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EXPERIENCE OF GROWING THE MICROALGA *TISOCHRYSIS LUTEA* (HAPTOPHYTA) UNDER CONDITIONS OF A LABFORS BIOREACTOR FOR THE PRODUCTION OF CAROTENOIDS AND NEUTRAL LIPIDS

© 2024 **Zh. Markina, A. Zinov, and T. Orlova**

A. V. Zhirmunsky National Scientific Center of Marine Biology, FEB RAS, Vladivostok, Russian Federation
E-mail: *zhannav@mail.ru*

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The results of the experiment on the use of a Labfors 5 Lux LED flat panel bioreactor (Infors HT, Switzerland) for *Tisochrysis lutea* (Haptophyta) cultivation are presented. During the three-week study, growth and size structure of the microalga population were assessed, and the content of chlorophyll *a*, carotenoids, and neutral lipids was estimated. The highest cell abundance, 5.3×10^4 cells·mL^{-1}, was recorded at the end of the experiment, on the 21st day. An increase in the proportion of 4–6-μm cells was registered on the 11th day. The maximum accumulation of carotenoids occurred on the 18th day (3.3 mg·L^{-1}), and neutral lipids (Nile Red fluorescence was of 5.3×10^6), on the 14th–21st day. As revealed, Labfors 5 Lux LED flat panel bioreactor can be successfully used for cultivation of the microalga *T. lutea*.

Keywords: *Tisochrysis lutea*, biotechnology, bioreactor, carotenoids, neutral lipids

For a long time, *Tisochrysis lutea* Bendif & Probert, 2013 (Haptophyta) is widely used in algal biotechnology as a food item for larvae of invertebrates [Alkhamis, Qin, 2016; Araújo et al., 2020]. This species is the most promising producer of the prevailing carotenoid, fucoxanthin (up to 98% of the total carotenoids content) [Mohamadnia et al., 2021]. Moreover, *T. lutea* is an important producer of neutral lipids. The development of conditions for cultivation in bioreactors is the basis for biotechnological processes, and this is especially relevant for *T. lutea* [Mohamadnia et al., 2021].

For biotechnology purposes, this species is cultivated in flasks [Mohamadnia et al., 2020], but more often, *T. lutea* is cultivated in bioreactors [Falinski et al., 2018; Gao et al., 2020; Ippoliti et al., 2016; Leal et al., 2020].

To date, various modifications of bioreactors have been developed. Panel bioreactors are among the most convenient models for microalgae cultivation. Their advantages, which together ensure intensive growth of microalgae, are good mixing of an algal suspension, large area of illuminated surface, and low accumulation of oxygen in the medium [Guedes, Malcata, 2011; Tan et al., 2020].

Not only fluorescent lamps, but also light-emitting diodes (LED) are used as a light source. The benefits of LED are low energy consumption, very low heat generation during work, stable luminous flux, long lifespan, and constant luminous flux parameters over time with regular on/off cycles [Posten, 2009].

The aim of the work was to study the dynamics of population growth and the content of carotenoids and neutral lipids in *Tisochrysis lutea*, strain MBRU_Tiso-08, in the flat panel bioreactor Labfors 5 Lux LED (Infors HT, Switzerland), which was used for microalgae cultivation for the first time.

The main indicators determined in the research were cell abundance and size structure of *T. lutea* population, as well as the content of carotenoids and neutral lipids in its biomass as substances of certain interest for biotechnology. The values of optical density obtained by various methods served as additional indicators proposed for rapid assessment of *T. lutea* abundance. To better describe the physiological processes of the microalga, chlorophyll *a* was analyzed as well.

MATERIAL AND METHODS

The object of the study was the culture of the unicellular alga *T. lutea* (Haptophyta) – strain MBRU_Tiso-08 from the Marine Biobank resource collection at A. V. Zhirmunsky National Scientific Center of Marine Biology, Far Eastern Branch of the Russian Academy of Sciences (http://marbank.dvo.ru/). The alga was cultivated in the medium *f* [Guillard, Ryther, 1962] prepared on filtered and sterilized seawater with a salinity of 32‰ in Labfors 5 running in fed-batch mode. Seawater temperature was +20 °C. Light intensity was 50 μmol·m^{-2}·s^{-1} (a LED panel served as a light source) within the range of photosynthetically active radiation. The light–dark period was 12 h : 12 h (light : dark). Air supply was 0.2 L·min^{-1}. The suspension layer in the panel was 45 mm thick. The working flask of Labfors 5 has the volume of 1.8 L and is made of carbonate glass. Out of all materials for algae cultivation, this one has the highest light permeability (95%) and high chemical resistance. It can be sterilized. Carbonate glass is inextensible, unlike polyethylene and polypropylene. It does not transmit ultraviolet radiation, like polycarbonate glass [Guedes, Malcata, 2011].

As an inoculum, a culture at the exponential growth stage was used. The initial concentration of the microalga cells in the experiment was 0.75 × 10^6 cells·mL^{-1}. The experiment lasted for 21 days.

A CytoFLEX flow cytometer (Beckman Coulter, the USA) was used to determine the abundance of cells and their diameter, as well as the content of neutral lipids. For analysis, 10,000 events (recorded in the particle sample) were registered during each measurement. Algal cells were selected from the total number of events recorded by the flow cytometer in accordance with chlorophyll *a* fluorescence [Hyka et al., 2013] determined on PC 5.5 channel. Cell diameter was established using calibration beads (Molecular Probes, the USA) based on direct light scattering.

Optical density (OD_{750}) was determined on a Spark 10M multimode microplate reader (Tecan).

In the flask of Labfors 5, optical density was established using a Dencytee sensor (Hamilton). It provides real-time measurements of the optical density of cell suspensions.

Chlorophyll *a* concentration and the total carotenoids content were determined by a standard technique of their extraction in acetone, followed by measurement of the optical density on a Spark 10M multimode microplate reader. Pigment concentrations were calculated in accordance with standard formulas [Jeffrey, Humphrey, 1975].

The content of neutral lipids was determined by the fluorescence of Nile Red fluorochrome (N3013-100MG, Sigma-Aldrich) at a concentration of 1 μg·mL^{-1}; staining was carried out for 15 min at room temperature in the dark. The excitation wavelength was of 488 nm, and the emission wavelength was of 580 nm. Determination of lipid content by flow cytometry is characterized by a high speed. Moreover, its data are consistent with data obtained applying other methods which was confirmed on various microalgae [Alemán-Nava et al., 2016].

RESULTS AND DISCUSSION

The abundance of *T. lutea* cells increased with rising exposure time until the end of the experiment (Fig. 1). Cell abundance correlated with optical density data obtained by spectrophotometric method (OD_{750}) and by using a bioreactor sensor and analyzing changes in the turbidity of the cell suspension (see Fig. 1). Cells of 4–6 μm prevailed in the suspension, especially from the 11th day. This fact must be taken into account when planning the diet for invertebrate larvae at different stages of their development.

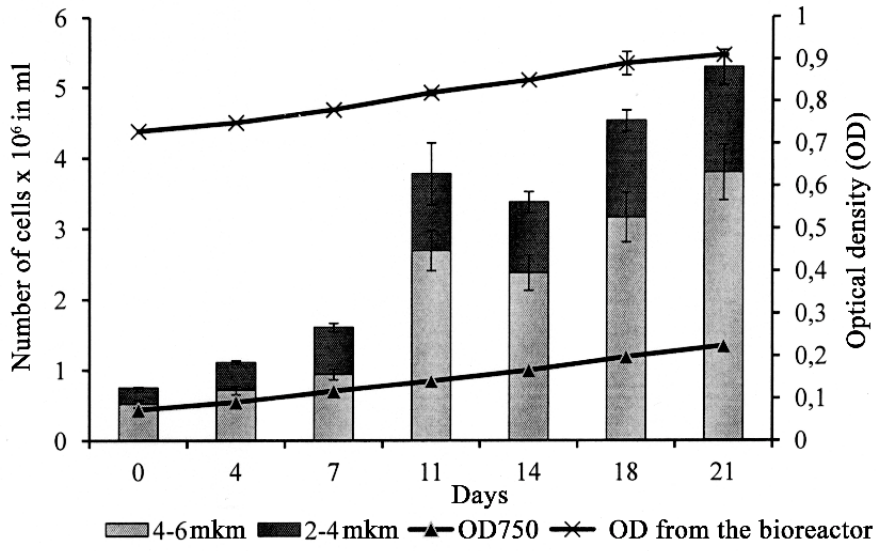

Fig. 1. Cell abundance (× 10^6 cells·mL^{-1}) and optical density (μm) of *Tisochrysis lutea* culture

Similar growth dynamics was described for *T. lutea* CCAP 927/14: for this strain, an increase in cell size was also recorded. The authors explain this fact by higher rates of cell division at the beginning of the experiment [Costa et al., 2017]. In the work [Rasdi, Qin, 2015], it is noted that *Tisochrysis* culture (without specifying the strain) entered the stationary growth stage on the 6th day of cultivation. *T. lutea* from the Roscoff Culture Collection (France) entered the stationary growth stage on the 7th day of the experiment; the alga entered the dying stage on the 21st day [Gnouma et al., 2017].

In a cylindrical reactor, the abundance of *T. lutea* cells, against the backdrop of the initial concentration of 0.4 × 10^6 cells·mL^{-1}, accounted for only 0.45 × 10^6 cells·mL^{-1} after 14 days [Falinski et al., 2018]. In a 500-L bioreactor, the abundance of cells reached its maximum, 6.92 × 10^6 cells·mL^{-1}, after 12 days of experiment, with the initial value of 0.2 × 10^6 cells·mL^{-1} [Leal et al., 2020]. For *T. lutea* cultivated in flasks, cell abundance was higher than that for the microalga in reactors and amounted to 4.3 × 10^8 cells·mL^{-1} after 4 days [Mohamadnia et al., 2020], against the backdrop of the initial concentration of 1.2 × 10^7 cells·mL^{-1}. In our experiment, cell abundance reached 1.1 × 10^6 cells·mL^{-1} after 4 days, with the initial value of 0.75 × 10^6 cells·mL^{-1}. However, bioreactors allow growing microalgae on a larger scale, and this is an important advantage.

Over 14 days, the concentration of photosynthetic pigments in *T. lutea* rose slightly (Fig. 2). On the 18th day, it sharply increased, and carotenoids content became higher than chlorophyll *a* concentration.

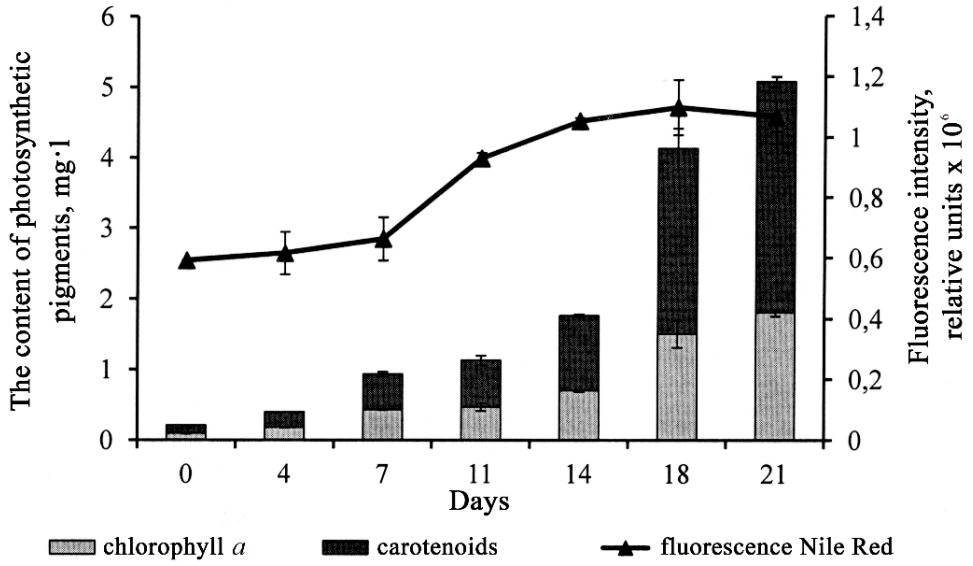

Fig. 2. Content of photosynthetic pigments and neutral lipids (Nile Red fluorescence) in *Tisochrysis lutea*

A decrease in chlorophyll *a* concentration is associated with a drop in nitrogen content during cultivation which results in a decline in the abundance of enzymes required for chlorophyll synthesis in algae cells [Costa et al., 2017]. By the 21st day, the content of pigments continued to increase but less intensely. The content of photosynthetic pigments depends on conditions of the microalga cultivation and characteristics of its physiology. Thus, in *T. lutea* CCMP 1324 cultivated under the same conditions as in this work, in a mixotrophic culture, 4,500 μg of chlorophyll *a* was recorded *per* 1 L on the 16th day of the experiment; in a heterotrophic culture, the value was 5,200 $\mu g \cdot L^{-1}$ [Hu et al., 2018].

Until the 7th day, the content of neutral lipids increased slightly; from the 7th to 14th day, the value rose significantly; and then, it remained at the same level (see Fig. 2). An increase in the content of neutral lipids with the age of the culture is described in other works as well [Costa et al., 2017; Huang et al., 2019]. Interestingly, in most algae, reserve neutral lipids are triacylglycerides, while in *T. lutea*, along with other representatives of the family Isochrysidaceae, those are alkenones [Costa et al., 2017].

The selection of research methods is the cornerstone of scientific work; its key criteria are accuracy and ensuring the reliability of the results obtained. When assessing the state of a microalgal culture, the speed of analysis is added to the listed criteria in routine biotechnological processes. Our data show that optical density indicators can be used to analyze the dynamics of *T. lutea* population growth. Moreover, it was revealed for *Chlorella vulgaris* Beijerinck, 1890 as follows: at the lag stage and exponential growth stage, OD_{750} correlates with cell abundance established by flow cytometry and by direct counting under a light microscope in a counting chamber; however, light microscopy is more accurate [Chioccioli et al., 2014].

The data obtained showed that a Labfors 5 Lux LED flat panel bioreactor can be successfully used for cultivation of the microalga *Tisochrysis lutea*.

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ОПЫТ ВЫРАЩИВАНИЯ МИКРОВОДОРОСЛИ *TISOCHRYSIS LUTEA* (HAPTOPHYTA) В УСЛОВИЯХ БИОРЕАКТОРА LABFORS ДЛЯ ПРОДУЦИРОВАНИЯ КАРОТИНОИДОВ И НЕЙТРАЛЬНЫХ ЛИПИДОВ

Ж. В. Маркина, А. А. Зинов, Т. Ю. Орлова

Национальный научный центр морской биологии имени А. В. Жирмунского ДВО РАН, Владивосток, Российская Федерация

E-mail: *zhannav@mail.ru*

Приведены результаты эксперимента по использованию биореактора панельного типа Labfors 5 Lux LED flat panel (Infors HT, Швейцария) для культивирования *Tisochrysis lutea* (Haptophyta). В ходе трёхнедельного исследования оценивали рост и размерную структуру популяции микроводоросли, содержание хлорофилла *a*, каротиноидов и нейтральных липидов. Максимальная численность клеток, $5{,}3 \times 10^4$ кл.·мл$^{-1}$, зафиксирована к концу эксперимента, на 21-е сутки. Увеличение доли клеток размером 4–6 мкм регистрировали на 11-е сутки опыта. Наибольшее накопление каротиноидов происходило на 18-е сутки эксперимента (3,3 мг·л$^{-1}$), нейтральных липидов (флуоресценция Nile Red составляла $5{,}3 \times 10^6$) — на 14–21-е сутки. Выявлено, что биореактор панельного типа Labfors 5 может быть успешно использован для культивирования микроводоросли *T. lutea*.

Ключевые слова: *Tisochrysis lutea*, биотехнология, биореактор, каротиноиды, нейтральные липиды