage of this method is the possibility to perform long-term continuous cultivation of algae maintaining constant suspension density at optimum value when there is maximum productivity of culture. The disadvantages of standard technology are red-ox potential increasing during cell division of chlorella to positive values, leading to slower growth processes of chlorella and its maximum density of 3.5 g/l, and the need in expensive foreign equipment.

The aim of work was the development and testing of bioreactor for algae intensive cultivation with fully controlled conditions within the operating parameters according to the selected evaluation criteria of chlorella cultivation.

Materials and Methods

The object of the study was algologically pure culture of green algae *Chlorella vulgaris* Beij. from the collections of the Institute of Hydrobiology of the NAS of Ukraine. It was cultured in Fitzgerald medium in Zehnder and Gorham No11 modification which, according to the protocol, contained among other cations 0.058 mg/dm^3 of Mn^{2+} and 0.023 mg/dm^3 of Zn^{2+}, which are chlorella growth activators without ions of copper, lead and other non-essential metals, at 22–25 °C and illumination with daylight electric lamps (intensity of 2 500 Lx) for 16 hours a day [15]. Carbon dioxide supply was ensured automatically with bottled CO_2 of a purity of 99.5% (Ukrainian State Standard 4817: 2007, Sort 1). Indicators of ambient temperature, pH, content of O_2 and CO_2 were controlled automatically with the electrodes embedded in cultivator corpus (Fig. 1). CO_2 consumption by algae cells was calculated by the difference between its income and content in the culture medium. Algae biomass sampling was performed weekly in the course of experimental cultivation. The number of cells was estimated with Goryaev chamber and biomass was determined by stereometric method [16]. Chlorella cell homogenates were obtained by rubbing with pieces of quartz glass. Proteins were precipitated with 10% trichloroacetic acid solution and centrifuged at 2 500 rev/min for 20 min. Protein content was determined according to [17]. Carbohydrates were separated with 75% ethanol solution, and then centrifuged, washed twice, again precipitated by centrifugation [18] and dried. Lipids were extracted with chloroform-methanol mixture at a ratio of 2:1 by the Folch method [19], adding to one mass fraction of 20 parts of extraction mixture for 12 hours, and determined by weighing method after extraction mixture stripping. Statistical processing of data was performed by Student t-test using the program Statistica 5.0.

In experimental studies the reagents of Sigma, Reanal and Chimreactive companies (qualification “pure for analysis p.f.a.”), carbon dioxide of Ukrainian State Standard 4817: 2007, Sort 1 (JSC “Lviv Chemical Plant”) were used.

Results and Discussion

For chlorella continuous cultivation, the photobioreactor of flow-through type is developed (Fig. 1).

Technical characteristics of the reactor: the total volume is 75 liters (height 1 000 mm, width 500 mm and depth 150 mm); working volume — 65 liters; the possibility of artificial up-lighting by four channels: white 1, red, blue, white 2; maximal source power on one channel — 10 A at 250 V; it is established the up-lighting power as 0.8 A at 12 V on one channel. It is possible to mix culture automatically depending on algorithm prescribed conditions using electric engine of 10 W power; to measure the pH within 0–14 with accuracy of ± 0.1 pH (at 25 °C) in temperature range of 0–60 °C, measurement speed is up to 30 seconds; to maintain automatically the pH using electromagnetic valve within the prescribed limits of pH (6.0–6.5) with inertia of ± 0.1 pH, minimal operation time of valve is 0.5 seconds; to measure automatically the temperature in the range of −10 °C to +85 °C with an accuracy of ± 0.1 °C; to maintain automatically the temperature at ambient temperature of 5–40 °C by preset algorithm within 22–25 °C with inertia of ± 0.2 °C; to measure automatically the intensity of light; to connect cylinders with carbon dioxide with pressure of up to 150 atm; to pump the exhaust gases with pressure of 200 mbar in a range of speeds of 200–400 l/min. Real-time clock is embedded; PC connection is via the USB interface to 3 m and 10/100 Base-t interface to 100 m; there is possibility to store measurement data and logs of reactor operation every second in magazines

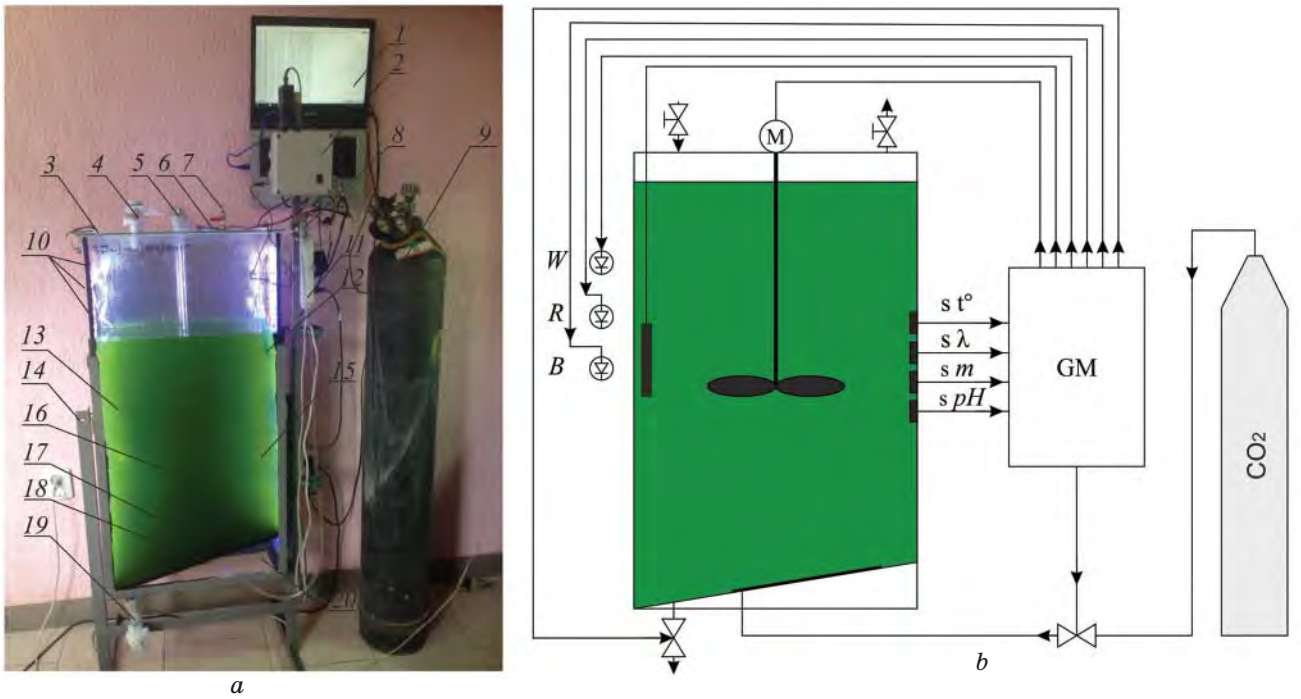

Fig. 1. **General view of a flat vertical photobioreactor (*a*):**
1 — laptop; *2* — gage module (GM); *3* — glass cover; *4* — nutrient medium delivery cock; *5* — mixing system; *6* — light sensor; *7* — gases rejection cock; *8* — electromagnetic valve 220 V; *9* — carbon dioxide cylinder; *10* — additional lighting system with LED strips; *11* — power systems (electricity); *12* — pH sensor ; *13* — electric heater; *14* — photobioreactor (PhBR) moving metal corpus; *15* — temperature sensor; *16* — microalgae concentration sensor; *17* — aerating tube; *18* — CO_2 delivery in PhBR cock; *19* — substrate drain cock; *20* — PhBR corpus made of glass;

Scheme of photobioreactor vertical plane (*b*):
W, R, B — white, red and blue LED strip; st° — cultivation medium temperature sensor; s λ — photobioreactor light level sensor; *s m* — microalgae concentration sensor; *s pH* — pH sensor

throughout the year; to regulate the up-lighting and mixing with the remote control; there is disinfector filter for incoming gases.

The structure of the cultivator has a number of advantages over other closed systems: simple design; large light receiving part for natural lighting with the possibility of artificial up-lighting; possibility of thermoregulation and regulation of gas supply; feature of the base, which is made in an inclined form that facilitates simple culture collection for further research and the relatively large volumes of culture medium in relation to the net cost of work area production (Table).

For this reactor the certain actuation method is used. Sterilization of working volume is carried out with 96% ethanol solution, it is sprayed in the working area, and then working area is washed with distilled water (three times), followed by work area and corresponding sensors sterilization with bactericidal lamps BUV-40 (micribicide lamp with ultraviolet glass) for 2 hours and one more washing with distilled water. The reactor pour with pre-prepared and sterilized nutrient medium, tightly cover the outer cap. Plug the automatic control system that tests the level of ambient temperature, acid status, according to the time of day control the system of mixing and lighting level. This system forms medium indicators to defined parameters of temperature, pH and light. At a time when the working area temperature reaches the required level, in reactor introduce the culture suspension through a hole of nutrient medium delivery (Fig. 1, *a*).

During the introduction of algae growing technology in large-scale production the basic control function belongs to gage module (*GM*), which according to information from the sensors provides the necessary photobioreactor action mode (Fig. 1, *b*). Before actuation in prepared by the method mentioned above photobioreactor, put cock (*18*) in the closed position for the culture medium will not

The equivalent ratio of materials and prices per unit of photobioreactor volume

Name	Light-conductor capacity, %	Diameter, mm	Length, mm	Volume, l	Price, UAH	UAH /l
Glass tube	85	92	3000	80	540	6.75
Acrylic tube	90–92	200	4000	125	4500	36.0
Glass aquarium	90	–	–	75	375	5.0

get in pipe sockets with the gas mixture. Fill previously prepared medium in a glass aquarium (*20*); hermetically close glass cover (*3*) that serves as a support for the pH-meter (*12*), temperature sensor (*15*), and electric heater (*13*). In addition, openings are provided with cocks for entering additional culture medium (*4*) and gases rejection (*7*) and for stirring construction (*5*).

The carbon dioxide flow from cylinder adjusted by reducer (*9*) is injected through the pipe socket to the electromagnetic valve (*8*) which opens at pH 6.0 (± 0.1) and does not stop up to 6.5 (± 0.1). After system calibration, turn the cock (18) and introduce gases mixture into the culture medium through aeration tube fixed on the bottom (*17*) that grinds gas bubbles and improves gas-mass-exchange and does not damage the culture. Fastening and corpus (*14*) are made with anticipation of possibility for changing of slope angle of light receiving part to the light source, which will allow to capture more light and heat energy. *GM* (*2*) monitors light level using sensor (*6*) and, if necessary, switches up-lighting system constructed of LED strips (*10*) located on the sidewalls of the cultivator. LED strips of required spectrum are located along the system in order of "white — red — blue — white" on both sides with total power of 80 W. The mixing of culture occurs every 4 hours for 240 s by an electric motor (5) with power of 10 W. Temperature control is carried out by sensor (*15*) in the desired temperature range (in our experiment 22–25 °C (± 0.2 °C)), if necessary, the system switches electric heater (*13*) with power of 50 W. Sensor (*16*) controls the number of cells (biomass) with respect to the volume. The drain cock (*19*) allows taking out and rejecting the necessary part of the culture in the continuous mode. After the system of cultivation reaches the stationary mode, elimination the suspensions is carried through the cock (*19*). The system data (time, light level, pH, temperature, duration of electric heater work, up-lighting and the valve of CO_2 supply open mode) measured every second are recorded in external memory and visually displayed on the computer (*1*).

To implement these functions, appropriate gage module was created based on microcontroller ATmega328 and its control algorithm was developed through which corresponding software was created. Stability of the cultivator functioning is confirmed by the dynamics of the main indicators of algae cultivation (Fig. 2).

Throughout the entire period of cultivation, the temperature regime was maintained within 22–25 °C (± 0.2 °C), pH 6.6–7.4. The oxygen content is also changing within 23–25 mg/l. By the system of automatic switching of CO_2 supply, CO_2 content was maintained at the level of 9–12 mg/l with peaks of delivery that relate to the algae multiplication intensity and biosynthetic processes activity (Fig. 3), consistent with the increasing the intensity of carbon dioxide consumption with a maximum on the 15^{th}- the 20^{th} day — 12 ± 1 g/l • day (Fig. 2, curve *5*).

The proposed system has allowed carrying out the long-term cultivation of chlorella in a stationary mode, as evidenced by the dynamics of cells content in the culture medium (Fig. 3).

During the first 18 days of cultivation the exponential growth of cells number (5.3 times, up to $269.2 \pm 3.0 \cdot 10^9$ cells/l) compared to their number in the initial culture — $51.2 \pm 1.6 \cdot 10^9$ cells/l ($P < 0.05$) was observed, and then for the duration of entire cultivation the number of cells was constant within $110.1 \pm 5.2 \cdot 10^9$ cells/l (2.1 times more compared to their number in the initial culture, $P < 0.05$).

It was shown that for *Chlorella* sp. from different natural habitats, Fitzgerald medium is the best for cultivation when compared with Tamiya, Chu-10 or Yelenkina media with or without barbotage for 30 days in lyuminostat (23–25 °C, lighting of 2 000 lux) [20]. On the 15^{th} day of cultivation it was obtained $5.9 \cdot 10^9$ cells/l. At further increase in cultivation terms up to 25 days the cell concentration increased 2.5 times, reaching a value of $14.7 \cdot 10^9$ cells/l. Note that the number

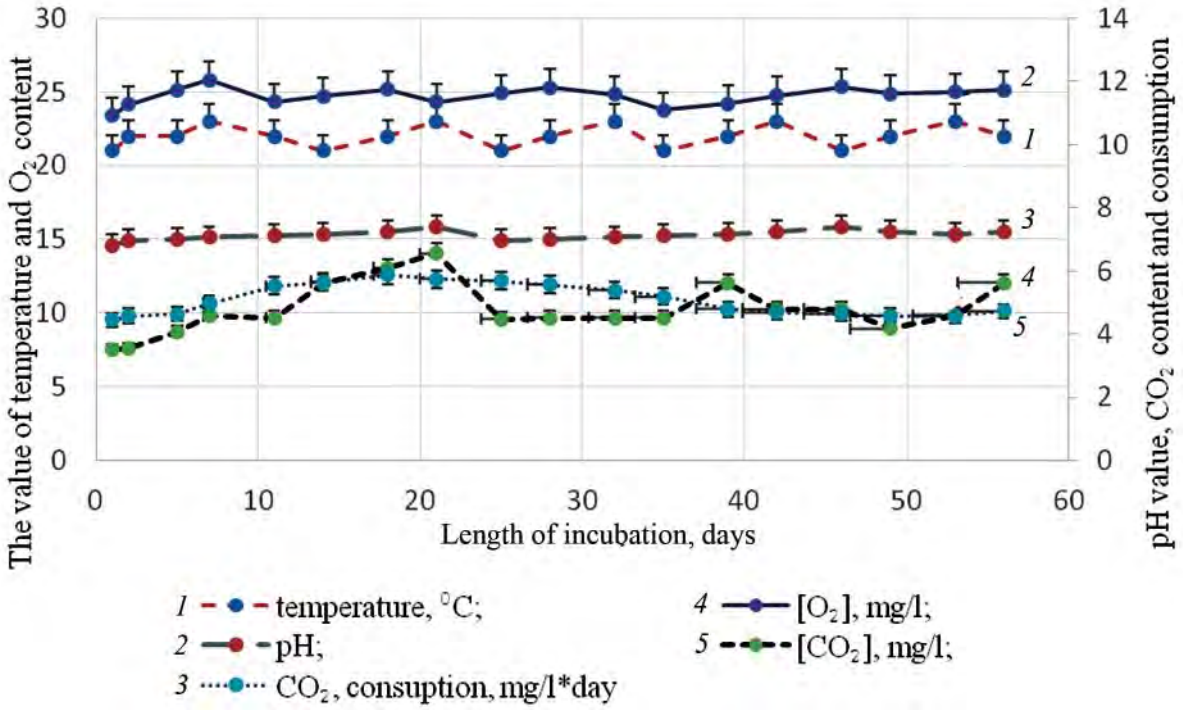

Fig. 2. **Dynamics of culture medium temperature and acidity, and dissolved O_2 and CO_2 content, and its consumption**

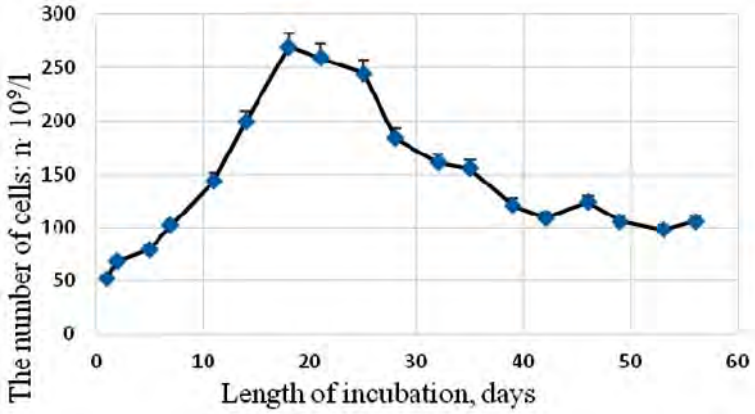

Fig. 3. **Dynamics of Chlorella cells number during incubation in the reactor**

of cells in comparison with the experiment beginning under these conditions has increased more than 5 times ($P < 0.05$). These data almost correlate with our data when on the 25th day of cultivation the cell number compared with the initial has increased 4.6 times.

In our experiment, the effectiveness of Fitzgerald medium for chlorella cultivation in continuous mode is confirmed. However, the number of cells compared with the results of mentioned work [20] is order of magnitude more.

Cells number dynamic, their total biomass and the main organic components mass were analogous (Fig. 4).

The curve of biomass increase is also characterized by exponential growth during the first two weeks of cultivation — 3.4 times since the beginning of cultivation ($P < 0.05$), which correlates with the usual terms of organisms adaptation to existence conditions forming [21]. The second peak is detected on the 45th–47th day of cultivation (2.2 times since the beginning of cultivation — $P < 0.05$), but by 34% less than in the first maximum index ($P < 0.05$).

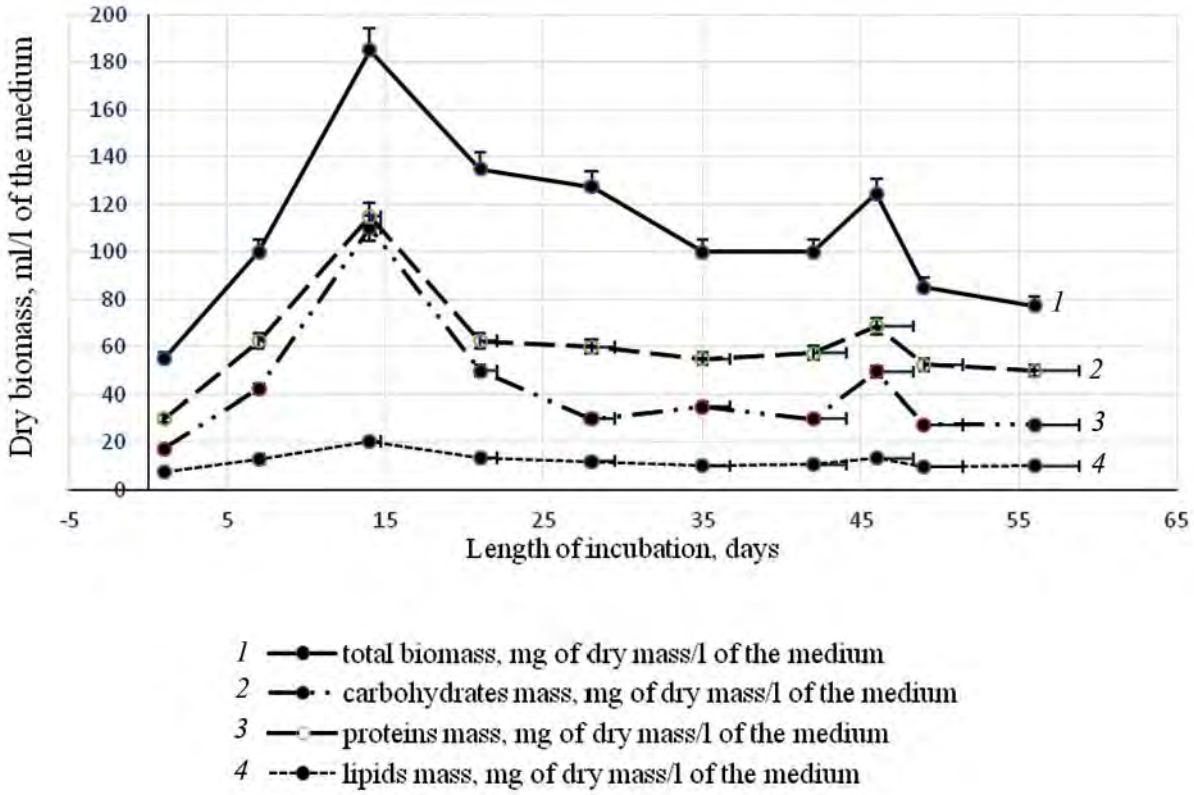

Fig. 4. **Dynamics of the main components of cell mass**

As for the content of basic organic components of cells, it has been found the most amounts of proteins — 54%, carbohydrates — 32%, lipids — 14% of the total biomass. During the exponential phase the proteins content increased 3.8 times, carbohydrates — 6.3 times, lipids — 3.0 times ($P < 0.05$). Further up to the 21^{th}–25^{th} day of cultivation the proteins content was about of 60 mg of dry weight/l, carbohydrates — 35 mg of dry weight/l and lipids — 12 mg of dry weight/l. The ratio carbohydrates-proteins-lipids was: at the start of cultivation — 5.4: 3.2: 1.4; at the final stage of exponential growth — 4.7: 4.5: 0.8; at the stage of stationary growth — 5.6: 3.3: 1.1.

Thus, the proposed system of chlorella cultivation contributes to the accumulation of proteins and carbohydrates, less of lipids. However, in case of possible use of chlorella for biofuels, heat capacity of solids is high due to protein and carbohydrates, and lipids content increase can be achieved by their biosynthesis activation by stimulating factors [22, 23]. At the same time, it is possible to change the ratio of proteins, carbohydrates and lipids by the usage of stimulant substances for biosynthesis of individual classes of organic compounds that is the prospect for further research. In addition, sunlight usage for photobioreactor lighting and cultivation under natural weather conditions are interesting.

Analysis of the results of the study has shown that in designed reactor at stabilization and automatic control of culture conditions, the algae cultures reached the maximum density in Fitzgerald medium on the 18^{th} day of cultivation. Cells content was $269.2 \pm 3.0 \cdot 10^9$ cells/l with the stabilization in stationary phase within $110.1 \pm 4.9 \cdot 10^9$ cells/l, which enables chlorella growing in continuous mode with an average productivity of about 110 ± 4 mg of dry weight/l, with protein content of about 60 mg, carbohydrates — 35 mg, lipids — 12 mg/l. These amounts of received quantities of biomass in general and organic substances in particular give grounds for further optimization the process with practical perspective of chlorella usage.

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ОПТИМІЗАЦІЯ КУЛЬТИВУВАННЯ *Chlorella vulgaris* Beij. У БІОРЕАКТОРІ НЕПЕРЕРВНОЇ ДІЇ

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Метою роботи було розроблення та апробація біореактора інтенсивного культивування водорості *Chlorella vulgaris* Beij. з повністю контрольованими умовами у межах режимних параметрів за обраними критеріями оцінювання процесу культивування. Для перевірки ефективності функціонування оригінального фотобіореактора досліджували ріст у ньому *Chlorella vulgaris* Beij. (*Chlorophyta*) у середовищі Фітцджеральда в модифікації Цендера і Горхема №11 за 22–25 °С та штучного освітлення лампами денного світла (інтенсивність 2 500 лк) упродовж 16 год на добу. Методами підрахунку клітин та визначення кількості протеїнів, вуглеводів і ліпідів встановлено, що за стабілізації та автоматичного контролю умов культивування максимальну щільність культури у розробленому реакторі спостерігали на 18-ту добу культивування із вмістом клітин $269{,}2 \pm 3{,}0 \cdot 10^9$ /л з кількістю у стаціонарній фазі в межах $110{,}1 \pm 4{,}9 \cdot 10^9$ /л. Це дає змогу вирощувати хлорелу в безперервному режимі із середньою продуктивністю у стаціонарному режимі близько 110 ± 4 мг сухої маси/л із вмістом протеїнів близько 60 мг, вуглеводів — 35 мг, ліпідів — 12 мг сухої маси/л. Співвідношення вмісту протеїнів, вуглеводів і ліпідів можна змінювати, використовуючи сонячне світло та речовини-стимулятори біосинтезу окремих класів органічних речовин, що становить перспективу подальших досліджень.

Ключові слова: хлорела, фотобіореактор, безперервне культивування.

ОПТИМИЗАЦИЯ КУЛЬТИВИРОВАНИЯ *Chlorella vulgaris* Beij. В БИОРЕАКТОРЕ НЕПРЕРЫВНОГО ДЕЙСТВИЯ

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Целью работы была разработка и апробация биореактора интенсивного культивирования водоросли *Chlorella vulgaris* Beij. с полностью контролируемыми условиями в пределах режимных параметров по выбранным критериям оценки процесса культивирования. Для проверки эффективности функционирования оригинального фотобиореактора исследовали рост в нем *Chlorella vulgaris* Beij. (*Chlorophyta*) в среде Фитцджеральда в модификации Цендера и Горхема №11 при температуре 22–25 °С и искусственном освещении лампами дневного света (интенсивность 2 500 лк) в течение 16 час в сутки. Методами подсчета клеток и определения протеинов, углеводов и липидов показано, что при стабилизации и автоматическом контроле условий культивирования максимальная интенсивность роста в разработанном реакторе наблюдалась на 18-е сут культивирования с содержанием клеток $269{,}2 \pm 3{,}0 \cdot 10^9$ /л и их количеством в стационарной фазе около $110{,}1 \pm 4{,}9 \cdot 10^9$ /л. Это дает возможность выращивать хлореллу в непрерывном режиме со средней производительностью в стационарном режиме около 110 ± 4 мг сухой массы/л с содержанием протеинов около 60 мг, углеводов — 35 мг, липидов — 12 мг сухой массы/л. Возможно изменение соотношения содержания протеинов, углеводов и липидов за счет использования солнечного света и веществ-стимуляторов биосинтеза отдельных классов органических веществ, что является перспективой дальнейших исследований.

Ключевые слова: хлорелла, фотобиореактор, непрерывное культивирование.