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Analysing the Influence of Cultivation Conditions on the Activity of Metabolic Pathways of Bcaa Biosynthesis in Chlorella Vulgaris Microalgae

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The influence of cultivation conditions on the productivity of strains of *Chlorella vulgaris Beijer* IPPAS C-1 *(Chlorella sorokiniana)*, *Chlorella vulgaris Beijer* IPPAS C-2, *Chlorella kessleri Fott et Nov* C-9 *(Parachlorella kessleri)*, *Scotiellopsis Vinatzer* IPPAS C-35, *Chlorella vulgaris Beijer IPPAS* C-38 has been studied experimentally. The microalgae strain *Chlorella vulgaris Beijer* IPPAS C-1 *(Chlorella sorokiniana)* was found to be promising in terms of protein production. Under autotrophic cultivation conditions at PAR = 150 μmol photons/m^2•s and temperature 30 ± 2 °C the amount of the biomass has increased by 10-15 times during 4-5 days of cultivation. When the cultivation temperature is lowered under mixotrophic conditions, *Chlorella vulgaris Beijer* IPPAS C-1 increases the synthesis of intracellular proteins and branched-chain amino acids.

1. Introduction

The world's population is steadily on the rise and, according to the latest UN data, if current trends in society persist, the amount of people suffering from hunger will reach 840 million by 2030 (UN World Food Program, 2020). In view of this, research aimed at finding alternative sources of raw materials for the production of foodstuffs that can quickly make up for the shortage of food is as relevant as ever.

Microalgae cells are a good source of protein, fats, complex carbohydrates, vitamins A, B1, B2, B3, B6 and C, as well as macro- and microelements (magnesium, potassium, iron, phosphorus, calcium) and antioxidants. They have a flexible metabolism: by changing the conditions of cultivation it is possible to obtain a product with a desired composition and a different ratio of proteins, fats, carbohydrates, antioxidants. In addition, these organisms have a higher photosynthetic efficiency than higher plants (Chisti, 2013). More than 70,000 species of algae and cyanobacteria are currently known, of which about 4,600 microalgae species are available to researchers in pure form, but only a few dozen strains belonging mainly to the genera *Arthrospira* are industrially cultivated: *Aphanizomenon (Cyanophyta)*, *Chlorella*, *Haematococcus*, *Dunaliella*, *Scenedesmus*, *Tetraselmis (Chlorophyta)*, *Nannochloropsis*, *Phaeodactylum (Ochrophyta)* (Sinetova et al., 2019). Thus, the biotechnological potential of microalgae remains largely unexplored and studying new strains, their biochemistry and physiology is an urgent research task.

The proteins of most microalgae contain almost all essential amino acids, except for the sulphur-containing methionine and cysteine. Protein derived from algae is of the same quality as other plant proteins and can be used in a certain dose in human and domestic animal diets (Graziani et al., 2013). The amount of proteins in microalgae and their amino acid composition can vary widely depending on the cultivation conditions (type of nutrition, temperature, illumination level). Depending on the cultivation conditions microalgae proteins may contain different ratios of essential and non-essential amino acids, including branched-chain amino acids (BCAAs). The search for strains capable of synthesizing proteins containing these amino acids has high potential.

One of the problems complicating industrial cultivation of microalgae is the fact that once the culture reaches a certain density, its growth rate is inhibited by the lack of illumination due to the self-shadowing effect (Sinetova et al., 2012). Therefore, since microalgae are capable of autotrophic and mixotrophic metabolic pathways

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(Brennan et al., 2010), it is advisable to investigate the potential of these cultivation regimes in order to increase the economic efficiency of the process.
The aim of the study was to determine the regularity of the influence of cultivation conditions of five *Chlorella vulgaris* strains on the protein and BCAA content in the biomass.

2. Methods and materials

2.1 Biomass cultivation

Experiment 1.
The aim of Experiment 1 was to identify the microalgae strain with the highest potential for producing protein and BCAAs, and to analyse their growth and metabolism under the cultivation conditions presented below. The following strains were used for this study: *Chlorella vulgaris Beijer* IPPAS C-1 *(Chlorella sorokiniana)*, *Chlorella vulgaris Beijer* IPPAS C-2, *Chlorella kessleri Fott et Nov* C-9 *(Parachlorella kessleri)*, *Scotiellopsis Vinatzer* IPPAS C-35, *Chlorella vulgaris Beijer* IPPAS C-38, obtained from Timiryazev Institute of Plant Physiology of the Russian Academy of Sciences. Each microalgae strain was cultivated on Tamiya nutrient medium under autotrophic and mixotrophic conditions (Tamiya medium with the addition of glucose at a concentration of 5 g/l). The process of cultivation was carried out in 5-litre photobioreactors under the following conditions (Table 1): 1) the amount of seed material, taken at a stationary growth stage, was 10% of the total suspension volume; 2) the samples were grown at the temperature of 30 ± 2 °C and photosynthetically active radiation level 150 (μmol photons)/(m^2•s); 2) pH level varied in the range of 6. 2..8.0; 3) aeration of the suspension (80 L/h) was carried out with an air-gas mixture containing 0.03% of carbon dioxide.

Table 1: Experimental modes

Mode	Strain	Photosynthetically active radiation (PAR), μmol photons/m^2•s	Temperature (T), °C	Type of nutrition
1	*Chlorella vulgaris Beijer* IPPAS C-1	150	30±2	Autotrophic
2	*Chlorella vulgaris Beijer* IPPAS C-1			Mixotrophic
3	*Chlorella kessleri Fott et Nov* C-9			Autotrophic
4	*Chlorella kessleri Fott et Nov* C-9			Mixotrophic
5	*Chlorella vulgaris Beijer IPPAS* C-2			Autotrophic
6	*Scotiellopsis Vinatzer IPPAS* C-35			Autotrophic
7	*Chlorella vulgaris Beijer IPPAS* C-38			Autotrophic

Experiment 2.
Based on the results of Experiment 1, the strain most promising in terms of producing the highest amount of biomass and water-soluble proteins was selected. The cells of the selected microalgae were cultured under the conditions presented in Table 2. Each sample was cultured in 5-litre photobioreactors under the following conditions (see Table 2): 1) the seed material was taken at a stationary growth stage and amounted to 10 % of the total suspension volume; 2) the nutrient medium was Tamiya under autotrophic and mixotrophic conditions (Tamiya medium with glucose added at a concentration of 5 g/L); 3) the suspension was aerated (80 L/h) with an air-gas mixture containing 0.03 % of carbon dioxide; 4) pH level varied in the range of 6.2...8.0.

Table 2: Experimental modes

Mode	Photosynthetically active radiation (PAR), μmol photons/m^2•s	Temperature, °C	Type of nutrition
1	150	30±2	Autotrophic
2	150	30±2	Mixotrophic
3	150	20±2	Autotrophic
4	150	20±2	Mixotrophic
5	30	30±2	Autotrophic
6	30	30±2	Mixotrophic
7	30	20±2	Autotrophic
8	30	20±2	Mixotrophic

2.2 Processing and analysis of microalgae cells

Calculation of microalgae cell concentration in the suspension was carried out by the method of direct count in the Goryayev chamber.

Sampling of microalgae biomass (500 mL) for cell mass determination and intracellular water-soluble protein extraction was performed prior to the experiment, as well as on the 2nd, 4th, 6th, and 9th day of culturing. Separation of fugate from microalgae biomass was carried out using a Sigma 2-16 RK/2-16P centrifuge at a rotation speed of 4000 r/min for 10 minutes.

Microalgae cells were disintegrated in the form of paste with humidity of 98 - 99 % using an ultrasonic disintegrator Scientz IID at ultrasound frequency of 25 kHz and power of 100 W over the period of 1 minute.

Microalgae cells were dried to determine the cell concentration in the suspension (g/L) in a dry-air oven "HS-121A" at 80 °C, to reach the constant weight Δ=0.01 g.

Protein extraction from microalgae biomass was carried out for 24 hours at 4 °C using phosphate buffer as solvent (pH 7.2-7.4).

The protein content of the extract was determined using the spectrophotometric method (Aitken et al., 2020) and the Bradford method (Bradford, 1976). The average value of the concentrations obtained using the two techniques was used for the calculations.

The concentration of glucose in the culture fluid (cultured under mixotrophic conditions) was determined using the ferricyanide method (Krishnaveni et al., 1984).

The amino acid content in equivalent to BCAA in microalgae protein was analysed according to the following procedure: 1) 1 mL of protein solution was placed in a 100 mL volumetric flask, adding 4 mL of phosphate buffer solution with pH 7.4, 2 mL of 1% alcohol ninhydrin solution and 2 mL of 0.05% ascorbic acid solution; 2) the reaction mixture was heated in a boiling water bath for 30 min, quickly cooled, the volume of solution was brought to the mark with water and stirred; (3) simultaneously, the same test was performed with 1 mL reference solution (BCAA solution at a concentration of 1 μL/ml) and 1 mL water (control sample); (4) the optical density of solutions obtained was measured at 568 nm in a 10 mm-thick liquid cell against 1 mL water as a control sample.

The water-soluble protein content (mg) per 1L of microalgae suspension was calculated by the formula: $m_l = M \cdot x$, where m_l is the concentration of the water-soluble proteins per 1L of microalgae suspension, mg/L; M is the concentration of microalgae biomass, mg/L; x is the weight percentage of water-soluble proteins in microalgae biomass.

The culture fluid was tested with reference to contaminants using the method of optical microscopy (fixed smear).

The specific growth rate of microalgae cells was calculated using the formula: $= \frac{\ln(x_n) - \ln(x_0)}{t_n - t_0}$, where x_l is concentration of microalgae cells prior to and on the n-th day of cultivation, mln cells/mL; t is the time of cultivation, days.

3. Results and discussion

Analysis of the results of Experiment 1 showed that strains cultured under autotrophic conditions had a high reproduction rate: samples 1, 3 and 5 (Figure 1). The highest specific growth rate and cell concentration were observed for *Chlorella vulgaris Beijer* IPPAS C-1 *(Chlorella sorokiniana)* - sample 1. The strains cultured under mixotrophic conditions (samples 2, 4) actively proliferated at the exponential growth phase, which was associated with active glucose uptake by cells (Figure 2): the cell concentration increased by 3-5 times compared with the initial seed concentration and reached a value of 3-5 million cells/mL. The death of microalgae cells (samples 2, 4) observed on the 4^{th}-9^{th} days of cultivation can be explained by the fact that under mixotrophic conditions the main pathway of glucose assimilation for microalgae is the Embden-Meyerhoff pathway, which is implemented in the cell cytosol (Sun et al., 2018). The presence of large amounts of organic carbon in the medium, with a presumably resulting oxygen deficit for a large number of cells, leads to the accumulation of intracellular products of this metabolic pathway: acetaldehyde and ethanol. The presence of these intracellular products leads to the death of a significant number of microalgae cells, which are sensitive to increased concentrations of these compounds (Perez-Garcia et al., 2015).

The only strains cultivated under autotrophic conditions which showed a high level of biomass accumulation were *Chlorella vulgaris Beijer* IPPAS C-1 *(Chlorella sorokiniana)* - sample 1- 0.7 g/L, and *Chlorella vulgaris Beijer* IPPAS C-38 - sample 7- 0.58 g/L (Table 3). This is 1.3 times higher on average than biomass accumulation of the other autotrophic strains.

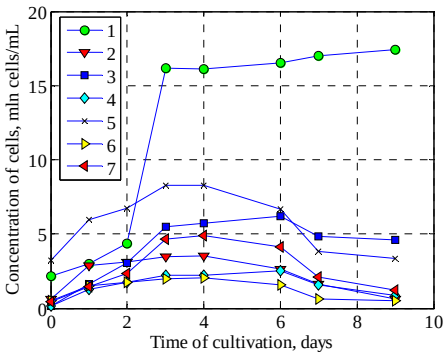

Figure 1: Changes in cell concentration (Experiment 1)

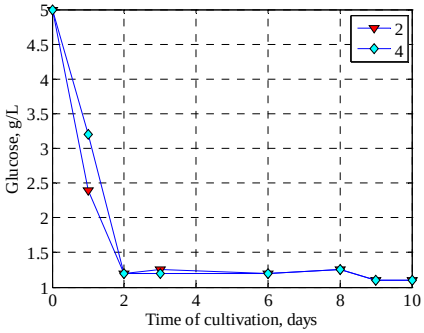

Figure 2: Changes in glucose concentration (Experiment 1)

Table 3: The study of the influence of cultivation conditions on strains (A – autotrophic nutrition; M – mixotrophic nutrition)

Parameter	Day	*C-1 (A)*	*C-1 (M)*	*C-9 (A)*	*C-9 (M)*	*C-2 (A)*	C-35 (A)	*C-38 (A)*
		1	2	3	4	5	6	7
Biomass, g/L	4	0.08	0.10	0.16	0.08	0.16	0.02	0.04
	6	0.24	0.20	0.38	0.20	0.22	0.31	0.10
	9	0.70	0.58	0.38	0.58	0.08	0.44	0.58
Water-soluble protein, mg/L	4	14.9	11.7	38.6	6.4	11.8	1.5	11.7
	6	21.6	7.5	57.0	8.0	10.0	3.7	4.0
	9	45.5	28.8	14.0	7.5	24.5	26.8	16.8
Amino acids (BCAA equivalent), µg/mg of protein	4	12.3	11.7	12.4	17.2	24.6	48.9	8.3
	6	4.9	31.6	6.7	17.5	12.0	27.9	34.2
	9	12.3	7.1	23.8	21.3	11.7	4.2	9.7
Amino acids (BCAA equivalent), µg/L	4	183.3	136.9	478.6	110.1	290.3	73.4	97.4
	6	105.8	237.0	381.9	140.0	120.0	103.2	136.8
	9	559.7	204.5	333.2	159.8	286.7	112.6	163.0

Strains cultured under mixotrophic conditions are characterized by the accumulation of large amounts of biomass: *Chlorella vulgaris Beijer* IPPAS C-1 *(Chlorella sorokiniana)* - sample 2 (mixotrophic conditions) - 0.58 g/L, and *Chlorella kessleri Fott et Nov* C-9 - sample 4 - 0.58 g/L.

The maximum amount of water-soluble protein was extracted from microalgae cells of the strain *Chlorella vulgaris Beijer* IPPAS C-1 cultivated in autotrophic conditions - 45 mg/L on the 9th day of cultivation, and from the strain *Chlorella kessleri Fott et Nov* C-9 - 57 mg/L (autotrophic conditions) - on the 6th day of cultivation. Low content of water-soluble proteins is caused by the fact that on days 6-9 of batch cultivation (without addition of nutrient medium) a deficit of nitrogen-containing compounds is observed. Such shortage inhibits protein biosynthesis and activates metabolic pathways of lipid and starch biosynthesis. This is indicated by a decrease in the percentage of water-soluble protein per unit of biomass dry weight, which drops from 10-35% prior to cultivation to 1-7% on the 9th day of cultivation.

The maximum content of water-soluble proteins in samples cultured under mixotrophic conditions was 28.8 mg/L (Table 3). Apparently, the lower concentration of proteins in the cells of mixotrophic strains is due to the suboptimal ratio of organic carbon to nitrogen.

The content of BCAAs in water-soluble proteins varied considerably, their maximum amount was observed on day 4 of cultivation and was 31.6 µg/mg for *Chlorella vulgaris Beijer* IPPAS C-1 *(Chlorella sorokiniana)* - sample 2 (mixotrophic conditions). Among autotrophic strains, the high content of BCAAs in protein was observed in sample 6 (*Scotiellopsis Vinatzer* IPPAS C-35), 27.9-48.9 µg/mg, and in sample 7 (*Chlorella vulgaris Beijer* IPPAS C-38), 34.2 µg/mg. Decrease in concentrations of these amino acids on day 9 can be explained presumably by restructuring of cell metabolism in nitrogen deficit: pyruvic acid, oxalic acid and acetyl-CoA, the precursors of BCAAs (isoleucine, leucine and valine) are directed into tricarboxylic acid cycle for energy production or into biosynthesis cycle of storage substances - lipids, starch. A possible spike in BCAA concentrations on days 4-6-9 can probably be explained by a saturation of the metabolic pathway of starch and lipid biosynthesis, and the storage of energy in the form of BCAAs, which can be used as energy

source or acetyl-CoA. This fact may also explain the tendency of increasing the content of BCAAs in protein amid an overall decrease in the amount of protein in the biomass.

The highest absolute content of BCAAs was observed in a suspension of *Chlorella vulgaris Beijer* IPPAS C-1 *(Chlorella sorokiniana)* cultivated in autotrophic and mixotrophic conditions, and was 559.7 μg/L and 237.0 μg/L, respectively (Table 3).

Based on the results of Experiment 1, it can be concluded that the strain *Chlorella vulgaris Beijer* IPPAS C-1 *(Chlorella sorokiniana)* cultivated under both autotrophic and mixotrophic conditions is most promising as a producer of water-soluble protein.

At the second stage of the study, the objective was to determine such cultivation conditions for *Chlorella vulgaris Beijer* IPPAS C-1 *(Chlorella sorokiniana)* as to obtain the maximum amount of water-soluble protein and BCAAs. The maximum amount of biomass was observed for sample 1 - 1.1 g/L, and sample 6 (Table 4) - 0.98 g/L. Thus, the temperature optimum for this strain is 30±2 °C. Apparently, *Chlorella vulgaris Beijer* IPPAS C-1 *(Chlorella sorokiniana)* is a photophilic strain, which explains the high growth rate in autotrophic conditions at high levels of photosynthetically active radiation (150 μmol photons/(m^2·s)).

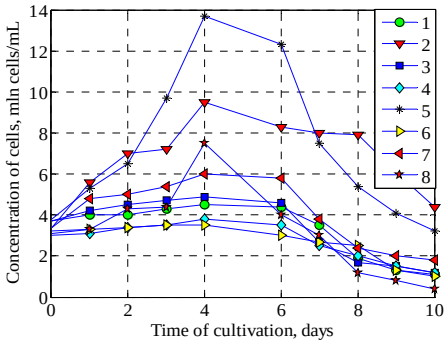

Figure 3: Changes in cell concentration (Experiment 2)

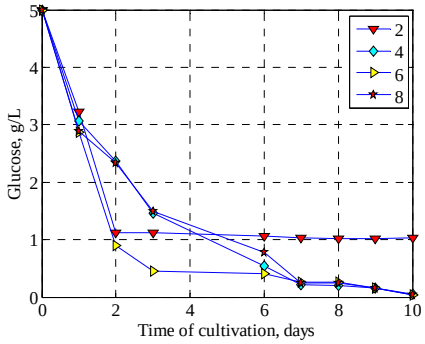

Figure 4: Changes in glucose concentration (Experiment 2)

When cultured under mixotrophic conditions, a significant decrease in photosynthetically active radiation is possible, because part of the cells located in the darkened area of the photobioreactor will actively consume organic carbon from the nutrient medium, which explains the high level of biomass accumulation for sample 6. In Figure 4 it is shown that sample 6 consumes glucose at a higher rate than others during the days 0-2. Notably, neither sample 1 nor sample 6 are characterized by high rates of proliferation (see Figure 3), which suggests that glucose absorbed from nutrient medium (sample 6) and synthesized during photosynthesis (sample 1) are not actively used in respiration process but are stored inside cells as starch, i.e. these conditions are not optimal for cell division.

Table 4: The study of the influence of cultivation conditions on the Chlorella vulgaris Beijer IPPAS C-1 (Chlorella sorokiniana) strain (A – autotrophic nutrition; M – mixotrophic nutrition)

Parameter	Day	Sample no., PAR/T							
		1 150/30 *(A)*	2 150/30 *(M)*	3 150/20 *(A)*	4 150/20 *(M)*	5 30/30 *(A)*	6 30/30 *(M)*	7 30/20 *(A)*	8 30/20 *(M)*
Biomass, g/L	4	1.10	0.60	0.30	0.80	0.5	0.80	0.20	0.56
	6	0.10	0.26	0.10	0.80	0.08	0.98	0.06	0.88
	9	0.06	0.28	0.04	0.40	0.10	0.18	0.06	0.8
Water-soluble protein, mg/L	4	47.3	32.3	11.8	157.5	22.5	86.3	10	37.0
	6	9.0	21.0	5.5	55.0	3.0	17.5	6	46.5
	9	0.5	10.5	2.0	49.5	3.5	29.5	3	56.5
Amino acids (BCAA equivalent), μg/mg of protein	4	29.2	26.5	4.5	13.3	38.2	20.8	10	18.9
	6	21.5	7.5	7.3	30.2	33.3	5.1	10	46.9
	9	23.3	3.5	56.7	10.9	26.2	2.7	9	14.2
Amino acids (BCAA equivalent), μg/L	4	1381.2	885.9	53.1	2094.6	859.5	1795.0	100.0	669.3
	6	193.5	157.5	40.2	1661.0	99.9	89.3	60.0	2180.9
	9	11.7	36.8	113.4	539.6	91.7	79.7	27.0	862.3

The active process of cell division on days 0-4 was observed in sample 5 with a maximum specific growth rate of 0.35 $days^{-1}$ (days 2-4) and in sample 2 with a maximum specific growth rate of 0.5 $days^{-1}$ (days 0-1). The highest amount of water-soluble protein was observed on day 4 when the strain *Chlorella vulgaris Beijer* IPPAS C-1 (*Chlorella sorokiniana)* was cultivated under the conditions of sample 4 - 157 mg/L and under the conditions of sample 6 - 87 mg/L. Nitrogen deficit observed on days 4-8 of cultivation leads to an increase in the amount of lipids inside the cells and a decrease in protein content (Table 4) (Pick et al., 2017).
The high content of BCAAs in protein was noted in sample 8, with 46.9 μg/mg on day 7, and in sample 5, with 38.2 μg/mg on day 4. Samples 4 and 8, cultured under mixotrophic conditions, exhibited a high content of BCAAs in 1 litre of suspension, 1661.0-2094.6 μg/L and 2180.9 μg/mL, respectively (Table 4). Nutrient deficiencies occurring at about the 4th day without the addition of new portions of nutrient media lead to the degradation of the microalgae culture under both autotrophic and mixotrophic conditions. Microbial contamination by bacteria and micromycetes was observed in samples cultured under mixotrophic conditions. This might be due to the fact that they use residues of organic carbon from the initial nutrient medium (glucose), residues of dead mycobacteria cells as well as microalgae endometabolites (amino acids, vitamins) as food substances.

4. Conclusions

Experimental and theoretical research has shown that the studied microalgae strains significantly differ in growth rate on Tamiya nutrient medium under conditions of autotrophic and mixotrophic nutrition at the photosynthetically active radiation level of 150 μmol photons/(m^2·s) and temperature 30 ± 2 °C (batch cultivation). Under these conditions, the microalgae strain *Chlorella vulgaris Beijer* IPPAS C-1 *(Chlorella sorokiniana)* is a promising producer of protein and branched-chain amino acids, capable to significantly increase its biomass by 10-15 times in 4-5 days at high PAR and temperature. A rise in protein and BCAA content in cells is observed at high PAR and lower cultivation temperature under mixotrophic conditions.

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