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INTENSIVE CULTURE OF *CYLINDROTHECA CLOSTERIUM* (EHRENBERG) REIMANN ET LEWIN ON THE NUTRIENT MEDIUM WITH SODIUM BICARBONATE

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The possibility is shown experimentally of using sodium bicarbonate in a nutrient medium to provide *C. closterium* culture with carbon under conditions of intensive cultivation without supplying CO_2 to the suspension. After *C. closterium* adaptation to a nutrient medium with sodium bicarbonate with a concentration of 1.2 g·L^{-1}, active growth is observed, with a maximum productivity of 0.6–0.7 g·(L·day)$^{-1}$ of dry weight. Carbon penetrates into diatom cells both in the form of carbon dioxide and bicarbonate ions. However, all nutrient media for artificial cultivation of diatoms still require using CO_2 from the atmosphere or from a gas cylinder. The aim of this work is to assess the possibility of using sodium bicarbonate to provide *C. closterium* with carbon under conditions of intensive cultivation without supplying CO_2 to the suspension. The culture was grown in the mode of accumulative cultivation in a 1-L flask on the RS nutrient medium prepared with sterile Black Sea water; its composition was as follows (g·L^{-1}): $NaNO_3$ – 0.775; $NaH_2PO_4{\cdot}2H_2O$ – 0.0641; $Na_2SiO_3{\cdot}9H_2O$ – 0.386; Na_2EDTA – 0.0872; $FeSO_4{\cdot}7H_2O$ – 0.045; $CuSO_4{\cdot}5H_2O$ – $0.2{\cdot}10^{-3}$; $ZnSO_4{\cdot}7H_2O$ – $0.44{\cdot}10^{-3}$; $CoCl_2{\cdot}6H_2O$ – $0.2{\cdot}10^{-3}$; $MnCl_2{\cdot}4H_2O$ – $0.36{\cdot}10^{-3}$; and $NaMoO_4{\cdot}H_2O$ – $0.12{\cdot}10^{-3}$. Previously, 1.2 g·L^{-1} of sodium bicarbonate was dissolved there. Cell suspension was stirred with a magnetic stirrer (250 rpm). On the 4th day of the experiment, 1 g of $NaHCO_3$ and 2 mL of 0.1 N hydrochloric acid were added to the culture in order to lower the medium pH down to 8.6. From the 2nd day of the experiment, active growth was observed, with a maximum productivity of 0.6 g·(L·day)$^{-1}$. After adding 1 g·L^{-1} of sodium bicarbonate to the actively growing culture and lowering pH down to 8.6, the growth rate approached almost zero, but considering the increase rate of the medium pH during adaptation, the culture actively absorbed bicarbonate ions. The possibility of cultivating the benthic diatom *C. closterium* on a nutrient medium with a high sodium bicarbonate content is experimentally shown. As found, on the RS nutrient medium with 1.2 g·L^{-1} of sodium bicarbonate added under conditions of intensive cultivation, *C. closterium* maximum productivity reaches 0.7 g·(L·day)$^{-1}$, with a significant increase in the medium pH. According to our data, optimal medium pH for *C. closterium* growth is in the range of 8.4–9.4. At higher values (pH > 9.4), the growth of diatoms slows down; at pH = 9.9, the culture enters the dying phase.

Keywords: nutrient medium, cultivation, diatoms, sodium bicarbonate

Diatoms have a rather efficient carbon-concentrating mechanism (Lebeau & Robert, 2003 ; Matsuda et al., 2017 ; Matsuda & Kroth, 2014). In terms of quantitative and qualitative composition of carbonic anhydrases, diatoms are superior to other algae species; therefore, they inhabit various water bodies with different CO_2 and HCO_3^- concentrations (Lebeau & Robert, 2003 ; Roberts et al., 2007).

Being representatives of secondary endosymbiosis (Keeling, 2010), diatoms have inherited the ability to synthesize ten unique carbonic anhydrases of α-, β-, γ-, and θ-class; those are located along the entire pathway of inorganic carbon transport from the environment to the chloroplast (Berges et al., 2002 ; Jensen et al., 2019 ; Matsuda & Kroth, 2014). This structure, combined with the urea cycle and the ability of diatoms to C_4 carbon fixation, significantly reduces CO_2 loss by a cell and allows diatoms to survive under unfavorable conditions (Horne, 1972 ; Obata et al., 2013 ; Reinfelder et al., 2004).

Inorganic carbon is known to penetrate into cells mainly in the form of CO_2, by free diffusion, as well as by active HCO_3^- transport due to ATP energy (Lebeau & Robert, 2003 ; Matsuda et al., 2017). Despite the fact that the ability of diatoms to use hydrocarbons has been known for a long time (Matsuda & Kroth, 2014 ; Matsumoto et al., 2017 ; Obata et al., 2013), all nutrient media for artificial cultivation of diatoms still require using CO_2 from the atmosphere or from a gas cylinder, *inter alia* for industrial growth of high-density cultures (Lebeau & Robert, 2003 ; Matsumoto et al., 2017 ; Reinfelder et al., 2004). In the literature, there is no information on the adaptive ability of diatoms to a medium with high concentration of hydrocarbons and high pH values; there is also no data on the use of nutrient media with hydrocarbons for intensive cultivation of high-density cultures.

Out of variety of marine diatoms, *C. closterium* is one of the most convenient study objects. Moreover, *C. closterium* is a promising object for industrial cultivation. Therefore, the aim of this work was to assess the possibility of using sodium bicarbonate to provide *C. closterium* with carbon under conditions of intensive cultivation without supplying CO_2 to the suspension.

MATERIAL AND METHODS

C. closterium from IBSS culture collection was adapted to the conditions of intensive cultivation on a luminostat for two weeks. The culture was grown in the storage cultivation mode in a 1-L flask, on the RS nutrient medium prepared with sterile Black Sea water. The composition was as follows ($g \cdot L^{-1}$): $NaNO_3$ – 0.775; $NaH_2PO_4 \cdot 2H_2O$ – 0.0641; $Na_2SiO_3 \cdot 9H_2O$ – 0.386; Na_2EDTA – 0.0872; $FeSO_4 \cdot 7H_2O$ – 0.045; $CuSO_4 \cdot 5H_2O$ – $0.2 \cdot 10^{-3}$; $ZnSO_4 \cdot 7H_2O$ – $0.44 \cdot 10^{-3}$; $CoCl_2 \cdot 6H_2O$ – $0.2 \cdot 10^{-3}$; $MnCl_2 \cdot 4H_2O$ – $0.36 \cdot 10^{-3}$; and $NaMoO_4 \cdot H_2O$ – $0.12 \cdot 10^{-3}$ (Zheleznova et al., 2015). The algae were grown at a constant temperature of (20 ± 1) °C and 24-hour illumination with LB 40 lamps with a mean illumination of the working surface of 27 $W \cdot m^{-2}$ (12 klx). During adaptation, the culture was bubbled with air by a compressor unit (0.5 L of air *per* 1 L of culture *per* minute).

The first stage of the experiment. Upon reaching a culture density of 1 $g \cdot L^{-1}$ of dry weight, the part of the culture volume was centrifuged (3 minutes at 1450 g). After removing supernatant, fresh RS nutrient medium was added to a raw biomass, where 1.2 $g \cdot L^{-1}$ of sodium bicarbonate was previously dissolved. The resulting suspension with a volume of 1 L and a density of 1.2 $g \cdot L^{-1}$ was placed in a flask mounted on a magnetic stirrer. The suspension surface area (phase separation) was of 50 cm^2. Throughout the experiment, the culture was grown in the storage cultivation mode at a constant stirring speed of 250 rpm. The experimental setup is shown in Fig. 1.

The culture density was determined daily by the method of iodate oxidation (Gevorgiz et al., 2015), and pH value was measured with an accuracy of 0.01 by the Aqua Medic pH Controller equipped with a combined electrode.

The second stage of the experiment. On the 4^{th} day of the experiment, 1 g of $NaHCO_3$ and 2 mL of 0.1 N hydrochloric acid were added to the culture to lower pH down to 8.6.

Fig. 1. *C. closterium* cultivation on a nutrient medium with sodium bicarbonate as the sole carbon source

RESULTS AND DISCUSSION

The dynamics of the culture density and the nutrient medium pH are shown in Fig. 2. At the first stage of the experiment, the culture was adapted to the nutrient medium with sodium bicarbonate within a day; some cells died, as evidenced by decreases in the culture density down to 0.9 $g \cdot L^{-1}$ and in the medium pH down to 8.77. From the 2nd day of the experiment, an active culture growth was observed, with a maximum productivity of 0.6 $g \cdot (L \cdot day)^{-1}$. This growth was accompanied by a significant increase in the medium pH: cells actively assimilated carbon in the form of HCO_3^-. After adding 1 $g \cdot L^{-1}$ of sodium bicarbonate to actively growing culture and lowering pH down to 8.6 at the second stage of the experiment, the culture growth rate decreased to almost zero, but considering the medium pH increase during adaptation period, the culture actively absorbed bicarbonate ions (Fig. 2). After adaptation, there was an active culture growth, with a maximum productivity of 0.7 $g \cdot (L \cdot day)^{-1}$, which was accompanied by a high rate of the medium alkalization as well. When the medium pH reached 9.4, the culture growth slowed down; at pH = 9.9, the growth stopped completely. A day later, the culture entered the dying phase.

Based on the results obtained and considering the fact that dissolved carbon dioxide is practically absent in the nutrient medium with pH > 8.4 (Kratkaya khimicheskaya entsiklopediya, 1961 ; Sonnenfeld, 1988 ; Horne, 1972), it can be argued as follows: *C. closterium* culture grew actively, absorbing HCO_3^- ions from the nutrient medium (Kupriyanova & Samylina, 2015). Thus, in nutrient media for intensive cultivation of marine diatoms, it is quite possible to use $NaHCO_3$ (1 $g \cdot L^{-1}$ or more) as the sole carbon source.

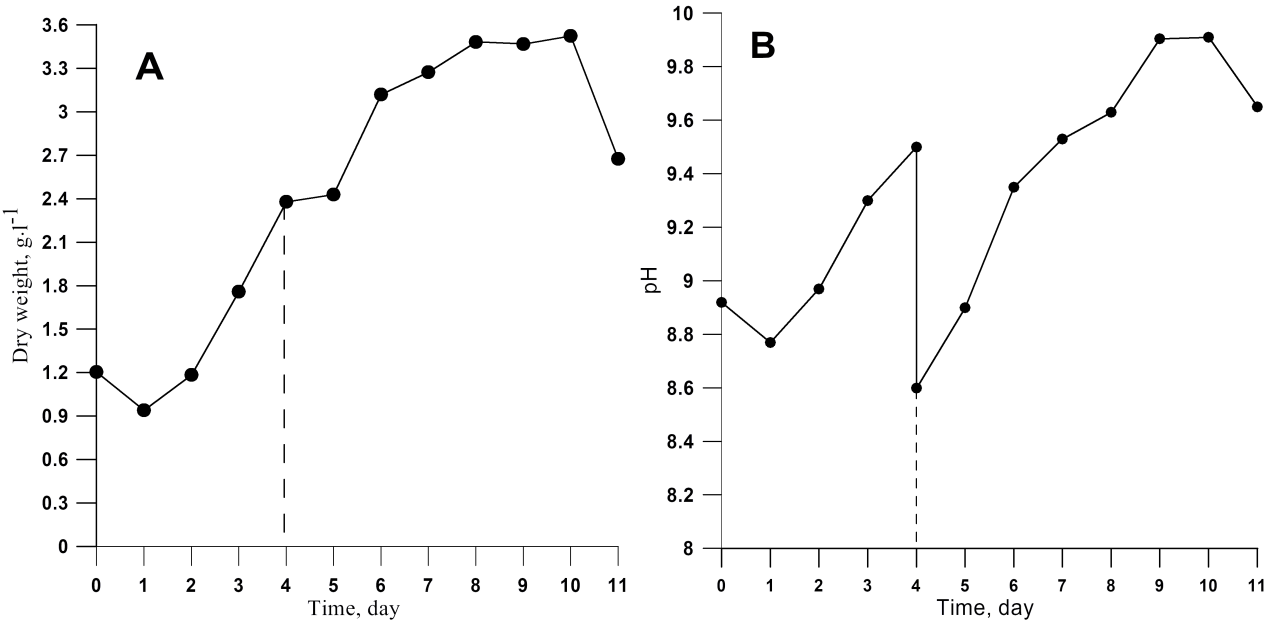

Fig. 2. Dynamics of the culture density when using sodium bicarbonate as the sole source of carbon (A) and pH dynamics during cultivation (B). The dotted line indicates the moment of adding 1 g of $NaHCO_3$ to the culture and lowering pH down to 8.6

Let us estimate limit values for an algal yield obtained on the nutrient medium with sodium bicarbonate. In general, when a nutrient from a dissolved inorganic salt is completely transformed into organic mass (without losses), and there are no losses related to the synthesis of extracellular metabolites, the maximum possible yield (B_{MAX}) is:

$$B_{MAX} = \frac{M(S)}{Y_S \cdot M(SX)} \cdot m(SX)\,, \tag{1}$$

where Y_S is nutrient content in the biomass;

M(S) and M(SX) are molar masses of a nutrient and salt containing a nutrient, respectively, g·mol^{-1};

m(SX) is the mass of salt dissolved in the nutrient medium, g·L^{-1}.

Carbon content in biomass of many microalgae species is about 50 % (Horne, 1972 ; Allen et al., 2011). However, due to high ash residue content in biomass of benthic diatoms, this value varies considerably (Anderson, 1995 ; Brown & Jeffrey, 1995). According to the literature, in the active growth phase, *Cylindrotheca* sp. microalgae biomass includes as follows: total proteins – 41 % of dry weight (Brown & Jeffrey, 1995 ; Brown et al., 1997); carbohydrates – 25 % (Gügi et al., 2015 ; Nesara & Bedi, 2019); and lipids – 1 % (Ying & Kangsen, 2005). Considering that carbon content in proteins averages 52 %; in carbohydrates, 40 %; and in lipids, 75 % (Kratkaya khimicheskaya entsiklopediya, 1961), we can assume that carbon content in the organic part of *C. closterium* biomass is 48 %.

Considering ash residue content in microalgae, the expression (1) takes the form:

$$B_{MAX} = \frac{M(C)}{(1-z) \cdot Y_C^{\mathrm{ORG}} \cdot M(NaHCO_3)} \cdot m(NaHCO_3)\,, \tag{2}$$

where z is ash residue content in the biomass;

Y_C^{ORG} is carbon content in the organic part of the biomass;

M(C) and M($NaHCO_3$) are molar masses of carbon and sodium bicarbonate, respectively, g·mol^{-1}; m($NaHCO_3$) is the mass of sodium bicarbonate dissolved in the nutrient medium, g·L^{-1}.

According to our data, the ash residue content in *C. closterium* biomass is 33 % (Gevorgiz et al., 2015); in the experiment, the weighed portion of $NaHCO_3$ dissolved in the nutrient medium is 1.2 g·L^{-1}. Therefore, substituting these values into (2), the maximum biomass increase (yield, B_{MAX}) is 0.53 g·L^{-1}. Considering carbon (total carbon in the form of HCO_3^- and CO_3^{2}) in Black Sea water, the concentration of which reaches 0.007 g·L^{-1} (Kratkaya khimicheskaya entsiklopediya, 1961), B_{MAX} is 0.575 g·L^{-1}.

For 4 days in the experiment, the yield was 1.2 g·L^{-1} (see Fig. 2A), which is more than double the limiting estimate. On the other hand, 1.2 g of $NaHCO_3$ was added to the nutrient medium. In the experiment, the increase was 1.2 g·L^{-1} of algal dry mass; then, taking into account carbon in Black Sea water, at least 2.66 g of $NaHCO_3$ should be consumed. This results from the fact as follows: in 1.2 g of biomass, the organic part is $1.2 \times (1 - 0.33) = 0.8$ g; carbon content in the organic part is $0.8 \times 0.48 = 0.384$ g; and carbon content in $NaHCO_3$ is 14.3 %. It has to be considered that in a nutrient medium at pH > 8.4, due to the hydrolysis of bicarbonate ions (Skopintsev, 1975 ; Sonnenfeld, 1988 ; Horne, 1972), the equilibrium in

$$HCO_3^- + HO^- + H^+ \rightleftarrows H_3O^+ + CO_3^{2-} \quad (3)$$

is shifted to the right, towards CO_3^{2-} formation; so, in the nutrient medium at high pH values, some carbon is in the form inaccessible to photosynthesis (Kupriyanova & Samylina, 2015 ; Jansson & Northen, 2010). Consequently, obviously, not all carbon from $NaHCO_3$ salt, which is dissolved in the nutrient medium, is absorbed by cells for photosynthesis; some carbon in the carbonate form remains in the nutrient medium.

Let us estimate the amount of $NaHCO_3$ to be used to increase biomass by 1.2 g at pH > 8.4. Phototrophic cells are known to release 1 mole of OH^- hydroxide ions into the nutrient medium (Jansson & Northen, 2010) when absorbing 1 mole of HCO_3^- hydrogen carbonate ions for photosynthesis. As a result, the equilibrium in (3) is shifted to the right, and 1 mole of CO_3^{2-} carbonate ions is formed. Thus, a decrease in HCO_3^- in the nutrient medium is associated both with the removal of bicarbonate ions by cells for photosynthesis and with the formation of carbonate ions in the nutrient medium. Therefore, in order to obtain 1.2 g of biomass, it is necessary to use at least $1.2 \times (1 - 0.33) \times 0.48 / 0.143 \times 2 = 5.4$ g of $NaHCO_3$, which is more than 4 times a weighed portion of sodium bicarbonate dissolved in the nutrient medium in the experiment.

Such an obvious discrepancy is likely to be due to the fact that atmospheric CO_2 was actively dissolved in the culture medium. Even though the interface-specific phase in the experiment was small, the dissolution rate of carbon dioxide in the nutrient medium was sufficiently high for *C. closterium* intensive growth. The 2nd to the 4th day of the experiment, the biomass increase was estimated at 1.2 g·L^{-1} (see Fig. 2A); 1.2 g of *C. closterium* dry biomass contained 0.387 g of organic carbon. Thus, 0.171 g·L^{-1} of inorganic carbon was added to the nutrient medium. Within two days of the experiment, at least $0.387 - 0.171 = 0.216$ g of carbon was dissolved in the nutrient medium, or 0.4 g·(L·day)$^{-1}$ of CO_2. Importantly, the estimate is approximate; to calculate the absorption rate of atmospheric CO_2 by *C. closterium* culture, further special research is required.

Conclusion. The possibility of cultivating the marine benthic diatom *C. closterium* on a nutrient medium with high sodium bicarbonate content has been experimentally shown. As found, on the RS nutrient medium with 1.2 g·L^{-1} of sodium bicarbonate added under conditions of intensive cultivation,

C. closterium productivity reaches 0.7 g·(L·day)$^{-1}$, with a significant increase in the medium pH. According to our data, optimal medium pH for *C. closterium* growth is in the range of 8.4–9.4. At higher values (pH > 9.4), the growth of diatoms slows down; at pH = 9.9, the culture enters the dying phase.

The development of nutrient media with sodium bicarbonate for intensive diatom cultivation is a promising task, since it greatly facilitates the supplying of culture with carbon, especially on an industrial scale. Adding bicarbonates to a culture medium increases the system buffer capacity and excludes sharp changes in pH, as well as the loss of carbon in the form of CO_2. Moreover, the use of culture media with hydrocarbons does not exclude the processes of CO_2 absorption from the atmosphere, even with a small interface area. According to the experimental data, during active growth, the culture received at least 50 % of atmospheric carbon.

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ИНТЕНСИВНАЯ КУЛЬТУРА *CYLINDROTHECA CLOSTERIUM* (EHRENBERG) REIMANN ET LEWIN НА ПИТАТЕЛЬНОЙ СРЕДЕ С ГИДРОКАРБОНАТОМ НАТРИЯ

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Экспериментально показана возможность использования гидрокарбоната натрия в питательной среде для обеспечения культуры *C. closterium* углеродом в условиях интенсивного культивирования без подачи CO_2 в суспензию. После адаптации *C. closterium* к питательной среде с гидрокарбонатом натрия с концентрацией 1,2 г·л$^{-1}$ наблюдался активный рост с максимальной продуктивностью 0,6–0,7 г·(л·сут)$^{-1}$ сухой массы. В клетки диатомовых водорослей углерод проникает как в форме углекислого газа, так и в форме гидрокарбонат-ионов. Однако все питательные среды для искусственного культивирования диатомей по-прежнему предполагают применение CO_2 из атмосферы или баллона. Цель работы — оценить возможность использования гидрокарбоната натрия для обеспечения *C. closterium* углеродом в условиях интенсивного культивирования без подачи CO_2 в суспензию. Культуру выращивали в режиме накопительного культивирования в колбе объёмом 1 л на питательной среде RS, приготовленной на стерильной черноморской воде, следующего состава (г·л$^{-1}$): $NaNO_3$ — 0,775; $NaH_2PO_4 \cdot 2H_2O$ — 0,0641; $Na_2SiO_3 \cdot 9H_2O$ — 0,386; Na_2EDTA — 0,0872; $FeSO_4 \cdot 7H_2O$ — 0,045; $CuSO_4 \cdot 5H_2O$ — $0{,}2 \cdot 10^{-3}$; $ZnSO_4 \cdot 7H_2O$ — $0{,}44 \cdot 10^{-3}$; $CoCl_2 \cdot 6H_2O$ — $0{,}2 \cdot 10^{-3}$; $MnCl_2 \cdot 4H_2O$ — $0{,}36 \cdot 10^{-3}$; $NaMoO_4 \cdot H_2O$ — $0{,}12 \cdot 10^{-3}$. Предварительно в ней растворили 1,2 г·л$^{-1}$ гидрокарбоната натрия. Суспензию клеток перемешивали посредством магнитной мешалки (250 оборотов в минуту). На 4-й день эксперимента в культуру добавили 1 г $NaHCO_3$ и 2 мл 0,1 н соляной кислоты, чтобы снизить pH до 8,6. Со 2-го дня эксперимента зарегистрирован активный рост с максимальной продуктивностью 0,6 г·(л·сут)$^{-1}$. После добавления в активно растущую культуру 1 г·л$^{-1}$ гидрокарбоната натрия и снижения pH до 8,6 наблюдали снижение скорости роста практически до нуля, однако, судя по скорости повышения pH среды́ за время адаптации, культура активно поглощала гидрокарбонат-ионы. Экспериментально показана возможность культивирования бентосной диатомовой водоросли *C. closterium* на питательной среде с высоким содержанием гидрокарбоната натрия. Установлено, что на питательной среде RS с добавлением 1,2 г·л$^{-1}$ гидрокарбоната натрия в условиях интенсивного культивирования максимальная продуктивность *C. closterium* достигает 0,7 г·(л·сут)$^{-1}$, при этом отмечено существенное повышение pH среды́. По нашим данным, оптимальное значение pH среды́ для роста *C. closterium* находится в диапазоне 8,4–9,4. При pH > 9,4 рост диатомовых водорослей замедляется, а при достижении в питательной среде значения pH 9,9 культура переходит в фазу отмирания.

Ключевые слова: питательная среда, культивирование, диатомовые водоросли, гидрокарбонат натрия