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Characteristics of Proteins Synthesized by Hydrogen-Oxidizing Microorganisms

T. G. Volova[a] and V. A. Barashkov[b]

[a] *Institute of Biophysics, Siberian Branch, Russian Academy of Sciences, Krasnoyarsk, 660036 Russia*
e-mail: volova45@mail.ru
[b] *Siberian Federal University, Krasnoyarsk, 660036 Russia*

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Abstract—The study was conducted to determine the biological value of proteins synthesized by hydrogen-oxidizing microorganisms—the hydrogen bacteria *Alcaligenes eutrophus* Z1 and *Ralstonia eutropha* B5786 and the CO-resistant strain of carboxydobacterium *Seliberia carboxydohydrogena* Z1062. Based on a number of significant parameters characterizing the biological value of a product, the proteins of hydrogen-oxidizing microorganisms have been found to occupy an intermediate position between traditional animal and plant proteins. The high total protein in biomass of these microorganisms, their complete amino acid content, and availability to proteolytic enzymes allow for us to consider these microorganisms as potential protein producers.

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INTRODUCTION

Search for effective ways of increase of protein resources for different spheres of use is one of the main goals of technological progress. Microbiological synthesis is an effective way for protein production, which, in comparison with traditional agricultural technologies, uses material and energetic resources more efficiently, does not acquire large land area, does not depend on climate and weather, and does not pollute the environment with pesticides. Use of biochemical activity of microorganisms, which are able to assimilate a lot of chemicals and synthesize biomass of a high biological value, is in the basis of microbiological technologies. In the 1970s–1980s, all over the world, including in the Soviet Union, microbiological technologies of protein synthesis by single-cell microorganisms in gaseous and liquid hydrocarbons, alcohols, natural gas, hydrogen, and others had been developing. Currently, a considerable increase of activity can be seen in this direction [1]. Different single-cell microorganisms and substrates are intensively studied as a protein producer: bacteria *Cellulomonas biazotea* cultivated in processed hydrolyzates of *Leptochloa fusca* [2], association of *Methylococcus capsulatus* with heterotrophic bacteria producing highly protein biomass in methane [3], microalgae *Spirulina* [4], yeast *Candida langeronii* cultivated in hydrolyzate of cane cake [5], *Cryptococcus aureus* utilizing earth apple extracts and hydrolyzates of soyabean meal [6, 7], *Debaryomyces hansenii* cultivated in malt waste of the brewing industry [8], and many others.

Promising outlook for hydrogen-reducing bacteria in comparison with other developed protein producers depends on their autotrophy, i.e., independence of scarce organic resources, rapid growth (doubling time 2.0–2.5 h), high content of amino acids' complete protein (to 60–70%), lack of extracellular intermediate organic metabolism products (the only by-product of hydrogen oxidation is water), high ecological purity of production process and product, as well as ability for growth on hydrogen of different origin including products of carbonaceous fuel processing. In the Biophysics Institute (Siberian Branch, Russian Academy of Sciences) in 1980s, the scientific bases of hydrogen-oxidizing bacteria biotechnologies were developed, i.e., a pilot production was constructed and started up, experiments on farm and fur animals were carried out feeding them with hydrogen bacteria biomass (HBB). HBB was shown to be useful for 25–50% of the diet of animal protein quota depending on species and age of animals [9, 10].

The aim of the work was investigation of proteolytic enzyme effect and amino acids and fractional content of proteins synthesized by hydrogen-oxidizing bacteria.

METHODS

Strains of hydrogen-oxidizing bacteria from the collection of lithotroph cultures of Winogradsky Institute of Microbiology (Russian Academy of Sciences) were given to the Biophysics Institute (Siberian Branch, Russian Academy of Sciences) by academician G.A. Zavarzin. They are hydrogen bacteria *Alcaligenes eutrophus* Z1 [11] and CO-resistant strain of carboxydobacteria *Seliberia carboxydohydrogena* Z1062 [12] and *Ralstonia eutropha* B5786 which is a

Table 1. Chemical composition of hydrogen-oxidizing bacteria biomass

Culture	Biomass, % to the dry substance of a cell			
	raw protein	RNA + DNA	carbohydrates	lipids
A. eutrophus Z1	74.2	12.8	5.0	6.0
S. carboxydohydrogena Z1062	71.3	11.2	6.7	9.1
R. eutropha B5786	75.6	12.3	6.4	7.2

highly growing variant of Z1 strain and was selected by growth rate in continuous culture [13].

Bacteria were cultured in strict sterile running conditions using a 15 l BioFlo 110 automatic fermentation system (New Brunswic, United States), which allows implementing aseptic conditions with stabilizing the main culture parameters (pH, temperature, and concentration of oxygen and nitrogen in culture) in mineral salt medium of the following composition: $N_2HPO_4 \cdot H_2O$—9.1, KH_2PO_4—1.5, $MgSO_4 \cdot H_2O$—0.2, $Fe_3C_6H_5O_7 \cdot 7H_2O$—0.25, NH_4Cl—0.2–1.0. Three milliliters of standard microelement solution according to Hoagland was added per 1 l of growth medium which contained H_3BO_3—0.228, $CoCe_2 \cdot 6H_2O$—0.030, $CuSO_4 \cdot 5H_2O$—0.008, $MnCe_2 \cdot 4H_2O$—0.008, $ZnSO_4 \cdot 7H_2O$—0.176, $NaMoO_4 \cdot 2H_2O$—0.050, $NiCe_2$—0.008 (g/l). Coefficient of fermenter filling was 0.3–0.5; number of revolutions of the mixer was 1000 rpm.

As a source of carbon and energy, gas mixture (CO_2, H_2, O_2) with a volume ratio 1 : 2 : 7 was used. CO-resistant strain Z1062 was cultured in the same gas mixture containing carbon monoxide in a concentration to 20 vol % as an additional source. Gas mixture was continuously run through the culture with 10–14 l/min rate using membrane type compressor. Gas mixture composition was controlled in continuous conditions using a serial gas analyzer and chromatograph LKhM-80 (Chromatograph, Russia) (detector—katharometer, carrier gas—argon).

Bacterial biomass was washed twice with distilled water and lyophilized. Lipids, total nitrogen, hydrocarbons, and nucleic acids in bacterial biomass were detected following the standard methods. Polyhydroxybutyrate was detected using a GSD plus chromatography-mass spectrometer (Hewlett Packard, USA) after preliminary sample methanolysis.

Total biomass protein, its fractional and amino acid composition, and digestion with proteolytic enzymes in vitro were analyzed. Simultaneously biologic activity of microalgae *Chlorella*, plant protein (field-grown wheat grain, grade 232), and casein (as an animal protein) were studied for comparison.

Protein hydrolysis was performed in 6 N HCl for 22 h at 110°C. Amino acid protein composition was detected using a KLA-38 automatic analyzer (Chitachi, Japan). Methionine and cystine were determined in a single protein sample after its preliminary oxidation with performic acid [14]. Protein fractioning and analysis of their fractional composition were performed based on known data about different solubility of cellular proteins in alkaline and salt solutions. Protein treatment was performed at 65°C with the following solutions: 0.03 M KCl (I), 0.6 M KCl + 0.04 M $NaHCO_3$ + 0.01 M Na_2CO_3 (II), 0.1 M NaOH (III), and 1.0 M NaOH (IV).

Availability of protein fractions extracted from bacterial biomass was detected following Pokrovskii–Ertanov's method with proteolytic enzymes [15]. Degree of proteolysis was judged by the increase of amine nitrogen content in a mixture received following the protein treatment with enzymes and was evaluated in percent. Product protein content was considered as 100%. The investigation was performed for 6 h; the first 3 h the proteins were treated with pepsin and for the next 3 h they were treated with trypsin.

Statistical processing of data was performed with the standard methods using the Microsoft Excel standard program package.

RESULTS AND DISCUSSION

Total yield, biomass chemical composition, total protein, and amino acids were investigated in 3 hydrogen-oxidizing strains as they are the criteria for estimation of efficiency of protists' protein synthesis [1]. Biomass of all strains mainly consists of nitrogen-containing components (proteins and nucleic acids) and a few carbohydrates and lipids (Table 1). Growth speed increase led to increase of intracellular concentration of nitrogen-containing components in strains; considerable increase of these fractions was registered with change of medium flow rate from 0.15 to 0.30 h^{-1}. Further increase of flow rate did not significantly influence protein synthesis. Influence of bacterial growth rate on biomass chemical content for strain Z1062 is shown in the figure (for strains Z1 and B5786, similar relationships were shown). Nucleic acid concentration increased with dilution rate increase as well. Nucleic acid amount increased mainly due to RNA synthesis intensification because DNA content at all dilution rates was 2.5–3.0% permanently. Intensification of protein synthesis in a cell with growth rate increase suggests an increase of ribosome activity where protein is synthesized. Increase of protein in a cell suggests increase of ribosome activity where pro-

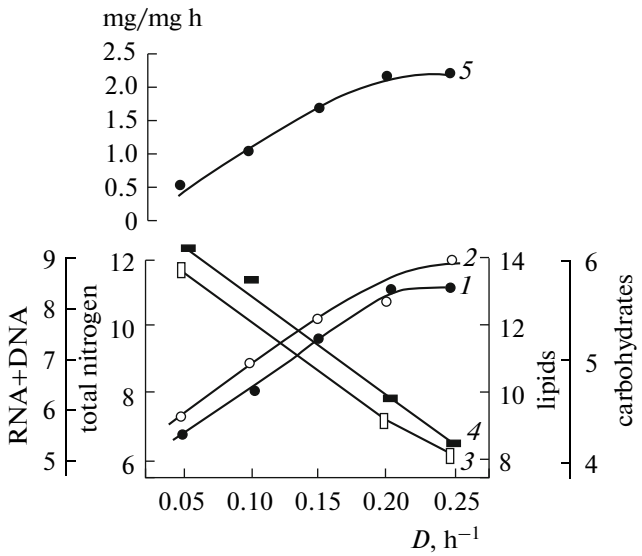

Figure. Dependence of chemical composition of carboxydobacteria *S. carboxydohydrogena* Z1062 from medium flow rate (D, h^{-1}). (*1*)—total nitrogen, (*2*)—RNA+DNA, (*3*)—lipids, (*4*)—carbohydrates (% from the dry substance of a cell), and (*5*)—protein synthesizing activity (mg/mg h).

tein is synthesized. The protein synthesizing activity of bacterial RNA naturally increased with growth rate increase (figure). Synthesis of reserve substances, carbohydrates, and lipids, on the contrary, decreased with growth rate increase. Content of these polymers was maximal at low growth rates. In a highly growing perfusion culture, when protein synthesis is maximal in cells, lipid and carbohydrate concentration decreased per 35–40% from the initial values to 4.0 and 8.2%, respectively. In a perfusion culture with bacterial specific growth rate higher than 0.15 h^{-1}, reserve macromolecules, such as hydroxybutyric acid polymers, did not accumulate. From the biological value point of view, chemical composition of the studied bacteria became more favorable with growth rate increase.

One of the primary indicators of biological value of microbial biomass is total protein and amino acid content including essentials. Results of comparison of protein amino acid content of strains B5786, Z1, and Z1062 with single-celled algae, yeast, and with full-value animal proteins (casein) are shown in Table 2, where it can be seen that the protein amino acid content of hydrogen-oxidizing bacteria, yeast, and microalgae is similar to casein. However, if content of essential amino acids of hydrogen bacteria is similar to yeast, then total biomass protein content (in % per dry mass weight) is different. For yeast, this value ($N_{total} \times 6.25$) is 50% on average and, for hydrogen-oxidizing bacteria, it is 70%.

Total essential amino acids in all studied strains was higher than content of essential amino acids in grain (more than 10%) and was close to this indicator in casein. Proteins of all strains have a full-value amino acid content presented with considerable amounts of Lys, His, Arg, Asp, Glu, Thr, Ser, Pro, Gly, Ala, Cys, Val, Meth, Isoleu, Leu, Tyr, and Phe. The studied proteins are rich in amino acids (their content in common fraction is 40%).

Values of chemical calculation (scora), determined according to the standard scale developed by the Food and Agriculture Organization of the United Nations (FAO scale), are shown in Table 3. Scora is based on the comparison of protein amino acid content of the estimated product with amino acid content of the standard (ideal) protein expressed in percent from content of this amino acid in a standard scale or in equal amount of standard protein. Favorable distribution of essential amino acids is typical for all studied proteins. This indicates the high quality of the studied proteins on the whole. Sulfur amino acids and isoleucine limit biological value of proteins synthesized by hydrogen-oxidizing bacteria. It was also mentioned that, with FAO scale, proteins contain some excess of threonine and aromatic amino acids. In comparison with traditional food animal proteins (casein) and plant proteins (wheat), the proteins of hydrogen-oxidizing bacteria are close to casein in content and distribution of amino acids. This testifies to a high biological value of the studied proteins.

It must be mentioned that, however important the characteristics are, information about protein content and their amino acid composition do not allow us to have a complete idea about the product nutritive value. Analysis of amino acid composition demonstrates only the relative potential protein nutritive suitability as amino acid content received after acid hydrolysis not always corresponds to the set of physiologically available amino acids. To estimate the biological value of the protein product, it is also necessary to take into account correspondence and availability of the proteins to proteolytic enzymes. The latter depends on protein type (reserve, transport, structural, etc.) and their ratio in a cell, strength of the cellular membrane and its content, and protein origin (plant, animal, and bacterial). The protein composition of a cell is heterogenous. Together with the soluble cytoplasmic proteins (albumin, globulin, enzyme proteins, and nucleoproteins), the cell contains proteins related to its structural elements (lipoproteins and glycoproteins of the cellular wall and membrane) [16].

Fractional composition is an important characteristic determining protein biological value. Results of the comparative research of proteins fractions and their amino acid content in hydrogen-oxidizing bacteria, meat, and wheat grains are shown in Table 4. Fractioning is based on different solubility of cellular proteins in salt and alkaline solutions. Fractional content of bacterial proteins differs considerably from traditional proteins. Unlike meat, which up to 80% consists of fractions I and II (most available for digestive enzymes), proteins' different distribution is typical for

Table 2. Amino acid composition

Amino acid, % from the dry substance of a cell	Hydrogen-oxidizing bacteria strain			Yeast	Algae	Casein (standard)
	Z1	Z1062	B5786			
Lysine	7.02	8.61	9.20	7.02	5.98	7.33
Histidine	1.96	2.48	1.40	1.96	1.81	2.20
Arginine	7.30	8.00	7.50	7.30	7.74	3.19
Asparaginic	10.08	9.57	9.10	10.08	9.49	7.11
Threonine	5.29	4.52	5.20	5.29	4.88	4.22
Serine	4.02	3.47	4.80	4.02	4.86	5.72
Glutamic	12.56	11.17	9.30	12.56	13.12	22.20
Proline	4.58	3.46	0.80	4.58	5.74	10.41
Glycine	6.05	5.47	10.20	6.05	6.34	1.88
Alanine	9.07	8.80	13.40	9.07	9.18	2.96
Cystine	0.56	-	0.30	0.56	1.37	0.42
Valine	6.38	7.13	7.50	6.38	5.41	5.72
Methionine	2.63	2.69	0.40	2.63	2.16	2.47
Isoleucine	4.47	4.58	4.50	4.47	3.55	4.10
Leucine	8.60	8.52	8.70	8.60	8.91	9.39
Tyrosine	3.62	3.26	2.40	3.62	3.13	4.75
Phenylalanine	4.42	3.96	3.60	4.42	4.41	4.62
Tryptophan	1.40	1.24	1.16	1.40	1.58	1.32
Total amino acids, %	100.01	96.93	99.46	100.01	99.66	100.01

hydrogen-oxidizing bacteria. Only half of the proteins belongs to fractions I and II and the other half includes fractions III and IV (mainly fraction III). They are the structural proteins extracted by alkali and less available to proteases; therefore, they are worse digested than fractions I and II proteins. Thus, in this characteristic, proteins synthesized by hydrogen-oxidizing bacteria slightly yield to animal proteins but surpass plant proteins.

The next important characteristic of biological protein value is their availability to proteolytic enzymes. Analysis of availability of the studied proteins to proteases is conducted in comparison with the proteins with high assimilation in human and animals. Such method of analysis allows for considering the role of structural organization of cellular components and influence of technological operations of preliminary treatment on protein availability.

Results in Table 5 show that protein assimilation of hydrogen-oxidizing bacteria of three strains is almost the same and it is slightly lower than that of casein; however, it excels wheat protein assimilation. It was shown that salt-soluble proteins synthesized by hydrogen-oxidizing bacteria are more available for pepsin than casein and their assimilation after the sequential effect of pepsin and trypsin is close to casein assimilation. However, it should be mentioned that bacterial structural proteins after the sequential effect of pepsin and trypsin were digested for not more than 50% of casein availability.

Investigation of proteolysis products of proteins synthesized by hydrogen-oxidizing bacteria showed that, after the protein treatment with pepsin, the heterogenous mixture of proteins, peptides, and free amino acids appears in which proteins with molecular weight (MW) of approximately 30 kDa and big

Table 3. Chemical count of proteins synthesized by hydrogen-oxidizing bacteria calculated according to the FAO scale

Amino acid	Hydrogen-oxidizing bacteria strain		
	Z1	Z1062	B5786
Lysin	103	101	100
Threonine	116	121	120
Valine	89	96	103
Cystine + Methionine	79	75	87
Isoleucine	83	80	85
Leucine	103	101	106
Tyrosine+ Phenylalanine	119	116	114

Table 4. Fractional composition of proteins

Protein type	Total content, % from the dry substance of a cell	Fractional composition of proteins, % from total protein content			
		I	II	III	IV
Meat	76.1 ± 3.2	30.5 ± 0.7	52.2 ± 0.7	16.6 ± 0.8	0.6 ± 0.1
Wheat grain	15.2 ± 0.8	25.9 ± 0.9	16.1 ± 0.9	39.0 ± 3.4	19.0 ± 0.4
Hydrogen bacteria, strain					
Z1	69.4 ± 1.2	28.0 ± 0.8	24.8 ± 0.8	32.8 ± 1.4	4.4 ± 0.1
Z1062	64.0 ± 1.4	29.0 ± 0.2	23.0 ± 0.2	33.0 ± 2.8	6.0 ± 0.9
B5786	68.0 ± 0.3	30.0 ± 2.2	31.0 ± 3.4	30.0 ± 2.7	7.0 ± 1.0

Table 5. Proteins availability for proteolytic enzymes in vitro

Protein	Protein proteolysis, %				
	pepsin			trypsin	
	1 h	2 h	3 h	5 h	6 h
Strain					
Z1	21.8	36.6	39.6	43.0	44.0
Z1062	20.9	35.9	40.1	40.6	41.2
B5786	22.5	37.0	37.9	42.8	43.1
Casein	23.0	43.5	44.0	54.1	55.0
Wheat grain	19.9	23.3	25.0	32.0	32.3

polypeptides with MW 8 kDa prevail. Chromatographic analysis of protein products appeared as a result of treatment of initial bacterial proteins with trypsin testifies to the pronounced degradation of protein structure under this enzyme. Thus, in 0.5 h of proteolysis, the main part of protein MW was approximately 10.5 kDa and in 4 h distribution of MW was shifted towards lower values to 7.3 kDa. At the same time, free amino acid fraction increased from 1.2% after 0.5 h of proteolysis to 4.6% after 4 h.

These analyses showed that proteins of hydrogen-oxidizing bacteria in some main characteristics determining the biological value occupy an intermediate position between traditional animal and plant proteins. High total content of protein in biomass of these bacteria, valuable amino acid content, and availability for proteolytic enzymes allow for us to consider hydrogen-oxidizing bacteria as a potential protein source.

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