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PROTEINS FROM *Spirulina platensis* BIOMASS

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The accumulation dynamics of total protein, its fractional composition, and the molecular forms of key oxidoreductases were studied during cultivation of Spirulina platensis *biomass.*

Key words: *Spirulina platensis* biomass, proteins, phycocyanin, molecular forms of enzymes, lactatedehydrogenase, malatedehydrogenase, glutamatedehydrogenase, electrophoresis.

Spirulina (*Spirulina platensis*) biomass (SB) is used as a general tonic in the form of biologically active additives. New effective medicinal preparations are based on it [1-3]. Water-soluble proteins and phycocyanin pigment in addition to the rich assortment of biologically active compounds (BAC) from spirulina biomass hold theoretical and practical interest [4-6].

The pigment phycocyanin possesses antioxidant, anti-inflammatory, immunomodulating, and hepatoprotective activity [7-9]. This is a biliproteid of molecular weight 275,000 D that contains phycobilins as prosthetic groups. These are tetrapyrrole compounds in an open chain of 20-30 units per pigment molecule. The protein part of phycocyanin is constructed from 17 mostly acidic amino acids. The N- and C-terminuses are threonine and serine [10].

The goal of our investigations was to develop a method for quantitative analysis of total protein and phycocyanin and to study the dynamics of change of fractional protein composition and the relative activity of key oxidoreductases during cultivation.

Protein in the aqueous extract from SB was determined after extraction of the lipid fraction with acetone by the biuret method, i.e., the total amount of water-soluble protein was analyzed.

A spectrophotometric method based on measurement of the optical density of the aqueous extract from SB with a fixed pH value was used to determine phycocyanin. The absorption spectrum of the aqueous extract from SB has a maximum at 620 nm due to phycocyanin in the extract (Fig. 1).

A calibration curve was constructed to determine the dependence of the optical density on the solution concentration of phycocyanin. The results showed that the optical density at 620 nm of a phycocyanin solution depends on the pH. Therefore, phosphate buffer at pH 7.0 was used to prepare the solution (Fig. 2). The Beer—Lambert law was obeyed for phycocyanin concentrations 0.01-0.1% at a fixed pH. The specific absorption coefficient was found from the calibration curve, which was then used to calculate the quantitative results.

Phosphate buffer at pH 7.0 was used to extract phycocyanin from SB. The effects of various factors on the yield of phycocyanin from SB are listed in Table 1. The extraction of phycocyanin was optimal if we used ground raw material with particles that pass through a sieve of diameter 0.5 mm with stirring on a magnetic stirrer at room temperature for 30 min at a raw material: extractant ratio of 1:100. Practically all phycocyanin was extracted after three extractions.

The degree of extraction from SB was monitored by electrophoresis [8]. Four samples from SB were obtained successively after extraction of the lipid fraction by acetone in a Soxhlet extractor. The raw material was extracted first by phosphate buffer at a 1:60 ratio; then at a 1:10 ratio. The resulting samples were analyzed by electrophoresis in PAAG.

The lack of colored fractions in electrophoregrams of the fourth sample was the criterion for completeness of phycocyanin extraction.

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TABLE 1. Effect of Extraction Conditions on Yield of Phycocyanin from Spirulina Biomass

Extraction conditions	Phycocyanin content (%) in samples of series				
	1	2	3	4	5
Particle-size of material, mm					
3	0.34	0.30	0.29	0.23	0.32
2	0.37	0.35	0.34	0.26	0.35
1	0.39	0.37	0.33	0.25	0.38
0.5	0.42	0.40	0.39	0.28	0.43
Extraction temperature, °C					
20	0.41	0.39	0.37	0.26	0.42
30	0.38	0.36	0.33	0.23	0.39
40	0.35	0.34	0.30	0.21	0.36
Extraction duration, min					
15	0.34	0.36	0.30	0.23	0.37
30	0.40	0.38	0.34	0.24	0.39
60	0.40	0.39	0.33	0.24	0.39
Material-to-extractant ratio					
1:10	0.37	0.36	0.36	0.25	0.35
1:50	0.36	0.38	0.35	0.24	0.36
1:70	0.42	0.40	0.37	0.25	0.38
1:100	0.43	0.40	0.38	0.26	0.39

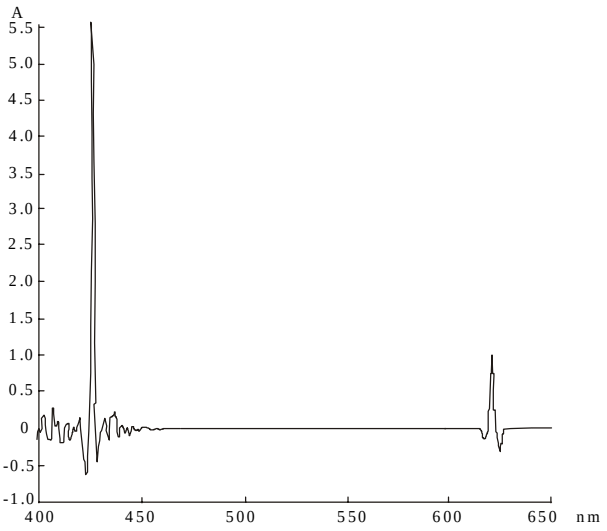

Fig. 1.

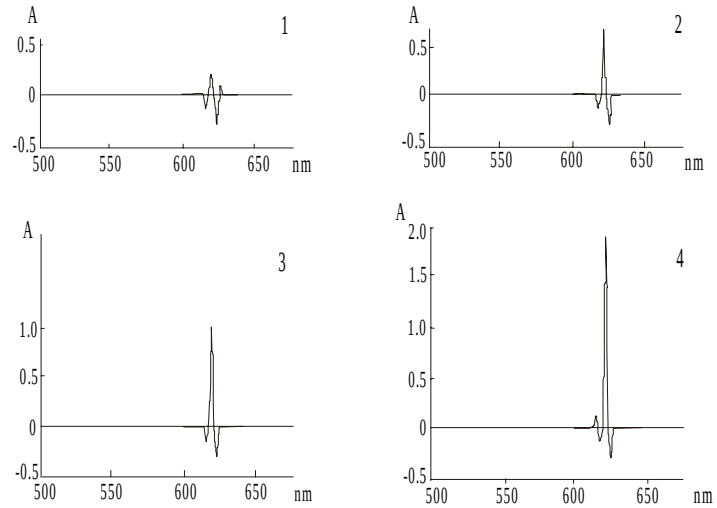

Fig. 2.

Fig. 1. Absorption spectrum of the aqueous extract of *S. platensis* biomass.

Fig. 2. Absorption spectra of phycocyanin isolated from *S. platensis* biomass at various pH values of the aqueous extract: 1.86 (1), 4.01 (2), 7.00 (3), 9.18 (4).

The working standard phycocyanin sample was obtained from SB. Most existing methods for isolating phycocyanin are based on ultrasonic destruction of biomass cells. Therefore, these methods are technically cumbersome and unsuitable for practical application without special installations [3, 11]. The method proposed by us includes isolation of phycocyanin from the aqueous extract of SB with subsequent chromatographic separation over a tricalcium phosphate column and precipitation by 80° ethanol.

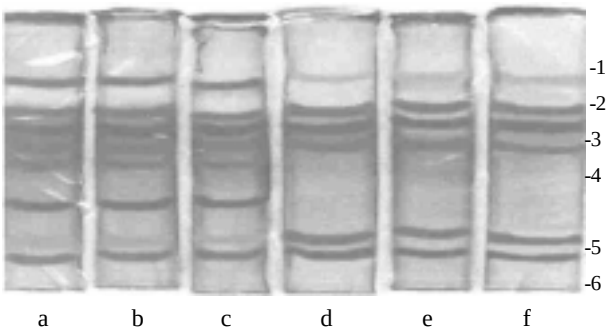

Fig. 3.

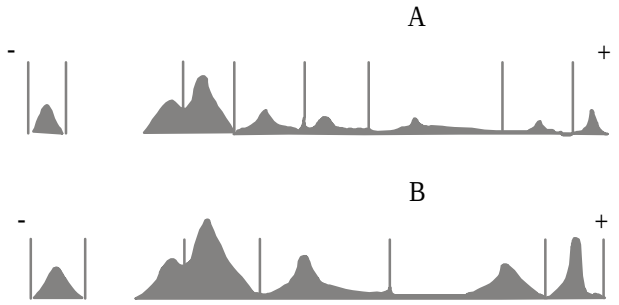

Fig. 4.

Fig. 3. Electrophoregrams of *S. platensis* biomass proteins from 3-day (a, b, c) and 4-day (d, e, f) cultures. Protein fractions: structural protein (1), phycocyanin subfractions (2, 3), water-soluble proteins (4-6).
Fig. 4. Densitograms of *S. platensis* biomass proteins from 3-day (A) and 4-day (B) cultures.

The degree of purification was monitored by electrophoresis in 7.5% PAAG. The purity criterion of the resulting sample was the presence in the electrophoregrams of two fractions with relative electrophoretic mobilities 0.33-0.34 and 0.35-0.36. This confirms the structural integrity of the phycocyanin [12].

We now present metrological characteristics for the developed quantitative methods for determining total protein and phycocyanin in SB.

$\overline{X}$	S	$\triangle x$	E, %
	Protein		
41.6	0.8027	±1.79	±4.31
	Phycocyanin		
9.52	0.1427	±0.32	±3.37

F = 10, P = 95%, t(p,f) = 2.23.

The analysis of total protein in SB during cultivation gives a limited picture of the specifics of *S. platensis* metabolic processes. The study of the fractional protein composition and the relative activity of the key oxidoreductases lactatedehydrogenase (LDH), malatedehydrogenase (MDH), and glutamatedehydrogenase (GDH) has theoretical and practical value.

We investigated the fractional protein composition of *S. platensis* grown in isolation at various ages and determined in parallel the total protein content and monitored the isoenzyme spectrum of LDH, MDH, and GDH.

The quantitative determination of protein showed that its content changes during biomass cultivation and reaches a maximum at 4-5 days (Figs. 3 and 4). From 6 to 10 protein fractions appear in the PAAG depending on the culture age. The relative protein content in each fraction changes over 24 h.

Protein fractions 1-6 appear regardless of culture age. Fraction 8 appears reliably up to the seventh day of growth. It disappears in the following days and is again observed at 21, 22, 28 and 40 days. Furthermore, additional fractions 9 and 10 are found on the 40th day of biomass cultivation (Table 2).

The changes in the fractional protein content in SB are due to the synthesis of molecular forms of enzymes as a function of development stage. This is confirmed by the variability in the molecular forms of LDH, MDH, and GDH (Table 3).

Molecular forms of LDH are not observed during the first three days of SB cultivation. Three molecular forms of 54.8, 29.2, and 16.0% were observed on the fourth day.

The specific activity of the enzyme appeared as two bands on the 5th (58.5, 41.5%), 26th (61.0, 39.0%), and 27th (86.4, 13.6%) days. The enzyme was present primarily as one molecular form after seven days of cultivation.

The isoenzyme spectrum of *S. platensis* MDH includes five molecular forms at 3 and 26 days of cultivation and four isoenzymes at 4, 5, and 25 days. The enzyme appeared as one molecular form on the remaining cultivation days.

GDH gives five forms (14.8, 11.8, 13.0, 53.6, 6.8%) on the 4th day of cultivation and two on the other days.

TABLE 2. Fractional Composition of Proteins from *S. platensis* Biomass as a Function of Culture Age

Culture age, days	Total protein, %	Fractional composition, % of fraction							
		1	2	3	4	5	6	7	8
3	38.01	7.1	14.8	10.4	11.0	28.8	3.6	6.8	17.5
4	41.62	6.0	10.5	20.6	11.3	9.7	20.0		
5	49.30	15.1	11.4	19.6	10.8	12.4	18.3	3.3	9.0
6	49.03	9.3	30.4	37.0	3.5	2.7	1.4	10.0	1.0
7	47.02	10.6	24.3	38.4	19.0	4.2	1.7	1.0	1.0
18	35.04	2.0	65.8	13.2	10.6	3.3	1.0	1.8	
19	33.01	4.8	17.5	62.4	5.0	2.0	5.4	2.0	
21	39.04	10.0	18.0	24.1	8.0	24.0	4.4	10.0	2.0
22	38.03	12.4	39.2	2.8	21.2	6.8	4.4	11.6	1.7
26	32.02	9.3	20.0	27.6	15.0	7.7	5.5	14.8	
27	31.02	15.3	36.3	15.1	14.0	18.4	1.0		
28	35.01	9.0	18.5	52.8	3.0	6.5	2.0	4.8	3.3
29	36.00	3.6	21.4	67.0	4.8	1.4	2.0		
40*	39.04	11.8	11.0	29.8	10.3	2.6	10.8	2.6	3.8
41	37.02	14.8	49.1	27.4	2.0	1.4	2.0	3.3	
43	36.01	2.0	18.0	63.0	7.3	6.8	1.4	1.7	
44	33.33	18.3	54.2	4.8	11.0	3.6	6.4	1.4	

*Fraction 9, 3%; 10, 13.2%.

TABLE 3. Relative Activity of Molecular LDH, MDH, and GDH of *S. platensis* Biomass as a Function of Culture Age

Culture age, days	Relative activity							
	LDH	MDH					GDH	
	1	1	2	3	4	5	1	2
3	-	5.0	43.0	14.2	33.1	4.8	18.6	81.4
4	54.8[a]	1.0	64.8	25.7	9.0	-	14.8*	11.8
5	58.5[b]	4.2	63.4	16.1	16.1	-	19.0	81.0
6	-	69.8	30.2	-	-	-	14.0	86.0
7	-	100.0	-	-	-	-	21.6	78.4
18	100	100.0	-	-	-	-	42.6	57.4
19	100	100.0	-	-	-	-	67.0	33.0
21	100	100.0	-	-	-	-	24.3	75.7
22	100	100.0	-	-	-	-	23.8	76.2
25	-	3.3	86.0	4.8	5.8	-	-	-
26	61.0[c]	3.0	35.5	40.1	20.2	3.3	16.3	83.7
27	86.4[d]	100.0	-	-	-	-	41.2	58.8
28	-	100.0	-	-	-	-	-	-
29	-	100.0	-	-	-	-	13.8	86.2
40	100.0	100.0	-	-	-	-	34.3	65.7
41	100.0	100.0	-	-	-	-	38.0	62.0
43	100.0	100.0	-	-	-	-	-	-
44	100.0	100.0	-	-	-	-	38.8	61.2

Observed: a) 3 bands (54.8, 29.2, 16.0); b) 2 bands (58.5, 41.5); c) 2 bands (61.0, 39.0); d) 2 bands (86.4, 13.6).
*Represented by five molecular forms (14.8, 11.8, 13.0, 53.6, 6.8).

It should be noted that only molecular forms of MDH are active at 7, 28, and 43 days of cultivation whereas LDH and GDH are inactive at these times. It can be hypothesized that these dehydrogenases at these times lack substrate specificity whereas MDH acts as the dominant dehydrogenase.

The results enabled us to propose a methodical approach to analysis of spirulina proteins that is simple and provides the maximum amount of information. We can recommend it as an objective method for monitoring protein accumulation in *S. platensis* biomass.

Thus, methodical approaches to the analysis of SB proteins are theoretically and experimentally justified. A method is proposed for preparing samples for quantitative analysis of total protein by the biuret method. A quantitative method is developed for determining phycocyanin in SB. Its biomass samples contain 33.0-49.3% protein and 7.1-10.6% phycocyanin for thoroughly dried material. The protein fractions and molecular forms of LDH, MDH, and GDH in SB vary. This can be used to determine the age and maturation time of the culture.

EXPERIMENTAL

We analyzed SB cultivated in isolation at NPP "Poisk" (Samara). We used strain *S. platensis* (Nords.) Geilt.-835 to obtain it.

Samples of SB were prepared by removing the lipophilic fraction (carotenoids, chlorophyll, other lipids) via extraction with acetone by various methods depending on the purpose of the analysis: in a Soxhlet apparatus (spectrophotometry of phycocyanine) or fractional extraction (analysis of total protein). The next step was drying SB at room temperature to constant mass to ensure complete removal of acetone.

Absorption spectra of phycocyanin were taken on a Perkin—Elmer Lambda 20 recording spectrophotometer in 1-nm steps at 240 nm/min. The solvents were standard buffers. The amounts of protein and phycocyanin were determined on a SF-46 spectrophotometer.

The fractional protein content and isoenzyme spectrum of oxidoreductases were established by electrophoresis in 7.5 PAAG with 1 M tris-EDTA-borate buffer at pH 9.2 as the electrode buffer [8]. Protein fractions were colored using amido black B (1%) in acetic acid (7%). The molecular forms of the enzymes LDH, MDH, and GDH were found by the phenazinemetasulfate—tetrazole reaction in Petri dishes in optimized incubation media [11]. The electrophoregrams were analyzed by densitometry on an AF-1 (L′vovpribor) analyzer.

Quantitative determination of protein in the supernatant liquid was performed by the biuret method [13].

Quantitative Determination of Phycocyanin. An analytical sample of raw material was extracted with acetone in a Soxhlet apparatus until the extract was no longer colored. Then the sample was dried in a drying chamber at room temperature to constant mass and ground to a particle size passing through a 0.5-mm sieve. Raw material (~0.5 g, accurate weight) was stirred on a magnetic stirrer for 30 min with phosphate buffer (30 mL) and centrifuged for 15 min at 8000 rpm. The supernatant liquid was transferred to a 50-mL volumetric flask. The solid was resuspended in phosphate buffer (10 mL, 0.1 M), thoroughly mixed, and centrifuged at 8000 rpm (twice). The supernatant liquid was transferred to a 50-mL volumetric flask, adjusted to the volume with phosphate buffer, and mixed. The optical density of the analyzed solution was measured at 620 nm in a 10-mm cuvette. The reference solution was phosphate buffer.

The phycocyanin content (X, %) in the SB was calculated per absolute dry weight using the formula:

$$X = D \cdot 50 \cdot 100 / 8.97 \cdot m \cdot (100 - W),$$

where D is the optical density of the analyzed solution, 8.97 is the specific absorption coefficient of phycocyanin at 620 nm, m is the raw material weight in g, and W is the mass loss of raw material on drying in %.

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