Microbiology, Vol. 70, No. 4, 2001, pp. 429–435. Translated from Mikrobiologiya, Vol. 70, No. 4, 2001, pp. 495–502.

EXPERIMENTAL ARTICLES

Investigation of the Physiological and Biochemical Characteristics of Bifidobacteria at the Late Stages of Their Development

G. I. Novik, N. I. Astapovich, and A. A. Samartsev

Institute of Microbiology, National Academy of Sciences of Belarus, ul. Kuprevicha 2, Minsk, 220141 Belarus

Received August 29, 2000; in final form, January 31, 2001

Abstract—An investigation of the physiological and biochemical characteristics of the *Bifidobacterium bifidum* no. 1, *B. adolescentis* MC-42, and *B. adolescentis* 94-BIM strains showed that bifidobacteria with a higher growth rate produced greater amounts of the end fermentation products, acetate and lactate. The growth of the strains in batch cultures was found to be inhibited by acidic fermentation products. The growth of *B. bifidum* no. 1 in a batch mode lasted 100 h at a population density of ~10^6 CFU/ml and the growth of *B. adolescentis* MC-42 and 94-BIM lasted 96–120 h at population densities from 10^4 to 10^7 CFU/ml. Analysis of the bifidobacterial populations by light and electron microscopy showed that they represent conglomerates of cells with a lysed cytoplasm in the cell center and an intact cytoplasm in the apical parts of the cells. The maximum production of extracellular and cell-bound proteinases was observed in the logarithmic growth phase. By the 120th h of cultivation, the metabolic activity of cells, the production of proteinases, and the protein content of bifidobacterial cultures considerably decreased. In the first, second, and third subcultures of 96-h-old bifidobacterial cells on fresh nutrient media, the population density of bifidobacteria and their normal physiological and biochemical characteristics were restored after 48 to 72 h of cultivation.

Key words: bifidobacteria, autolysis, retention of viability by bacterial populations, activity of proteinases.

Bifidobacteria are gram-positive, anaerobic, nonspore-forming microorganisms that inhabit the gastrointestinal tract of humans and animals [1]. Bifidobacteria are of great practical importance, as they are the primary components of effective remedies and prophylactics against acute and chronic intestine infections, allergic and immunodeficiency diseases, etc. [2].

In earlier publications, we showed that the development of bifidobacterial populations is associated with the morphological and structural differentiation of cells. The vegetative reproduction of bifidobacteria represents the successive transformation of rod-shaped and coccoid cells into budding cells and branching septated filaments. Autolytic processes in bifidobacterial populations are accompanied by the irreversible structural and functional rearrangements of cells [3–5]. It remains, however, unclear whether or not the nano- and resting forms of bifidobacteria generated during autolysis retain their ability to reproduce or to revert to the original forms.

According to modern concepts, the growth strategy of microbial populations transforms into a survival strategy through the stage of autolysis, whose degree and rate are controlled by some endogenous autoregulatory factors. Autolysis should be considered not only as the death phase of microbial populations but also as a normal developmental stage necessary for the formation of resting cell forms. During this phase, part of the population is induced to generate cystlike resting forms. In nature, the autolysis of microbial communities (populations) is frequently induced by the exhaustion of nutritional and energy sources [6].

In view of the foregoing, the aim of the present work was to study the physiological, biochemical, and morphological alterations in bifidobacteria at the late stages of their development, in particular, the rates of their reproduction and acidogenesis; their retention of viability during autolysis; the restoration of the populations and reversion into the original forms; their accumulation of proteins, metabolites, and proteinases; and the morphological and ultrastructural rearrangements of the cells.

MATERIALS AND METHODS

Bifidobacterium bifidum strain no. 1 and *Bifidobacterium adolescentis* MC-42 were obtained from the Gabrichevskii Moscow Research Institute of Epidemiology and Microbiology, Russia. The strain *Bifidobacterium adolescentis* 94-BIM was derived from *B. adolescentis* 91-BIM by selection with respect to proteolytic activity. This strain was deposited in the

0026-2617/01/7004-0429$25.00

Collection of Microorganisms at the Institute of Microbiology of the National Academy of Sciences of Belarus as *B. adolescentis* BIM B-87. The strains were cultivated at 37°C in liquid Blaurock medium under mineral oil or on Blaurock agar in an anaerobic jar as well as in a patented corn–lactose medium [7] and a casein hydrolysate–yeast extract medium. Material for inoculation represented physiologically active bifidobacteria of the third generation. Depending on the aim of the experiment, bifidobacteria were grown from 24 to 336 h.

To study the dynamics of growth, acidogenesis, proteolytic activity, and protein accumulation, the nutrient medium was inoculated with 18-h-old bifidobacterial cells in an amount of 5 vol % and incubated at 37°C. The samples were collected at 3-, 6-, or 24-h intervals for 3–10 days. The biomass was determined either by weighing washed and dried (to a constant weight at 105°C) bifidobacterial cells or by measuring the optical density of bacterial suspensions at 590 nm. The concentration of viable bifidobacterial cells was determined by the dilution method, inoculating cells onto a semisolid nutrient medium containing 0.2% agar. The results were expressed in colony-forming units (CFU) per 1 ml of cell suspension. The kinetic parameters of bacterial growth (the cell yield X, the specific growth rate μ, the division rate constant ν, and the generation time g) were determined by the standard procedures [8, 9]. The pH of the medium was measured potentiometrically.

Acetic acid was identified and quantified using a Chrom-5 gas chromatograph (Laboratorni Pristroje, Czech Republic). The concentration of lactic acid was calculated from the concentration of acetic acid, taking into account the glucose fermentation balance [10]. The proteolytic activity was assayed by a modified Anson's method [11], using casein (pH 9.0) as the enzyme substrate. One unit of proteolytic activity was defined as the amount of enzyme required to provide for an increase in the optical density of the casein solution supernatant comprising 0.0005 units in 20 min due to the hydrolysis of casein into peptides nonprecipitable with trichloroacetic acid. Proteolytic activity (E) was expressed in arbitrary units per 1 ml of medium. The specific rate of enzyme synthesis, ε, was calculated by the formula $\varepsilon = dE/(dt\ x)$, where x is the biomass expressed in mg/ml and dE is the increase in the enzyme activity (expressed in U/ml) in time dt [12]. To solubilize cell-bound proteinases and proteins, washed bifidobacterial cells were incubated in a 0.5% solution of Triton X-100 in 10 mM phosphate buffer (pH 6.9) for 24 h at 4°C under occasional stirring. After the removal of cells, the proteolytic activity and the protein content of the supernatant were determined according to the methods of Anson and Bradford [11, 13], respectively.

For microscopic studies, the biomass grown in Blaurock medium was treated with glutaraldehyde, osmic acid, and uranyl acetate, and it was dehydrated in a series of acetone solutions of increasing concentration. A portion of this preparation was placed in Canadian balsam on a specimen slide for examination under a light microscope, whereas another portion was embedded in epoxy resin and cut on a Reichert ultratome as described earlier [3–5]. Specimens were examined at a magnification of 10000 to 50000×. Thin sections were additionally contrasted with uranyl acetate and examined at a magnification of 18000 to 100000×.

The results presented in the paper are the means of experiments performed at least in triplicate. The results were statistically processed according to Rokitskii [14].

RESULTS AND DISCUSSION

An analysis of the logarithmic curves describing the growth of *B. bifidum* no. 1, *B. adolescentis* MC-42, and *B. adolescentis* 94-BIM in optimal media in a batch mode (Fig. 1a) showed that only *B. bifidum* no. 1 had a 6-h lag phase, whereas the two *B. adolescentis* strains grew without lag phases. The growth curves of all the strains exhibited 12- to 18-h logarithmic phases and 35-to 40-h stationary phases, which were followed by the death phases. The kinetic growth parameters of bifidobacteria are summarized in the table. The growth of *B. bifidum* no. 1 in a batch culture lasted 100 h at a population density of 10^6, CFU/ml, and the growth of *B. adolescentis* MC-42 and 94-BIM in batch cultures lasted 96–120 h at population densities from 10^4 to 10^7 CFU/ml. In the first, second, and third subcultures of 96-h-old bifidobacterial cells on fresh nutrient media, the population density of bifidobacteria and their normal physiological and biochemical characteristics were restored after 48 to 72 h of cultivation.

The growth of the bifidobacteria was accompanied by the accumulation of acetate and lactate in the cultivation medium (Figs. 1c and 1d). Taking into account that the proportion between the fermentation products in bifidobacterial populations is relatively constant [1, 10], their concentrations in the cultivation medium can be used to determine the degree of utilization of carbon sources, such as lactose, and the intensity of the cell metabolism in general. A comparison of the growth characteristics of bifidobacteria and the accumulation of fermentation products showed that more actively growing strains accumulated greater amounts of acetic and lactic acids. It is evident that the growth of bifidobacteria in batch cultures is subject to a feedback inhibition by the end fermentation products. Taking into account the fact that bifidobacteria are not acidotolerant organisms [1], their ability to remain viable at pH 4.0–4.2 for relatively extended periods of time suggests that they have developed mechanisms that allow them to tolerate unfavorable conditions.

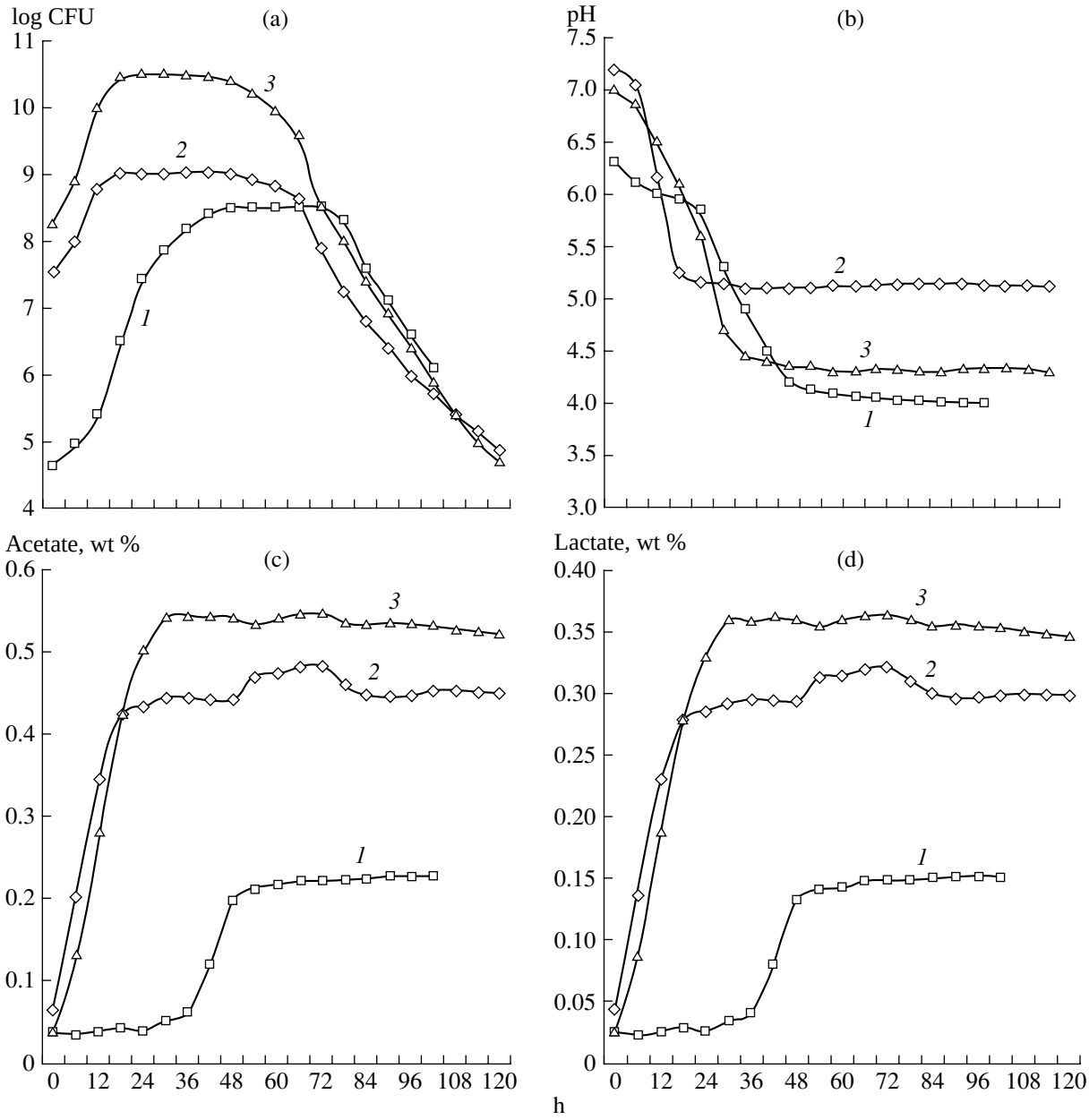

Fig. 1. (a) Growth, (b) acidogenesis (pH dynamics), and the accumulation of (c) acetate and (d) lactate by (*1*) *B. bifidum* no. 1, (*2*) *B. adolescentis* MC-42, and (*3*) *B. adolescentis* 94-BIM grown in, respectively, Blaurock medium, corn–lactose medium, and casein hydrolysate–yeast extract medium.

The morphological analysis of the bifidobacterial populations showed that they contain primarily rod-shaped and coccoid cells in the exponential and early stationary growth phases. In the developed stationary and the death phases, the bifidobacteria exhibited wide morphological diversity: in addition to rod-shaped and coccoid cells, the cultures contained budding cells, branched and septated filaments subject to fragmentation, and involutional club-shaped cell forms. The senescence of the bifidobacterial populations was accompanied by the structural and functional rearrangements of cells: the oversynthesis of the cell-wall constituents (including capsular polysaccharides and glycoproteins) the increase in the number of cell-to-cell contacts, the complication of the intracytoplasmic

Kinetic parameters of the exponential growth of bifidobacteria

Strain	Parameter			
	log$X$	μ	v	g
B. bifidum 1	3.86	0.33	0.46	2.17
B. adolescentis MC-42	2.6	0.46	0.66	1.52
B. adolescentis 94-BIM	2.69	0.54	0.77	1.29

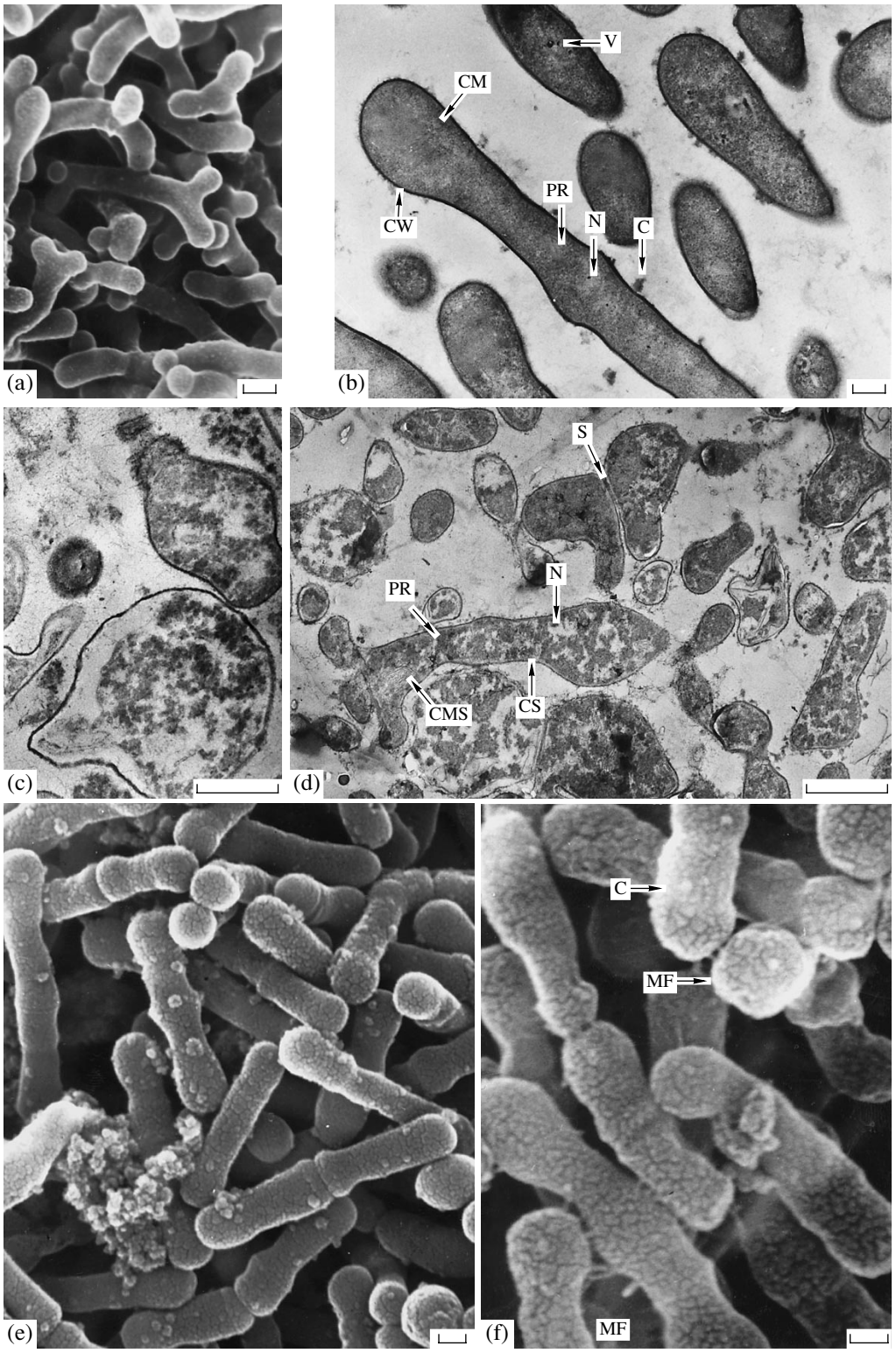

Fig. 2. Structure and morphology of bifidobacteria at the late developmental stages of their populations: (a and b) the population of stationary-phase bifidobacteria is dominated by physiologically active budding and dichotomously branching cells containing polyphosphate inclusions and intracytoplasmic membrane structures with bound polyribosomes; (c and d) the autolysing population contains cells of the normal morphotype and degrading club-shaped cells, whose morphotype is characterized by the alteration of the cytoplasm, nucleoid, and membrane apparatus and the accumulation of cytoplasmic inclusions; (e and f) the build-up of capsular polysaccharides in the late stationary and death phases and the microfibril-mediated cohesion of cells. The bars represent 0.1 μm. (a and d) *B. bifidum* no. 1 cells cultivated for 36 and 336 h; (b, c, e, and f) *B. adolescentis* MC-42 cells cultivated for 24, 120, 240, and 288 h. C, capsule; CW, cell wall; CM, cytoplasmic membrane; PR, polyribosome; N, nucleoid; V, volutin (polyphosphate) granule; CMS, cytoplasmic membrane structure (mesosome); S, septum; and MF, microfibril.

membrane system, the alteration of the nucleus compactness, and the accumulation of cytoplasmic inclusions. One could also observe the appearance of the clusters of autolysing cells and mycelial fragments and of nonviable, vacuolated cell forms resembling protoplasts (Fig. 2). In the death phase, bifidobacterial populations represented the conglomerates of cells with a lysed cytoplasm in the cell center and intact cytoplasm in the apical parts of the cells.

Protein content and proteolytic activity are important physiological characteristics of bifidobacteria, as proteinases play a role in providing the cells with the low-molecular-weight products of protein hydrolysis, which serve as the main or supplementary sources of nitrogen. The investigations showed that the maximum activity of the extracellular and cell-bound proteinases of *B. adolescentis* 94-BIM was in the logarithmic growth phase. In the 6th hour of growth, the activity of the extracellular and cell-bound proteinases increased at rates of 71.9 and 29.2 U/(mg h), respectively (Fig. 3). The specific rate of the synthesis of extracellular proteinases was also observed in the stationary growth phase. In the death phase, the proteolytic activity of bifidobacteria considerably decreased. The concentration of extracellular proteins correlated with the culture growth: this concentration decreased in the exponential growth phase, increased in the stationary and early death phases, and again decreased by the 120th to 240th h of cultivation (Fig. 3). The concentration of cell-bound proteins was minimum in the stationary phase (36 h of growth), exhibiting a correlation with the activity and the accumulation rate of extracellular proteinases. This was presumably due to the saturation of enzyme-binding sites on the cell surface. It is possible that the increase in the concentration of cell-bound proteins by the 210th h of cultivation represents the response of cells to unfavorable exo- and endogenous factors, toxic metabolites in particular. According to the suggested scale of metabolic activity (0% corresponds to the cell dormancy and 100% corresponds to the active metabolism of cells) [15], the late developmental stages of bifidobacterial populations correspond to 10–50% metabolic activity.

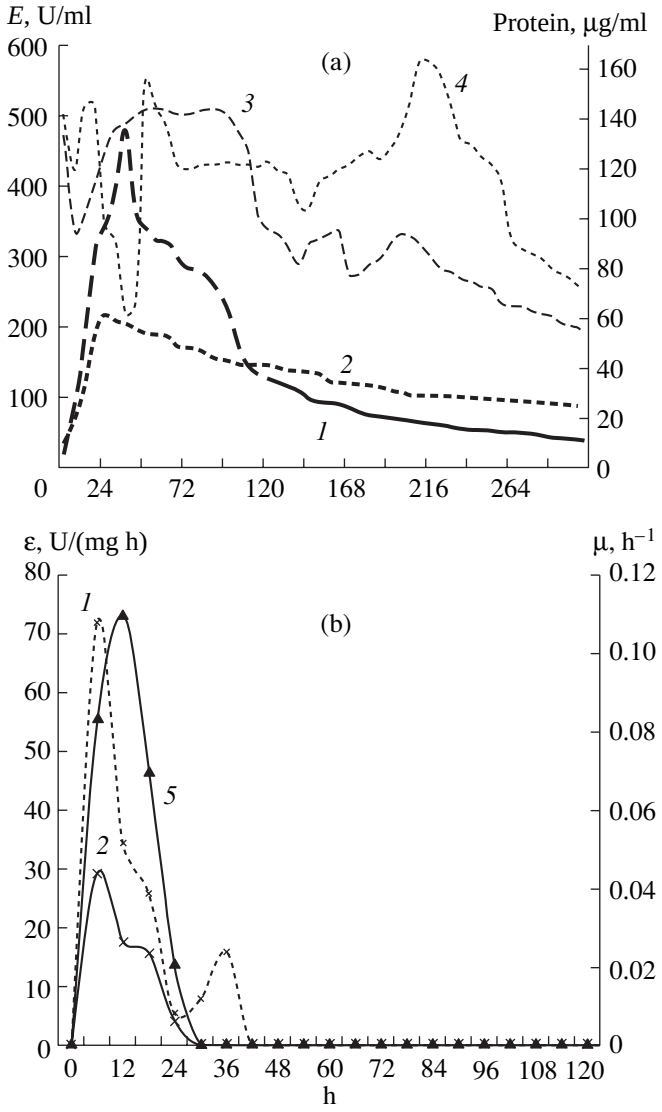

Fig. 3. (a) Activity (*E*) and (b) production of (*1*) extracellular proteinases, (*2*) cell-bound proteinases, (*3*) extracellular proteins, and (*4*) cell-bound proteins by *B. adolescentis* 94-BIM grown in casein hydrolysate–yeast extract medium. ε is the specific rate of proteinase synthesis expressed in U/(mg h) and μ (curve *5*) is the specific growth rate expressed in h^{-1}.

Summing up the experimental data presented here and published earlier [3–5, 16–21], we may conclude that the bifidobacterial populations occurring at the late stages of their development are characterized by the decreasing growth rate; the autolysis and death of part of cells; their increasing sensitivity to the population pressure, dehydration, and extreme values of temperature and pH; and autoinhibition by acidic end fermentation products [16, 20, 21]. Subculturing on fresh nutrient media promotes the viability of bifidobacterial cells and the reversion of part of the population to the original cell forms with the restoration of their physiological and biochemical activity. In this case, the processes of cell growth, division, enzyme synthesis, and acidogenesis are activated. In senescent cultures, rod-shaped and coccoid cells transform into budding cells and branched, septated filaments that are subject to fragmentation [19]. The lysis of the cells is accompanied by their structural and functional rearrangements, such as the formation of lamellar membrane structures, the alteration of the nucleus morphology and compactness, and the accumulation of polyphosphate and glycogen granules in the cytoplasm. The structure of the cytoplasm greatly changes: one can observe many cell envelopes containing the remnants of the cytoplasms, membrane fragments, storage polymer granules, etc. [3]. Along with nonviable partially degraded cells, the senescent bifidobacterial populations contain an

amount of physiologically active dividing cells [3, 16]. The cells develop thick envelopes with a three-layer cell wall, a polysaccharide capsule, and multiple polymorphic microfibrils. Such cells differ from the logarithmic-phase cells in that they possess an enhanced capability for cohesion between each other and for adhesion to solid surfaces [20]. The senescent bifidobacterial cultures are characterized by the retardation of acidogenesis; the accumulation of the end fermentation products such as acetate, lactate, and ethanol; the slowing down of assimilation processes; and the decrease in the activity and the production of extracellular and cell-bound proteinases, dextranase, β-galactosidase, β-fructofuranosidase, and other enzymes [16–18].

All these facts allow the suggestion to be made that bifidobacteria possess a mechanism that enables part of the autolysing population to revert to the original physiologically active cell forms. Such a mechanism is typical of different pro- and eukaryotic microorganisms [6]. For instance, non-spore-forming microorganisms occurring under the conditions that promote their spontaneous and induced autolysis produce cystlike dormant cells, whose number depends on the density of cell suspensions, the presence of Ca^{2+} ions in nutritionally deficient medium, pH, and the autolysis rate, the latter being, in turn, dependent on the concentration of the *d*2 factor (oleic acid), which is an autoinducer of autolysis. Microorganisms possess a specific system for the self-regulation of growth and development, which includes the autostimulants of autolysis (free unsaturated fatty acids) and the autoinducers of dormancy (alkyloxybenzene derivatives), which promote the formation of refractory cystlike cell forms [6]. Mulyukin *et al.* believe that autolysis may not only promote the formation of resting cystlike cells, but also trigger some other mechanisms responsible for the survival of unfavorable environmental conditions.

The data presented suggest that autolysing bifidobacterial populations retain cells that are capable of growth, reproduction, and reversion to their original physiologically active cell forms. Further investigations along this line of research should provide insight into other biosynthetic and regulatory capabilities of bifidobacteria in the state of dormancy. Such investigations are of great theoretical and practical importance from the standpoints of the biological significance of autolysis and the control of the biotechnological processes employing bifidobacteria.

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