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## DYNAMICS OF CHLOROPHYLL AND PARAMYLON ACCUMULATION IN *EUGLENA GRACILIS* CELLS AT MIXOTROPHIC CULTIVATION

V. M. Mokrosnop

M.G. Kholodny Institute of Botany, NAS of Ukraine, 2, Tereshchenkivska St., Kyiv 01601, Ukraine  
e-mail: VictoryM6@yandex.ua

Microalgae *E. gracilis* is capable of using ethanol as a carbon and energy source for growth both in the light and in the dark. Ethanol is efficiently utilized under illumination of the culture. At mixotrophic cultivation, the dynamics of accumulation of chlorophyll and paramylon – the main reserve polysaccharide of *E. gracilis*, was significantly different from the autotrophic control. Chlorophyll content in the mixotrophic cells at moderate intensities of light ( $100\text{--}250 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) at the beginning of the exponential growth phase decreased and then increased steadily to reach a stationary growth phase. On the contrary, the content of paramylon, was maximal at the lag phase, and decreased in an exponential growth phase. Thus, the synthesis of photosynthetic pigment-containing complexes was delayed at the beginning of cultivation, in the presence of ethanol, and cell growth was mainly due to the substrate uptake. Probably, after assimilation of exogenous ethanol, the observed intensive growth of the culture was provided by the photosynthetic conversion of light energy and usage of carbon deposited in paramylon.

**Keywords:** *Euglena gracilis*, mixotrophic cultivation, ethanol, chlorophyll, paramylon.

### INTRODUCTION

The photosynthetic efficiency of microalgae is much higher than land plants, its commercial cultivation is viewed therefore as a promising area of biotechnology, that can facilitate the production of many useful compounds [28, 29]. The metabolic flexibility enables microalgae to use different organic substrates for nutrition and to adapt to existence in wide range of environment conditions [20, 23]. Unicellular microalga *E. gracilis* attracts an increasing attention in this direction due to ability to simultaneous accumulation of several useful products: amino acids, vitamins, fat acids, paramylon [19, 21]. *E. gracilis* has hybrid photoautotroph-heterotrophic genome in which a lateral genes transfer and a fusion of genes was found, so that the organism is widely used in evolution investigations [1].

Microalga *E. gracilis* belongs to the Protista kingdom. The organism is able to photosynthesis and can also absorb different organic substrates from the environment in the light and in the dark. *E. gracilis* can use ethanol as organic substrate, despite the fact that it is toxic for majority of microorganisms [26]. The addition of ethanol in culture

medium of *E. gracilis* influences on the biochemical composition and the physiological parameters of the cells, because of the using of ethanol as substrate for *E. gracilis* is under active investigation [4, 8, 13, 20, 25]. The physiological effects of ethanol on the cells of *E. gracilis* are the stimulation of cell respiration, the prevention of losses of mitochondrial enzymes after transition heterotrophic cells on the photoautotrophic cultivation, the repression of light-induced synthesis of chloroplast enzymes and light-harvesting chlorophyll *a/b*-binding protein of photosystem II [13, 24]. Biotechnological value of the cultivation of *E. gracilis* in the presence of ethanol consist in the increase in the biomass, the stimulation of protein,  $\alpha$ -tocopherol and paramylon accumulation in the cells [2, 25]. The investigation of ethanol influence on the metabolism of heterotrophically cultivated cells of *E. gracilis*, showed that this alcohol rapidly oxidized in the cells to acetate, 50 % of which incorporated into paramylon [9]. There is evidence that ethanol as a substrate inhibits the glycolytic conversion of glucose into pyruvate and incorporation of these molecules into lipids and proteins, while a larger percent of glucose incorporates into paramylon [13].

Paramylon is the  $\beta$ -1,3-glucan – a storage polysaccharide of *E. gracilis*. Paramylon has highly crystalline and fibrillar structure and occurs as membrane-bound granules in the cytosol [12, 14, 22]. The crystallization level of the paramylon granules reaches 90 %, what distinguish this polysaccharide from the other storage products of plants and algae [6, 16]. Paramylon has promising prospects of application in medicine and veterinary medicine. The biological active and medicinal properties of paramylon include its ability to stimulate the immune system and protection against viral and bacterial infections, antitumor and radioprotective effects [5, 17]. It should be also noted that this polysaccharide helps to reduce cholesterol and regulates glycometabolism [12, 14]. Paramylon as the other  $\beta$ -glucans provides a remarkable range of health benefits, and is especially important against the two most common conventional causes of death in industrialized countries, i.e. cardiovascular diseases (where they promote healthy cholesterol and blood glucose levels) and cancer (where they enhance immune system functions). In this work we investigated the effect of ethanol as a substrate on the culture growth and the paramylon accumulation in the cells during the cultivation of *E. gracilis*. Changes of chlorophyll concentration were also studied.

## MATERIALS AND METHODS

*Microalgae and culture conditions.* *E. gracilis* var. *bacillaris* culture was obtained from the algae collection of the Institute of Biophysics and Cell Engineering of NAS of Belarus. The cells were grown without agitation and aeration in 250 mL Ehrlenmeyer's flasks containing 200 mL of salt medium [18] at 25 °C. The experimental types of cultures differed in the culture medium composition and intensity of continuous illumination. There were two types of photoheterotrophic cultures including culture with 100 mM ethanol (variant **Et**) and combination of 100 mM ethanol and 40 mM glutamate (variant **EtGt**). Photoautotrophic culture was used as a control variant. All variants of the cultures were grown under different light intensity: 20, 100 and 250  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ .

*Determination of the growth parameters of the cultures.* The number of the cells in ml of the cultural suspensions was counted in Goryaev's chamber using light microscope ( $\times 150$ ). The rate of cultures growth ( $r$ ) was determined by the formula [3]:

$$r = \frac{\ln(N_t / N_0)}{\Delta t},$$

where  $N_0$  – the amount of the cells in the volume unit at time 0;  $N_t$  – the amount of the cells after time period  $\Delta t$ .

**Total chlorophyll determination.** Chlorophylls were extracted from the cells with acetone. The optical density of extracts was measured at 662 and 646 nm and the content of pigments was calculated from the formulae given in [15].

**Paramylon determination.** Cells (aliquots of culture  $5 \times 10^6$ ) were harvested by centrifugation at 3000 rpm for 5 min, the supernatant was discarded and the pellet was washed twice by ddH<sub>2</sub>O. Pigments were extracted by 96 % ethanol to receive a cell's pellet of white color. The supernatant was discarded, 2 ml of 1 % sodium dodecyl sulfate was added to the pellet and this mixture was vortexed for 1 min before heating in boiling water for 10 min. Next, the sample was centrifuged at 10 000 rpm for 10 min and the supernatant discarded and this step was repeated twice. The final pellet was resuspended in 0.5 ml of 1 N NaOH. To determine the amount of free glucose in the final NaOH solution, a 100  $\mu$ l aliquot was mixed with 1 ml of 5 % phenol and 1.6 ml of concentrated (95–98 %) H<sub>2</sub>SO<sub>4</sub>. The absorbance was determined at 490 nm [11], after incubation the sample in thermostat (27–30 °C) for 10 min. Absorbance signal from experimental samples was transformed to glucose equivalents using the calibration curve, where minimal and maximal concentration limits of D-glucose were 50 and 400  $\mu$ g, respectively.

**Statistical analysis of the results.** Statistical analysis of the results was performed using the methods of variation statistics using the software package Excel.

## RESULTS AND DISCUSSION

**Growth of the mixotrophic cultures *E. gracilis* under different light intensity.** Among three variants of continuous illumination intensities (20, 100 and 250  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ), 100  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  was the most favorable for biomass accumulation by cultures of *E. gracilis* (Table 1). The largest increase in the cell concentrations of the cultures was at time 2–5 days of cultivation (Table 2). The exponential growth phase begun at 2–3<sup>th</sup> days of cultivation and continued up to the 10<sup>th</sup> day in the all variants of the cultures, when transition growth phase began. The ratio of the cellular concentrations in the cultures grown at light intensity 100  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  was: 1.0 : 4.8 : 5.54 (control : ethanol : ethanol+glutamate Na) at the 15<sup>th</sup> day. The duration of stationary growth phase was 15–20 days and the secondary growth of the cultures was observed at the 23<sup>th</sup> day.

Table 1. Cells' concentration in the stationary growth phase in cultures of *E. gracilis* grown at different light intensity

Таблиця 1. Концентрація клітин у культурах *E. gracilis* у стаціонарній фазі росту, вирощених за різної інтенсивності освітлення

Culture	Stationary growth phase, 15 <sup>th</sup> day of cultivation (cells $\times 10^9$ /ml)		
	20 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$	100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$	250 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$
Control	0.23	0.84	0.71
Ethanol	2.14	4.00	3.67
Ethanol + glutamate Na	3.24	4.65	4.21

The mixotrophic cultures grew faster during the early exponential growth phase (2–5 days). But in the next three days, the growth rate ( $r$ ) of the mixotrophic cultures

dropped almost in half. In the same time, the growth intensity of the autotrophic culture did not change up to stationary growth phase. The increase in biomass *E. gracilis* grown with ethanol and glutamate Na was larger in the first days of exponential growth phase, which was in whole longer than for the culture grown with ethanol only.

**Table 2. The growth rates ( $r$ ) of *E. gracilis* cultures at light intensity of  $100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  during 2–15 days of cultivation**

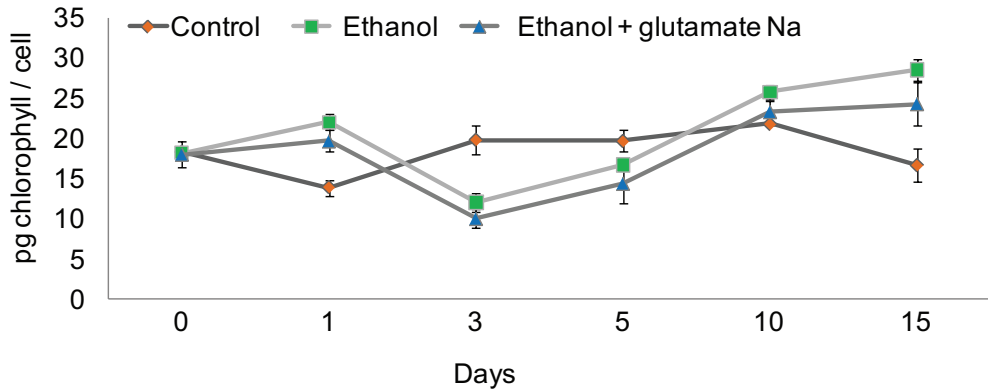
**Таблиця 2. Швидкості росту ( $r$ ) культур *E. gracilis* при освітленні  $100 \text{ мкмоль}\cdot\text{м}^{-2}\cdot\text{с}^{-1}$  протягом 2–15 діб культивування**

Culture	Duration of cultivation			
	2–5 <sup>th</sup> days	5–8 <sup>th</sup> days	8–10 <sup>th</sup> days	10–15 <sup>th</sup> days
Control	0.22	0.21	0.22	0.054
Ethanol	0.63	0.30	0.09	0.048
Ethanol + glutamate Na	0.7	0.31	0.11	0.056

*E. gracilis* in the mixotrophic cultural conditions accumulated sufficient reserve of carbon in the form of organic substrates and ATP, formed in the photochemical reactions to support anabolic processes in the cells and culture growth. In the absence of organic carbon sources, the rate of the culture growth was significantly lower as it was observed in the autotrophic culture [27]. The low light intensity ( $20 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) did not promote biomass accumulation in mixotrophic cultures because the cells lacked enough energy in result of photosynthesis restriction. In these conditions, mitochondrial oxidation of available organic substrates perhaps was also inhibited due to the reduced activity of water photolysis and the lowered oxygen concentration in the medium.

*Accumulation of chlorophylls in the cells of mixotrophic cultures of E. gracilis under different light intensity.* The main difference between control and mixotrophic variants of the cultures was noted in a level and dynamics of photosynthetic pigments accumulation. The decreasing in chlorophylls content in comparison to the initial values in the cultures with ethanol or the combination of ethanol and glutamate Na by 34 and 45 % respectively, was observed in the exponential growth phase at the 3<sup>rd</sup> day of cultivation with the light intensity of  $100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  (Fig. 1). Declines in chlorophyll content were ~60–80 % at higher light intensity ( $250 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) (data not presented). Such changes in pigment amount in the mixotrophic variants were not observed at the low light intensity ( $20 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) and the dynamics of accumulation was similar to the control variant. Increasing of chlorophylls content in the mixotrophic cultures occurred at 5–10 days and continued to the last 15<sup>th</sup> day of cultivation. The ratio of chlorophylls concentrations in the cultures at the 3<sup>rd</sup> and 5<sup>th</sup> day were: 1.97 : 1.19 : 1 and 1 : 1.71 : 1.46. According to the published and our data, the level of chlorophylls in the cells of autotrophic culture of *E. gracilis* negatively correlated with intensity of light during its growing [7]. Comparison of mixotrophic cultures that were grown at 100 and  $250 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  showed the same trend. Observed decline of chlorophylls after the lag phase and early exponential growth phase is likely consequence of the presence of organic substrate in the medium. It is known that the induction of the synthesis of chlorophyll-containing proteins of light-harvesting complexes is catabolite-sensitive [4, 24]. The light intensity  $250 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  caused a decrease in chlorophylls content in the cells due to the more intensive process of photodestruction of these pigments [10]. Increasing chlorophylls content at the 5<sup>th</sup> day in the mixotrophic cultures may indicate an activation of photosyn-

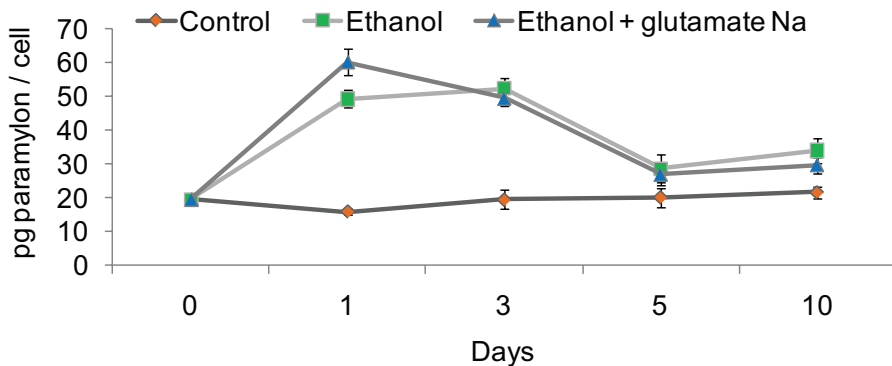
thetic apparatus development after assimilation of available organic substrate by the cells. At the low light intensity the level of chlorophylls remained almost unchanged during the most of the cultivation time, what may be linked with much lower probability of photodestruction and rebuilding processes of photosynthetic apparatus in this conditions.



**Fig. 1.** Dynamics of chlorophyll accumulation in cells of the mixotrophic cultures of *E. gracilis* at light intensity of  $100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$

**Рис. 1.** Динаміка накопичення хлорофілів у клітинах міксотрофних культур *E. gracilis* на світлі інтенсивністю  $100 \text{ мкмоль}\cdot\text{м}^{-2}\cdot\text{с}^{-1}$

*Accumulation of paramylon in the cells of mixotrophic cultures of E. gracilis in light intensity  $100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ .* Paramylon accumulation in the cells of the mixotrophic cultures showed a variable dynamics in comparison with control (Fig. 2).



**Fig. 2.** Dynamics of paramylon accumulation in cells of the mixotrophic cultures of *E. gracilis* at light intensity of  $100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$

**Рис. 2.** Динаміка накопичення парамілоу у клітинах міксотрофних культур *E. gracilis* на світлі інтенсивністю  $100 \text{ мкмоль}\cdot\text{м}^{-2}\cdot\text{с}^{-1}$

Sharp increase in the amount of the storage polysaccharide in the mixotrophic cells was observed at the first day of cultivation, and succeeded by the decreasing its content at the 5th day. The maximum content of the polysaccharide was registered in the cells of the culture, which was grown in the presence of ethanol and glutamate Na. The con-

tent of paramylon in this mixotrophic culture exceeded 18 % the mixotrophic culture supplemented with ethanol only. An increase in paramylon content after 1–3 days of mixotrophic cultivation can be explained by active assimilation of carbon of organic substrates from the nutrient medium in lag phase of microalgae growth. Only the slight increase in paramylon content was observed during the first 5–10 days of autotrophic cultivation of *E. gracilis* cells. It is known, when ethanol is used as nutrient substrate, the metabolism of the cells of *E. gracilis* changes toward synthesis of glucose, 70 % of which incorporated into paramylon [13]. Our data suggest that the exogenously added ethanol affects the growth and the accumulation of chlorophylls and paramylon dynamics not only as a source of carbon and energy for the cells of *E. gracilis*, but also as a regulatory factor of the cellular metabolism.

## CONCLUSIONS

The obtained results suggest that in the presence of exogenous ethanol the cells of mixotrophic culture of *E. gracilis* accumulate the storage polysaccharide paramylon at the lag growth phase. During a subsequent cultivation, the content of chlorophylls increases and an intensive growth of the culture is observed due to photosynthetic conversion of light energy and usage of carbon stored in the paramylon.

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## ДИНАМІКА НАКОПИЧЕННЯ ПАРАМІЛОНУ ТА ХЛОРОФІЛІВ У КЛІТИНАХ *EUGLENA GRACILIS* ЗА МІКСОТРОФНИХ УМОВ КУЛЬТИВУВАННЯ

**В. М. Мокросноп**

Інститут ботаніки ім. М.Г. Холодного НАН України  
вул. Терещенківська, 2, Київ 01601, Україна  
e-mail: VictoryM6@yandex.ua

Мікрородорість *E. gracilis* здатна використовувати етанол як джерело вуглецю та енергії під час вирощування як на світлі, так і в темряві. Етанол ефективно утилі-

зується мікроводорістю в умовах освітлення, у цьому разі динаміка накопичення хлорофілів і парамілону – основного запасного полісахариду *E. gracilis*, суттєво відрізнялася від автотрофного контролю. За помірних інтенсивностей освітлення ( $100\text{--}250$  мкмоль·м<sup>-2</sup>·с<sup>-1</sup>) на початку експоненційної фази росту в міксотрофно культивованих клітинах вміст хлорофілів знижується, а потім стабільно зростає до досягнення культурою стаціонарної фази росту. У цьому разі вміст парамілону, навпаки, сягає свого максимуму в лаг фазі росту культури, знижуючись в експоненційній фазі. Отже, на початку культивування за наявності етанолу утворення фотосинтетичних пігментовмісних комплексів гальмується, і живлення клітин здійснюється переважно завдяки поглинанню субстрату. Подальший інтенсивний ріст культури відбувається завдяки фотосинтетичному перетворенню світлової енергії та використанню вуглецю, депонованого у парамілоні, вірогідно, після поглинання етанолу зі середовища.

**Ключові слова:** *Euglena gracilis*, міксотрофне культивування, етанол, хлорофіл, парамілон.

## ДИНАМИКА НАКОПЛЕНИЯ ПАРАМИЛОНА И ХЛОРОФИЛЛОВ В КЛЕТКАХ *EUGLENA GRACILIS* ПРИ МИКСОТРОФНЫХ УСЛОВИЯХ КУЛЬТИВИРОВАНИЯ

**В. М. Мокросноп**

Институт ботаники им. Н.Г. Холодного НАН Украины  
ул. Терещенковская, 2, Киев 01601, Украина  
e-mail: VictoryM6@yandex.ua

Мікроводоросль *E. gracilis* способна использовать этанол в качестве источника углерода и энергии при выращивании как на свету, так и в темноте. Этанол эффективно утилизируется микроводорослью при освещении, причем динамика накопления хлорофиллов и парамилонна – основного запасного полисахарида *E. gracilis*, при миксотрофном культивировании существенно отличалась от автотрофного контроля. При умеренных интенсивностях освещения ( $100\text{--}250$  мкмоль·м<sup>-2</sup>·с<sup>-1</sup>) в начале экспоненциальной фазы роста содержание хлорофиллов в миксотрофно культивируемых клетках снижалось, а затем стабильно возрастало до достижения стационарной фазы роста. При этом содержание парамилонна, напротив, достигало своего максимума в лаг фазе, снижаясь в экспоненциальной фазе роста. Таким образом, в начале культивирования в присутствии этанола подавляется синтез фотосинтетических пигментосодержащих комплексов, и рост клеток обеспечивается преимущественно за счет поглощения субстрата. Последующий интенсивный рост культуры происходит за счет фотосинтетического преобразования световой энергии и использования углерода, депонированного в парамилоне, вероятно, после ассимиляции этанола из среды.

**Ключевые слова:** *Euglena gracilis*, миксотрофное культивирование, этанол, хлорофилл, парамилон.

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