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EXPERIMENTAL ARTICLES

Sorption of Microorganisms by Wide-Porous Agarose Cryogels Containing Grafted Aliphatic Chains of Different Length

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Abstract—The possibility of fractionation of heterogeneous bacterial populations using wide-porous agarose cryogels containing grafted aliphatic groups with the chain lengths of 4, 7, and 12 carbon atoms was demonstrated for the first time. The maximum sorption of vegetative cells of gram-positive bacteria (60%) was shown for the polymeric carrier with the chain length of 4 carbon atoms, while the hypometabolic cells appearing in the population after prolonged (60-day) cultivation were trapped by wide-porous affinity sorbents with C_7- and C_{12}-aliphatic groups much better than vegetative cells.

Key words: hydrophobized agarose cryogels, heterogenic populations, sorption, gram-positive bacteria, gram-negative bacteria, hypometabolic forms.

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The problems associated with the study of heterogeneous microbial populations are of interest to the scientific community due to the necessity for discrimination between different affiliation morphotypes of strains in bacterial population, and aging or responding to various stressors. The analysis of heterogeneous aging populations is concerned, first of all, with determination of the diagnostic criteria of the dormant forms of microorganisms with the accent on elucidation of the mechanisms responsible for the possibility or impossibility of reversion of such cells to metabolic activity and propagation [1]. Stresses, including those caused by aging, induce considerable changes in the physiological and biochemical status of bacteria, which manifest themselves in modification of properties of the cell surface, in particular, its hydrophilic-hydrophobic characteristics. In principle, it means that such microbial cells, due to not only interspecies but also intraspecies “age-specific” differences, must be differently adsorbed on the surface (i.e., interact with it with different binding energy). Therefore, it may be quite reasonably assumed that, when a heterogeneous cell population is run through a column with a special adsorbent “tailored” for fractionation of sorbates by their hydrophilic–lipophilic balance (HLB), the above differences in the hydrophilic–hydrophobic characteristics of the cells in a microbial population will result in their separation.

Such a process is essentially analogous to the well-known hydrophobic chromatography extensively used for isolation and purification of protein substances [2, 3]. However, the bioaffinity sorbents conventionally used for this purpose, e.g., Phenyl Sepharose or Octyl Sepharose matrices developed by Pharmacia Fine Chemicals (Sweden), are not suitable for work with whole cells, because such suspension cannot pass through a sufficiently long column with these sorbents. Hence, hydrophobic cell chromatography needs the carriers of other morphology, which can provide the required hydrodynamics of the flow of liquid containing suspended micrometer-sized particles, in particular, suspension of microbial cells.

One of the types of such matrices is so-called cryogels: wide-porous polymer gels formed in not-deeply frozen solutions of respective precursors [4]. Two types of cryogels are known: macroporous and supermacroporous ones, with the pore section of several μm and tens to hundreds of μm, respectively [5]. Supermacroporous cryogels proved to be the promising materials for this purpose, “passable” for cell suspensions; they were successfully used for development of biocatalytic systems containing immobilized cells [6, 7], at creation of chromatographic sorbents for cell separation [8, 9] and spongy carriers for 3D-cultivation of animal cells [10–12]. Hence, in this work the bioaffinity sorbents for the separation of microorganisms according to the differences in their surface HLB were prepared on the basis of a supermacroporous polymeric cryogel, namely hydrophilic agarose cryogel, followed by grafting of aliphatic chains in order to obtain a series of hydrophobized matrices with alkyl groups of a different length.

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The main goal of this work was to assess the possibility of separation on such sorbents of a mixture of vegetative cells of gram-positive and gram-negative bacteria, as well as vegetative and hypometabolic forms within the same bacterial population.

MATERIALS AND METHODS

The following bacterial strains were used in the work: *Escherichia coli* K1 (All-Russian Collection of Microorganisms (VKM), Moscow), *Salmonella typhimurium* TA100 (Research Institute for Biological Tests of Chemical Compounds, Kupavna), *Micrococcus luteus* (museum of the Department of Microbiology, Kazan State University, Kazan), *Lactobacillus plantarum* 8P-A3 (preparation "Lactobacterin dry", Biomed Research and Production Association, Perm), and *Staphylococcus epidermidis* (Institute of Ecology and Genetics of Microorganisms, Perm').

The cultures of *E. coli, S. typhimurium, L. plantarum, M. luteus* and *S. epidermidis* were grown in L-broth (per 1000 ml of distilled water: NaCl, 5 g; yeast extract, 5 g; peptone, 10 g). The grown (1- or 60-day) cultures were centrifuged; the supernatant was removed and the cells were resuspended in the physiological saline (0.9% NaCl) to the optical density of 1.4 (SF 2000, Spektr Experimental Design Office, λ = 590 nm, l = 1 cm).

Initial cryogels were prepared from Type VII-A agarose (Low Gelling Temperature, Sigma, United States) according to the patented procedure [13]. The preparations were formed inside the plastic 5-ml syringes as cylinders of 3 ml in volume, which then were washed in the flow mode followed by grafting of the aliphatic groups, using the solutions of butyric (C_4), enanthic (C_7), or dodecyl (C_{12}) aldehydes (Aldrich, United States) in 0.05 N HCl. Thus obtained, the hydrophobized cryogels were washed with distilled water and stored in 0.2% sodium azide solution at 4–6°C.

Experiments on the sorption of microorganisms by hydrophobized agarose cryogels were carried out as follows. The surface of a cryogel column was first washed with 5 ml of sterile 0.9% NaCl solution, and then, blown with sterile air; 0.4 ml of 0.9% NaCl solution was run through the column for 40 s to fill the pores of the upper part of the polymer matrix. Then, 0.4 ml of bacterial suspension was applied to the cryogel surface and run through the porous bioaffinity sorbent for 2.5 min. The column was then washed 10 times with 0.4 ml of 0.9% NaCl solution to elute the cells that were not retained on the carrier groups but nonspecifically settled on the walls of the cryogel pores. In all cases, the running of the suspension and saline solution through the gel was carried out by gravity flow. The optical densities of the initial suspension and obtained filtrates were measured. The degree of cell trapping by the sorbent containing the grafted aliphatic groups with hydrocarbon chains of specified length was assessed by the difference in optical density between the effluent and the initial suspension. The dilution obtained as a result of gel washing was taken into account. After the work, the carrier was washed with 20% ethanol solution and covered with 2% sodium azide solution to prevent contamination with the cultures under study.

The trapping of vegetative and hypometabolic cells by the bioaffinity sorbents was compared for the 1- and 60-day cultures of the same bacteria grown on 10% L-broth; each type of microorganisms was analyzed separately by the same sequence of sorption–desorption operations. The 60-day cultures were heterogeneous populations containing dormant, nonculturable, and dead bacterial cells. The presence of cells in the viable but nonculturable state was determined by the modified Kogure test [14]. This test is based on the assumption that viable cells forming no colonies on a solid medium elongate when supplemented with the nutrient substrate in the presence of nalidixic acid (an inhibitor of DNA gyrase and, therefore, cell division). Under the microscope, it is easy to distinguish between the elongated viable cells and the small cells "unresponsive to the substrate". The modification of the test used in this work consisted in staining of the preparations with vital dyes (methylene blue) to determine not only the number of elongated ("responsive to substrate") cells but also of small ("unresponsive") unstained (probably viable) and small stained (dead) cells [15]. The total number of microorganisms in the culture and the number of cells in subpopulations (in the Kogure test) were determined under an XS-90 universal binocular microscope in 10–15 fields of vision.

Data processing was carried out using the standard Microsoft Excel software package.

RESULTS AND DISCUSSION

Hydrophobic interactions are known as one of the universal types of molecular and supramoleular interactions between the substances in aqueous media as a whole and in living systems particularly [16]. Therefore, sorption processes based on the differences in HLB of sorbates are widely used for working with biopolymers. In the present work, such an approach was applied to the problems associated with fractionation of microbial cells, using bioaffinity sorbents based on supermacroporous agarose cryogel hydrophobized by aliphatic groups with the chain lengths of 4, 7, and 12 carbon atoms.

Figure 1 shows a microphotograph of a thin section of agarose cryogel demonstrating its wide-porous morphology with pore sections many times exceeding the size of microbial cells. It is quite obvious that, in the absence of marked specific (bioaffinity) interactions between the cell surface and the material of macropore walls, cell suspensions must run through such supermacroporous matrices without any steric obstacles [8].

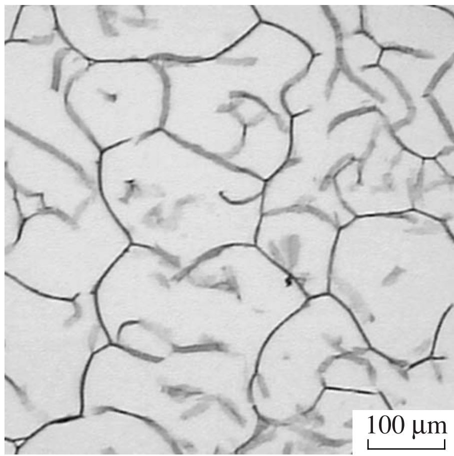

Fig. 1. Microphotograph (light microscope Axiolab Pol, Carl Zeiss Jena GmbH, Germany) of a thin section (10 μm) of agarose cryogel stained with cresyl violet.

The study of the binding of different microorganisms by hydrophobized agarose cryogels, did not reveal the "optimal" sorbent was for the 1-day culture of gram-negative *E. coli*; C12 was the "nonoptimal" sorbent (Fig. 2). Since the cell surface's HLB in gram-negative microorganisms is determined mainly by the properties of the outer hydrophobic phospholipid membrane, this is probably responsible for the trapping of such cells by cryogels. The outer surface of the *E. coli* membrane, due to a thick layer of protein pili [17], is sterically poorly accessible for direct interaction with the aliphatic groups on the surface of the walls of cryogel macropores. This is probably the reason why the values of sorption capacity for these bacteria on C_0–C_{12} were not statistically reliably different. Together with the high mobility of *E. coli*, this fact may explain the low percentage of cells trapped in cryogel.

The C_4-bioaffine matrix retained a higher percentage of cells than other carriers (C_7 and C_{12}), in case of gram-negative rods *S. typhimurium* TA100 (Fig. 2). However, the quantity of sorbed cells was generally not high (about 45%), thus indicating the low affinity of the surface of gram-negative microorganisms to hydrophobized agarose cryogel.

A somewhat different situation in respect to the sorption capacity of the same bioaffinity matrices was observed in the experiments with gram-positive cells. It was found that the cocci *M. luteus* (up to 50% of the cells applied to the column) were trapped by the cryogel with butyl substitutes. Besides, these bacteria exhibited statistically reliable dynamics of dependence between the quantity of cells retained in the column and the length of graft's aliphatic groups: with elongation of the hydrocarbon chain, the quantity of microbial cells sorbed by hydrophobized agarose cryogels decreased, i.e., the quantity of cells in a breakthrough increased (Fig. 2). The cell wall's surface of a gram-positive

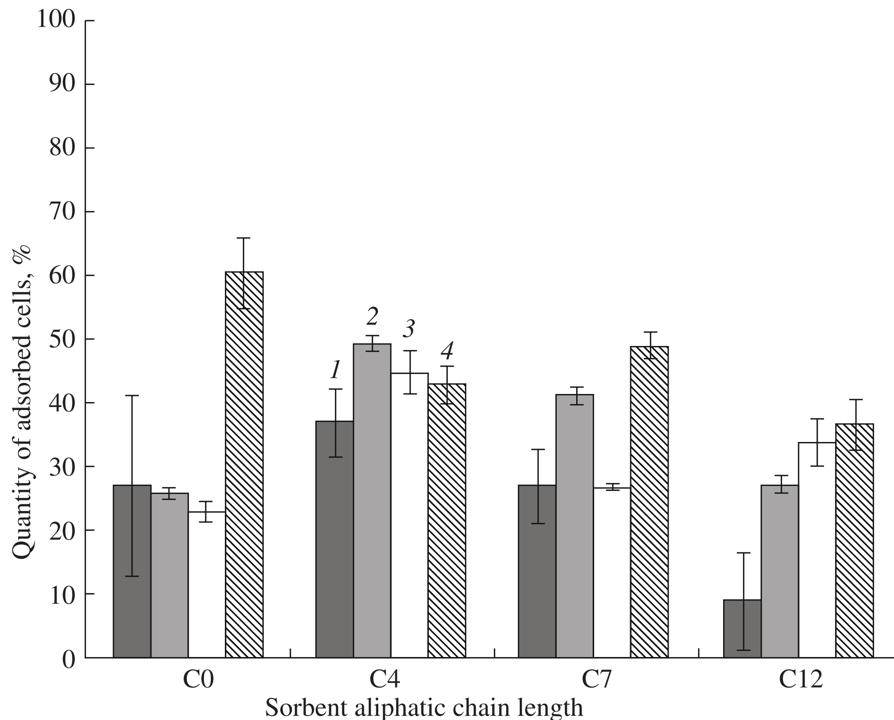

Fig. 2. Percentage of the cells of gram-positive and gram-negative microorganisms adsorbed at the running of cell suspensions through columns by bioaffinity sorbents containing grafted aliphatic chains of different length. The content of cells in the initial suspension is taken as 100%. *E. coli* (*1*); *M. luteus* (*2*); *S. typhimurium* (*3*); *L. plantarum* (*4*).

The content of cells in 60-day suspensions of microorganisms responding to introduction of exogenous substrate (Kogure test)

Microorganism	Cell quantity, % of total number		
	Responsive to substrate	Unresponsive to substrate	Dead
E. coli K12	14 ± 1	64 ± 2	22 ± 1
S. typhimurium TA100	9 ± 1	82 ± 1.5	8 ± 1
M. luteus	14 ± 1	72 ± 2	13 ± 1.5
L. plantarum 8P-A3	20 ± 1.5	5 ± 1	70 ± 6.3

micrococcus contains peptidoglycan and teichoic acids [18, 19] and, apparently, the presence of lipoteichoic acids results in realization of the hydrophobic sorbate–sorbent interactions responsible for retention of these cells by the affinity carriers used in the work.

In case of the culture of another gram-positive bacterium, the rod *L. plantarum* 8P-A3 isolated from the probiotic preparation “Lactobacterin dry”, the average values of cell sorption by hydrophobized agarose cryogels were 50 to 65% (Fig. 2). The dependence of retention of bacteria on the length of the aliphatic chain of the sorbents was not pronounced; it was, however, higher for the C_0 sorbent. Similar to *M. luteus*, the cell wall surface of lactobacilli includes peptidoglycans, teichoic acids, and an S-layer [18–20].

The culture of *S. epidermidis* showed no statistically reliable differences in the values of the quantity of sorbed cells at running of their suspension through hydrophobized agarose cryogels. Probably, it results from the fact that staphylococcus in the course of growth forms bunch-like aggregates, which are not decomposed at the introduction of the culture into saline solution. There are two variants of interpretation of the above results in sorption experiments with these cells: either the “bunches” are too big and cell suspensions “get stuck” on top of the cryogel column and move unevenly, thus blocking the liquid flow through the column, or too big particles of undefined shape cannot “approach” close to the aliphatic groups on the surface of the walls of macropores for effective interaction.

In general, the results obtained for young cultures show that further studies in optimization of the properties of sorbents, in particular, in respect to specific quantity of graft hydrophobic groups, are needed to reveal the possibility of using these gels for separation of microorganisms by Gram reaction.

The goal of the analyzed series of experiments was to compare the sorption of bacterial cells by the same affinity carriers at the early and late stages of their cultivation. The content of hypometabolic and dead cells in 60-day cultures was determined (using the modified Kogure test) prior to the experiments with cryogels. According to the data of some authors, the effect of nalidixic acid in complex with some other division inhibitors applies not only to gram-negative but also to gram-positive bacteria [21–24], which allowed us to use the Kogure test for all samples under study. The findings are presented in the Table, from which it follows that the composition of 60-day cultures significantly differed from the composition of 1-day (young) cultures, which contained at least 80–90% of proliferatively active cells, while in aging cultures this value did not exceed 20% and the rest of the population was represented by hypometabolic (test-positive) and dead (test-negative) cells.

Morphological evidence of the presence of hypometabolic cells, different from vegetative cells in the rounded shape and thickened envelopes, was obtained for aging bacterial cultures. Previously it was shown that *E. coli* cells could also take a coccoid shape under stress [25, 26].

Comparative assessment of the sorption of gram-negative *E. coli* cells by hydrophobized agarose cryogels showed that the cells of old cultures were sorbed by bioaffinity matrices (C_4, C_7, and C_{12}) to a much greater extent than the cells of 1-day cultures (Fig. 3a); the hydrophobized sorbents had a slightly greater sorption capacity than the C_0 matrix, though no statistically reliable dependence was found between the degree of cell retention by sorbents and the length of the graft’s aliphatic chain.

The experiments with another gram-negative bacterium, *Salmonella typhimurium* (Fig. 3b), showed that the cells of post-stationary 60-day culture were trapped in hydrophobized agarose cryogels better than the cells of 1-day culture, but the level of sorption by bioaffinity C_0 and C_{12} matrices actually was not different as in the case of *E. coli*. Undoubtedly, this fact points to the change in HLB of the surface of these cells in time towards the increase of hydrophobicity.

At the qualitative level, the study of sorption of gram-positive cells of *M. luteus* and *L. plantarum* by hydrophobized agarose cryogels yielded results analogous to those described above, i.e., “old” cells were reliably better retained by bioaffine matrices than “young” cells. One may note the slightly better retention of the 60-day culture of *M. luteus* by agarose cryogel with grafted heptyl (C_7) groups (Fig. 3c), whereas for *L. plantarum* cells no differences were revealed (Fig. 3d).

The Kogure test for 60-day cultures of lactobacilli revealed significant differences in the specific portion of hypometabolic and dead cells (Table). It should be

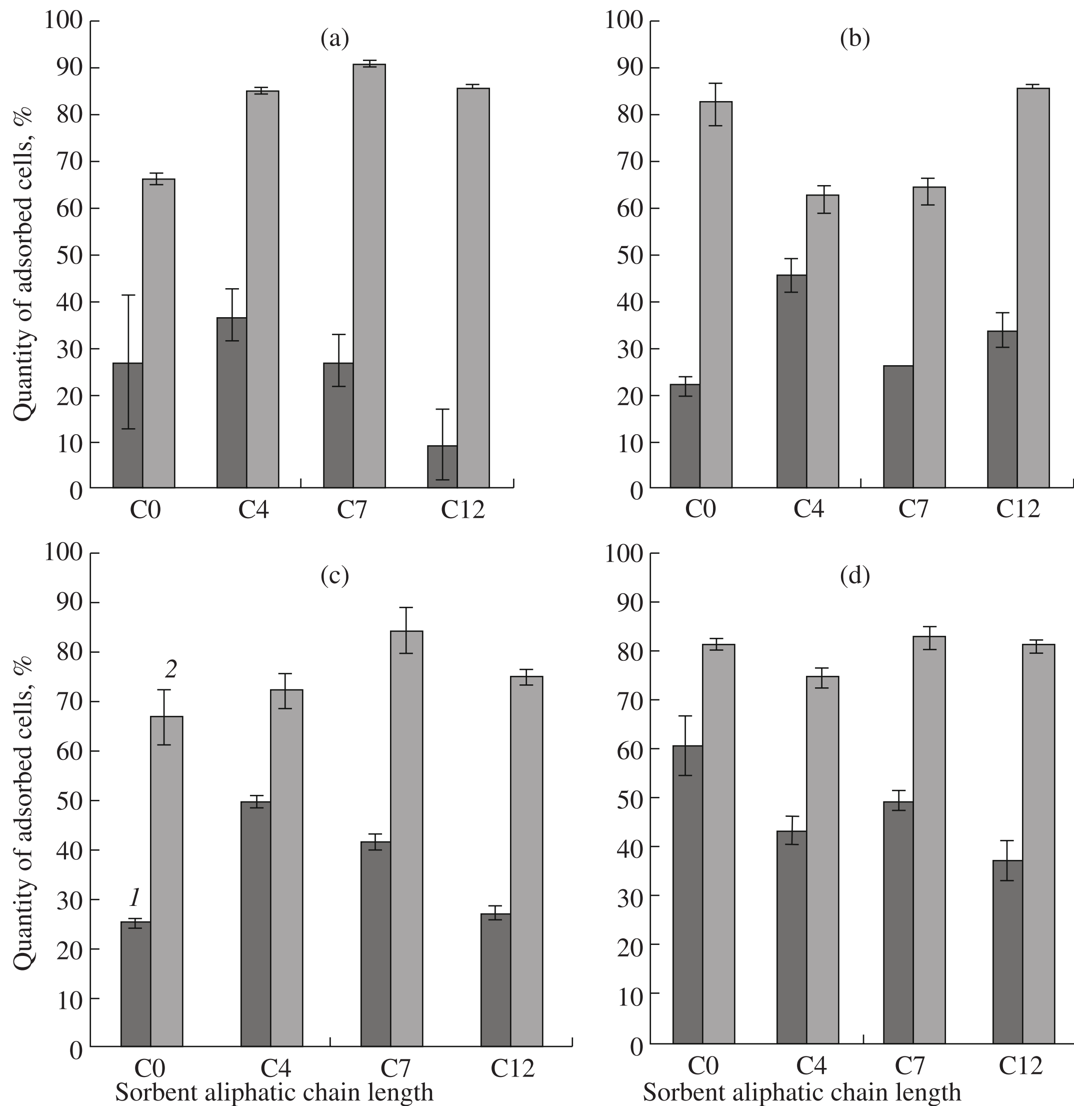

Fig. 3. Percentage of the cells of 1-day (*1*) and 60-day (*2*) cultures adsorbed at the running of cell suspensions through columns with bioaffinity sorbents containing grafted aliphatic chains of different length: *E. coli* (a); *S. typhimurium* (b); *M. luteus* (c); *L. plantarum* (d).

noted that dead cells have very high indices of sorption capacity and their surface is similar to the surface of a cell in the hypometabolic state [26]; probably, that is why hydrophobized agarose cryogels sorbed equally well both types of cells, hypometabolic and dead ones. Considerable sorption on unmodified cryogel, in our opinion, is also due to the high percentage of dead cells in the 60-day suspension of *L. plantarum.*

Thus, it was established for the studied gram-positive and gram-negative bacteria that the old and dead cells of post-stationary 60-day cultures are much better sorbed by macroporous hydrophobized agarose cryogels containing grafted aliphatic chains of different length than young vegetative cells. The changes in the cell surface HLB at transition to the state of hypometabolism result in the increase of affinity of surface structures to hydrocarbon groups and cell adhesion on the inner surface of the walls of macropores of a hydrophobized cryogel.

The results lead to the conclusion that wide-porous agarose cryogels, especially hydrophobized by grafted aliphatic chains of different length, can be used as indicators of the relative hydrophobicity/hydrophilicity of the cell surface and as an instrument for fractionation of vegetative and hypometabolic forms of microorganisms.

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