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TECHNOLOGY OF CULTIVATION OF THE MARINE MICROALGA *TETRASELMIS VIRIDIS* UNDER NATURAL LIGHT AND AT MINIMUM TECHNICAL COST

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The main reason for slow implementation of scientific developments of marine algotechnology into industrial practice is the lack of systems that allow obtaining microalgae biomass in quantities that are necessary for practical study of potential products and development of industrial technology for their production. Such systems can significantly reduce the economic cost of creating and maintaining favorable abiotic conditions for growing microalgae on an industrial scale, since solar energy is used as a light source. The article proposes a method for growing marine microalgae *Tetraselmis viridis* in natural light and at minimum technical cost. The authors developed a mobile unit for cultivating marine microalgae and studying their growth characteristics in natural light. This unit is proposed to be used in the transition from laboratory cultivation to cultivation on an industrial scale. The basic requirements for the mobile unit for industrial cultivation of algologically pure *T. viridis* are specified. The technology ensuring the organization of *T. viridis* cultivation process with a maximum productivity of 5.7 $g \cdot m^{-2} \cdot day^{-1}$ and a maximum culture density of 271.6 billion $cells \cdot m^{-2}$ ($R^2 = 0.99$) has been developed. The authors provide a comparative assessment of the biochemical composition and kinetic growth characteristics of *T. viridis* depending on growing conditions using either the mobile unit in natural light or the laboratory photobioreactor in constant artificial light.

Keywords: marine microalgae, *Tetraselmis viridis*, batch culture, productivity, industrial cultivation

Intensive cultivation of microalgae in open water bodies is aimed mainly at obtaining biomass and using it as a food supplement [Benemann, 1992; Chaumont, 1993; Gudvilovich, Borovkov, 2012], as a source of raw materials in producing chemicals for their further use in the pharmaceutical industry [Demmig-Adams, Adams, 2002; Zhondareva, Trenkenshu, 2019], and for wastewater treatment [de la Noüe et al., 1992; Dobrojan, 2010; Markou, 2015]. The main reason for slow implementation of scientific developments of algotechnology into practice is the lack of industrial systems that allow obtaining microalgae biomass in quantities necessary for study of potential products and development of technologies for their production.

In most cases, the technology of microalgae mass cultivation involves the use of open systems in natural light, consisting of pools and ponds. Mostly, these are shallow film-lined pools; sometimes, these are cemented trenches, trays of various shape, or tanks. Such systems can significantly reduce

the economic cost of creating and maintaining favorable conditions for microalgae cultivation on an industrial scale, since solar energy is used as a light source. However, when using open systems, there is a danger of biomass contamination by bacteria and other invasive organisms. Moreover, it is reasonable to place production facilities in areas with few cloudy and rainy days and small diurnal temperature fluctuations. One of such areas is the south of Russia; this is due to favorable climatic conditions which allow growing microalgae using only solar energy continuously for two–three seasons during the year [Abdulagatov et al., 2018; Borovkov et al., 2020; Peel et al., 2007].

The objective of this work was to develop a low-cost version of mobile unit, the conditions in which are close to industrial conditions for growing marine microalgae on the example of *Tetraselmis viridis* (Rouchijajnen) R. E. Norris, Hori & Chihara, 1980, and to compare the biochemical composition and kinetic characteristics of algae growth when cultivated in laboratory conditions under constant artificial light and in the mobile unit under natural light.

MATERIAL AND METHODS

The green microalga *T. viridis*, strain IBSS-25 from IBSS collection, served as a study object. A nutrient medium was prepared based on non-sterile Black Sea water with a salinity of 1.4–1.8‰. The composition of the medium for *T. viridis* cultivation in dense culture was given earlier [Trenkenshu et al., 1981]. To preserve the algologically pure culture of the microalga, the salinity level in the medium was increased to the Mediterranean one by adding 15 g·L^{-1} NaCl [Gorbunova, Trenkenshu, 2020]. To obtain an inoculum, *T. viridis* was grown for 5 days in laboratory conditions by the batch method in 3-L photobioreactors under 10 klx illuminance on the Trenkenshu nutrient medium. The pool was filled with an actively dividing culture with an initial density of 0.08 g·L^{-1} of dry matter.

The unit for the microalga cultivation was placed on the pier of IBSS laboratory building from 26 August to 7 September, 2020. It was a pool 1 × 1 m in size and 0.1 m in height (Fig. 1). Since the unit was in the open air 24 h a day, it was necessary to maintain the temperature there within optimum for *T. viridis* cultivation. For this purpose, a cooling system was connected. A foam sheet was fixed throughout the perimeter of the pool bottom, with a polyurethane tube placed on it, through which seawater continuously circulated. The tube was covered with sand, and the pool bottom was lined with plastic film.

Fig. 1. The pool with a cooling system

Using a water pump Air Pump ACO-008, with a power of 120 W and a pressure of less than 0.032 MPa, water was supplied from the sea to the pier. The daytime temperature of the suspension in the pool was lower than the air temperature by 3–7 °C. Throughout the experiment, the daily temperature in the unit was maintained within the range of +23…+28 °C. Without a cooling system, the culture overheated and died within 36–48 h. To protect the pool from debris and possible precipitation, a sloping roof covered with plastic film was installed (Fig. 1); this ensured natural ventilation of the unit. The working volume of the pool was 70 L. To compensate for water evaporation, this volume was maintained throughout the experiment by adding distilled water before measurements to the level of 7 cm.

Continuous mixing of the microalga in the pool was carried out by the water pump, with a suspension pumping rate of 2,800 $L \cdot h^{-1}$, which ensured round-the-clock gas exchange of the culture and uniform cell illumination throughout the unit. On the pool surface, illumination was monitored twice a day with a Yu-116 luxmeter. *Prior* to the pool launch, the culture was kept under different light conditions: in the laboratory, the illumination was constant, and its intensity was more than 6 times lower than the natural maximum daily light. During the first three days, while the culture density was low, the pool roof was shaded with a net. Otherwise, under cloudless conditions, *T. viridis* cells became completely discolored. On average, during the experiment, the maximum daily illumination in the area of photosynthetically active radiation was 300 $W \cdot m^{-2}$; for calculations, the data presented in [Chekushkin et al., 2020] were used. The value of illumination is given taking into account the absorption of 25% of solar energy by the pool roof.

At the same time, *T. viridis* was grown in the laboratory photobioreactor under the same conditions as the pool inoculum. The optical density was calculated by the formula $D = -\lg(T)$, where T is the transmission value determined using Unico 2100 (United Products & Instruments, the USA) at a wavelength of 750 nm, in cuvettes with a working length of 0.5 cm. Absolute error did not exceed 1.0%.

For the convenience of comparing the obtained results with the data presented in the publication [Zhondareva, Trenkenshu, 2019], the optical density of the suspension layer (7 cm) was determined by multiplying the density values in the cuvette (0.5 cm) by 14.

When calculating optical density units (o. d. u.) in terms of the microalga dry weight (hereinafter DW), an empirically determined coefficient k was used, equal to 0.8 $g \cdot L^{-1} \cdot o.\,d.\,u.^{-1}$: $DW = k \times D_{750}$ [Borovkov, Gevorgiz, 2005]. The cell concentration was determined by direct counting in the Goryaev chamber under a microscope in eight replications. Microscopic control of the culture was carried our under a light microscope Carl Zeiss Axiostar Plus (Germany).

Pigments (chlorophyll *a*, chlorophyll *b*, and carotenoids) were extracted from the microalga cells with acetone (100%) [Kopytov et al., 2015]. The absorption spectra of acetone extracts were recorded in the range 400–800 nm in quartz cuvettes with a 1-cm optical path. The pigment composition was estimated from the absorption spectrum of acetone extract in three replications according to the standard method, using linear equations for three points in the absorption spectrum of the extract [Wellburn, 1994]. All calculations were performed for the significance level $a = 0.05$. Mathematical processing and modeling of the experimental data were carried out using computer programs Grapher 3, MS Excel, and MATLAB.

RESULTS AND DISCUSSION

The growth of *T. viridis* culture in the pool with constant illumination was monitored for 12 days. Linear growth of the culture was recorded from the 2nd to the 10th day of the experiment (Fig. 2A). During this time, the culture density increased by 7.5 times.

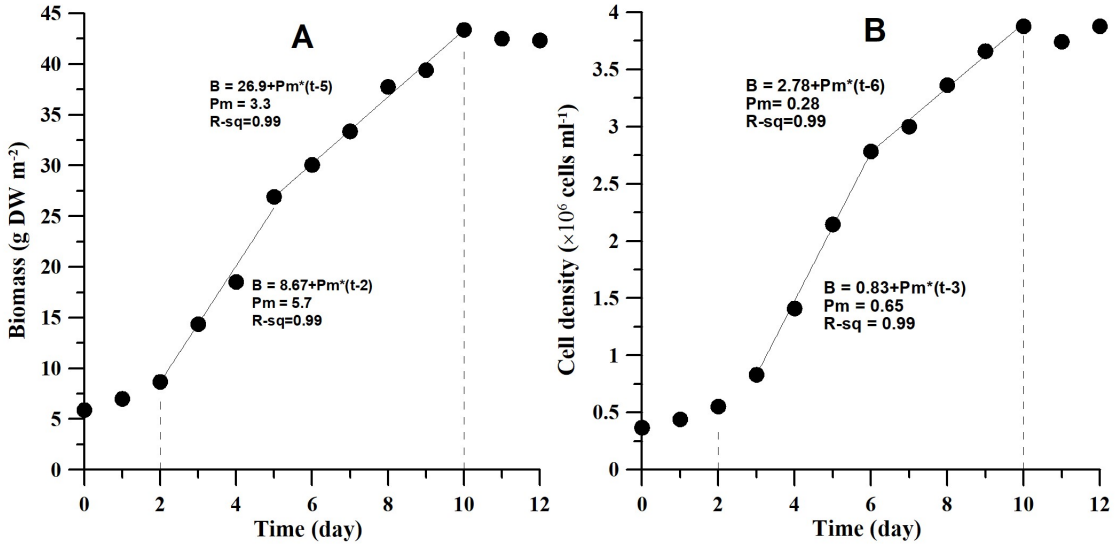

Fig. 2. Dynamics of *Tetraselmis viridis* density (A) and growth (B) in the pool under natural light

On the 5th day, the productivity of the culture by biomass (Pm) decreased from 5.7 to 3.3 g DW·m^{-2} (R^2 = 0.99). Apparently, this results from the beginning of limiting the microalga growth by carbon or light conditions, since in the linear plot, the growth rate is determined by the magnitude of the external flux (light or carbon dioxide), which is completely absorbed by the culture and limits its productivity [Trenkenshu, 2005]. On the 11th day, *T. viridis* growth stopped. The end of the linear growth phase indicates a change in the limiting factor [Lelekov, Trenkenshu, 2007], and both light and mineral medium conditions can limit the microalga growth. In our experiment, the mineral component cannot be a limiting factor, since the Trenkenshu nutrient medium, on which *T. viridis* was grown, is designed to achieve a culture density of 4–6 g DW *per* L [Trenkenshu et al., 1981]. Thus, we can assume that culture growth was limited by light conditions.

The obtained characteristics were compared with the results presented in [Zhondareva, Trenkenshu, 2019]. In that work, the linear growth phase of *T. viridis* was two times shorter than that according to our data and was recorded only from the 1st to the 5th day. Starting from the 6th day, the microalga growth stopped, while in our experiment, the linear growth phase was recorded from the 5th to the 10th day, on which the maximum productivity was 3.3 g DW·m^{-2}. Thus, we obtained the microalga yield of 43.4 g DW·m^{-2}, and this value is 2 times higher than in [Zhondareva, Trenkenshu, 2019]. This can be explained by providing optimal conditions for *T. viridis* cultivation: the presence of a cooling system, an increase in the salinity of the culture medium to the Mediterranean level, a 7-cm layer of the microalga in the pool, and an effective mixing system. A similar pattern was observed for the concentration dynamics of *T. viridis* cells in the culture (Fig. 2B).

As established, the linear growth of the culture occurs from the 3rd to the 10th day of the experiment, with a change in the curve slope on the 6th day, which confirms the theory about the beginning of the microalga growth limitation by carbon or light conditions. *T. viridis* productivity in the first part of the linear growth phase was 650 thousand cells·day^{-1}; in the second part (6th–10th days), the value was 280 thousand cells·day^{-1}. During the experiment, the cell concentration almost reached the value of 4 million cells·mL^{-1}. Fig. 3 shows the appearance of the pool at the beginning of the experiment and at the end (in 12 days).

Fig. 3. The pool with *Tetraselmis viridis* culture at the beginning of the experiment (left) and at the end (right)

As shown, with easy-to-use equipment and minimal capital investments, it is possible to organize *T. viridis* cultivation with a productivity of up to 5.7 g DW·m^{-2}·day^{-1}. When microalgae are grown in large volumes in natural light, productivity values are higher than theoretical maximum; also, the values may differ from those determined in laboratory conditions [Béchet et al., 2017; Bonnefond et al., 2016].

Data on the biochemical composition and kinetic characteristics of *T. viridis* grown in the laboratory photobioreactor under constant illumination and in a pool under natural light are given in Table 1.

Table 1. Biochemical composition and kinetic characteristics of the microalga *Tetraselmis viridis* growth (the end of the linear growth phase)

Parameter	*Tetraselmis viridis* in the laboratory photobioreactor	*Tetraselmis viridis* in the pool
Chlorophyll *a*, %	1.05 ± 0.05	1.01 ± 0.01
Chlorophyll *b*, %	0.58 ± 0.07	0.52 ± 0.01
Total carotenoids, %	0.23 ± 0.01	0.21 ± 0.004
Maximum density, g DW·L^{-1}	1.00 ± 0.05	0.62 ± 0.03
Maximum productivity, g DW·L^{-1}·day^{-1} (R^2 = 0.99)	0.122	0.08

In both cases, the culture was grown on the same nutrient medium and without additional carbon sources used. Sampling for the analysis was carried out at the same time. Fig. 4 shows the appearance of the laboratory photobioreactor at the beginning of the experiment and in 12 days.

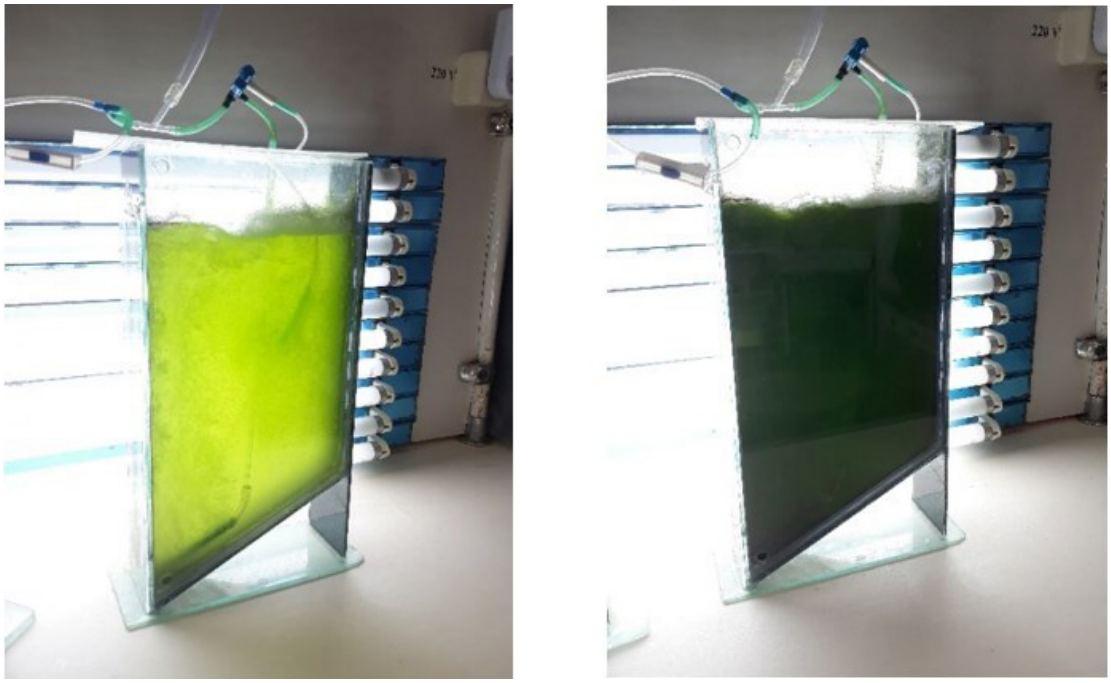

Fig. 4. Photo of the laboratory photobioreactor with the microalga *Tetraselmis viridis* at the beginning of the experiment (left) and after 12 days (right)

Models of native forms of chlorophyll *a*, chlorophyll *b*, and total carotenoids were used to assess the biological value of the obtained microalga and quickly calculate the concentrations of pigments in the culture [Chernyshev et al., 2020]. Our results on the chemical composition of *T. viridis* grown in laboratory conditions are in good agreement with the data of [Kharchuk, Beregovaya, 2019]. No significant differences in the microalga biochemical composition depending on the growing conditions were found. However, there was a trend towards a decrease in concentration of biochemical components in *T. viridis* cells when grown in the pool in natural light. This results from the shift in physicochemical parameters, the inability to maintain sterile conditions due to large volumes of pools, and diurnal changes in the illumination, accompanied by the reduction of some microalga biomass during the dark period [Avsiyan, 2018; Bonnefond et al., 2016; Xu et al., 2016].

Conclusion. A mobile unit for marine microalgae cultivation was developed. Microalgae growth characteristics in natural light were studied. This unit is proposed to be used in the transition from laboratory scales of marine microalgae cultivation to industrial ones. It was experimentally shown that the minimization of capital investments is ensured by the presence of easy-to-use equipment, the use of a cooling system, an increase in the salinity of the culture medium to the Mediterranean level, the choice of the optimal layer of the microalga in the pool, the absence of additional carbon sources, the use of an efficient mixing system, and the use of solar energy as a light source. The proposed approach allows *Tetraselmis viridis* cultivating with a maximum productivity of 5.7 $g \cdot m^{-2} \cdot day^{-1}$ and a culture density of 271.6 billion $cells \cdot m^{-2}$. Comparative assessment revealed no differences between biochemical and kinetic characteristics of *T. viridis* growth when cultivated in the mobile unit under natural light and in the laboratory photobioreactor under constant artificial light.

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ТЕХНОЛОГИЯ ВЫРАЩИВАНИЯ МОРСКОЙ МИКРОВОДОРОСЛИ *TETRASELMIS VIRIDIS* ПРИ ЕСТЕСТВЕННОМ ОСВЕЩЕНИИ И МИНИМАЛЬНЫХ ТЕХНИЧЕСКИХ ЗАТРАТАХ

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Главной причиной медленного внедрения научных разработок морской альготехнологии в промышленную практику является отсутствие систем, позволяющих получать биомассу микроводорослей в количествах, которые необходимы для исследования потенциальных продуктов и для отработки промышленной технологии их производства. Такие системы позволяют значительно снизить экономические затраты на создание и поддержание благоприятных абиотических условий для выращивания микроводорослей в промышленных масштабах, поскольку в качестве источника освещения используется энергия солнца. В статье предложен способ

выращивания морской микроводоросли *Tetraselmis viridis* при естественном освещении и минимальных технических затратах. Авторами разработана мобильная установка для культивирования морских микроводорослей и для исследования их ростовых характеристик в условиях естественного освещения. Данную установку предлагается использовать при переходе от лабораторных масштабов культивирования микроводорослей к промышленным. Приведены основные требования, которым должна удовлетворять мобильная установка, и обоснование её конструкции для промышленного выращивания альгологически чистой культуры *T. viridis*. Разработана технология, позволяющая обеспечить организацию процесса культивирования *T. viridis* с максимальной производительностью культуры 5,7 г·м$^{-2}$·сут$^{-1}$ и плотностью 271,6 млрд кл.·м$^{-2}$ (R^2 = 0,99). Дана сравнительная оценка биохимического состава и кинетических характеристик роста *T. viridis* при выращивании в мобильной установке в условиях естественного освещения и в лабораторных культиваторах при постоянном искусственном освещении.

Ключевые слова: микроводоросли, *Tetraselmis viridis*, накопительная культура, продуктивность, промышленное культивирование