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REVIEWS

Approaches to Cultivation of "Nonculturable" Bacteria: Cyclic Cultures

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Abstract—The work deals with more efficient procedures for the isolation and cultivation of "nonculturable" microorganisms (NM) from environmental sources. The techniques for NM cultivation in situ and under laboratory conditions are discussed. A new approach is considered, viz., cultivation under cyclically varying conditions with the cycle duration comparable to the duration of the cell cycle. Cyclic cultivation implies sequential changes of several cultivation phases with different growth conditions. An established sequence of growth phases provides for the competitiveness of the target microorganisms and for accumulation of their biomass. Cultivation of phosphate-accumulating bacteria, nonculturable microorganisms which have not been previously isolated in pure culture, in an SBR reactor is discussed as an example of cyclic cultures.

Keywords: nonculturable microorganisms, cyclic culture, phosphate-accumulating bacteria

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Despite the impressive achievements of modern microbiology, mainly due to the development of molecular biology techniques, the skill of isolating new microorganisms and growing them under laboratory conditions remains the key one for studies on the physiology of microorganisms and application of their properties in biotechnology. Taking into account that environmental objects still remain the major sources for new microorganisms, optimization of the conditions for successful cultivation and isolation of previously unstudied microorganisms from natural habitats may be considered the most important factor for successful development of both fundamental and applied research.

In microbial ecology to date, there is a paradox of considerable divergence between the results of direct count of microorganisms in natural samples and quantitative evaluation of the population density based on microbial cell cultivation under laboratory conditions. The phenomenon was termed the great plate count anomaly [1]. For example, in natural ecosystems (in freshwater and marine water bodies, silt deposits, and soils) high density of microbial population, typically within the range of 10^7–10^9 cells/g, is registered with direct counting methods, and molecular biology techniques reveal the great diversity of microorganisms. At the same time, classical methods of plating under laboratory conditions allow for cultivation of an insignificant fraction of cells visible under the microscope; the result of laboratory cultivation often shares no common features with the spectrum of dominant forms detected by molecular biology methods. As follows from the results of many studies, presently only 1% of prokaryotes may be grown under standard laboratory conditions while the overwhelming majority of the cells occurring in nature cannot be cultured outside their habitat for some reasons [2, 3]. Moreover, many major phylogenetic lines (phyla) of bacteria and archaea established in the recent years by methods of molecular genetics do not even have any known cultured representatives [4, 5]. Microorganisms that cannot be grown under artificial conditions are usually termed nonculturable microorganisms (NMs). With certain limitations, the terms unculturable, uncultivable, uncultured, uncultivated, and nonculturable organisms may be used as synonyms of NM.

Supposedly, there are two main reasons for the nonculturability problem:

(1) For most of the microorganisms, conditions for growth in laboratory have not yet been selected, which explains the existence of NMs. With improved understanding of ecology and physiology of microorganisms, prokaryotes that are considered nonculturable will be transferred to the group of culturable. From this point of view, we are to speak of yet-to-be-cultivated organisms [3].

(2) Emergence of the cells incapable of proliferation on laboratory media without a preliminary stage of resuscitation under natural conditions or upon lab-

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oratory cultivation. These cells are called viable but not culturable (VBNC), i.e. living cells that lose the ability to form colonies or grow in liquid media, but are capable of restoration of metabolic activity [6]. Both yet-to-be-cultivated cells and the cells of known organisms already grown under laboratory conditions but having lost the ability to restore growth without special resuscitation procedures may be referred to VBNC. Often, these cells are termed non-dividing, dormant, or resting cells. Emergence of such cells may be of adaptive nature, i.e., a response to sudden changes in the environmental conditions or the effect of unfavorable conditions (starvation, antibiotics, etc.) It was demonstrated that these forms differ from the normal cells by the organization of their genetic apparatus, morphological, and biochemical features (see reviews [6, 7]).

The cells belonging to VBNC may be divided into two groups. The first group is represented by the forms originating from actively growing cells struck by unfavorable conditions (low temperature, starvation, etc.). This group has been first described by Colwell et al. [8] by the example of enterobacteria in estuarine water. The debates on the nature, properties, and methods of revival of such cells are ongoing, e.g. [6, 9, 10, etc.]. The procedures used to reverse these cells include medium dilution, washing off possible inhibitors, introduction of antioxidants, etc. The second group is represented by specialized dormant cells formed in the course of a normal cycle of culture development and inherently capable of prolonged survival under unfavorable conditions. Reversion of these resting forms requires certain environmental stimuli which may be present, for example, in the culture liquid of a certain growth phase. For example, for a successful revival of dormant *Micrococcus luteus* cells, sterile (but not autoclaved) culture liquid of the lag or early exponential growth phase was used [11]. The signal waking the cells up is Rpf, a specific proteinaceous factor of the resting form waking [12]. Signals for germination of the resting (persistent) forms of pathogenic bacteria, for example, tuberculosis mycobacteria, may be the components contained in the fluids of a host organism [13]. An example of a comprehensive approach to resuscitation of NMs is successful germination of the dormant cells of NMs preserved in ancient soils and frozen subsoil deposits several million years old (in the samples considered sterile based on the absence of colony-forming cells in them). The procedure of resuscitation of the dormant forms included washing off the germination autoinhibitors and introduction of extracellular low molecular-weight growth regulators of both microbial and plant origin: alkylhydroxybenzenes of the alkylresorcinol group, indolylacetic acid, and wheat germ agglutinin [14]. Application of semisolid agar, diluted media, and addition of pyruvate may be efficient in resuscitation of resting forms (for example, those of *M. luteus* and *Arthrobacter globiformis*) [15]. Most probably, the successfully used signal components of the media and approaches to resuscitation simulate the conditions of natural bacterial habitats, which act for them as a signal for germination.

Despite the obvious biological importance of the existence of VBNC organisms, the approaches to cultivation of microorganisms reviewed in this work aim to overcome, in particular, the lack of growth in yet-to-be-cultivated organisms.

IN SITU CULTIVATION

The first choice among the methods to isolate new organisms is cultivation in their natural habitat or under laboratory conditions (obviously, combinations of the above are also possible). Cultivation of microbial cultures under natural conditions or those closest to natural seems the most straightforward and reliable way to obtain the proofs of viability and proliferation of microbial cells in a natural environment and to produce a culture enriched with the microorganisms living in the environment. It is assumed that under these conditions aboriginal microorganisms get all or most of the nutrients required for their growth, including those that are synthesized by the surrounding organisms, while they are relieved from the growth-suppressing products of their own metabolism.

Origination and initial development of the method of in situ cultivation of microorganisms living in soil and aqueous ecosystems are associated with the Russian works. The basics of the methods for microculture growth under natural or close to natural conditions were developed back at the rise of soil microbiology, when the Russian researcher N.G. Kholodnyi proposed the methods of fouling glasses and soil chambers [16, 17]. A prototype of the modern methods of growth (enrichment) of autochthonous microflora under natural conditions is the capillary method developed in the middle of the previous century by B.V. Perfil'ev and D.R. Gabe [18]. The authors developed a special technology to prepare glass capillaries with a profiled cross-section that allowed creating unique flow microchambers that simulated natural (or close to natural) conditions of microbial growth. The methods employing fouling glasses and capillaries (or pedoscopes) in their further modifications gained wide acknowledgment in soil microbiology [19].

Diffusion and fiber membrane chambers are examples of a modern development in this direction. A diffusion chamber for in situ cultivation is a ring-shaped microreactor limited by two semipermeable membranes covering the bottom and the top of the ring. Its interior is filled with agarized medium with microbial cells. Diffusion chambers provide for microcolony growth due to diffusion of the substrates from outer medium (environment) to the growth chamber and

backward diffusion of the metabolic products; this allows for a considerable increase in the numbers and diversity of cultured microorganisms [20, 21]. Hollow fiber membrane chambers are functionally similar to diffusion chambers: in these systems the objects grown are separated from the environment with a membrane permeable for the substrates and products of metabolism [22]. Their compact size and simplicity make it possible to construct working blocks of up to hundreds of hollow fiber membrane chambers functioning under the same conditions. Certain progress has been associated with the use of other methods of membrane cultivation. For example, growth of microorganisms on semipermeable membranes separating the substrate (soil suspension) and the inoculum was used to produce microcolonies of many previously uncultured bacteria, including the bacteria of the TM7 group [23, 24].

Despite the obvious progress reached by in situ cultivation of new aboriginal microorganisms belonging to NMs, the approach is associated with a number of inconveniences: low biomass yield of the enrichment culture, the dependence on a complex of unpredictably changing factors, complexity of the system, etc. Therefore, in most cases the final stage of "domestication" of microorganisms includes investigation and simulation of the conditions for their cultivation in the laboratory and, if needed, industrial settings, which provides for the possibility of detailed study of their physiology and utilization in scaled production.

CULTIVATION IN THE LABORATORY SETTINGS

The experience on prokaryote cultivation accumulated by now makes it possible to outline the major approaches defining the success of laboratory cultivation of previously uncultured organisms [25–28]. In general, the approaches to NM cultivation may be divided into three groups, although combinations of these approaches are typically used in practice.

(1) Modification of the media towards better simulation of natural nutrient solutions.

(2) Choice of the method and mode of cultivation (continuous or batch cultivation, substrate supply mode, etc.).

(3) Application of specific approaches increasing the selectivity of cultivation and the possibility of isolation of target microbial cultures: increase in the repeat number (for example, due to decreased bioreactor volumes and their increased numbers), isolation of individual cells using micromanipulators and microcapsules, introduction of inhibitors of growth of accompanying microorganisms, co-cultures, etc.

Let us take a closer look at the first two approaches.

Modification of the Media

The central principle of NM cultivation is the maximum similarity between the whole complex of cultivation conditions and the natural environment. This relates primarily to application of the media reproducing the natural substrate solutions in their composition and content of the components. On the rich media traditionally used in microbiology, fast-growing microorganisms develop at priority rates, while the slow-growing ones may be suppressed [29]; therefore low concentrations of nutrients are applied in the investigations of most terrestrial and aquatic microbial associations. Application of diluted media improves the efficiency of microbial cell germination due to a decrease in the probability of substrate-accelerated death [30]. To reproduce natural growth conditions, soil and sediment extracts, humic acids, and other components characteristic of natural substrates are used. Another successful approach is the addition of signaling metabolites and autoregulators, including cell-free extracts or culture fluids of combined cultures [31–34]. In microbiology of aquatic systems, media based on sterile natural water are widely used [35, 36]. A considerable enlargement of the spectrum of cultivated microorganisms in the past few decades has been due to media modification combined with other approaches of laboratory cultivation. Application of diluted media made it possible to isolate laboratory cultures of representatives of a number of groups that have been previously considered nonculturable: marine bacteria of the SAR11 cluster [35, 37], freshwater ultramicrobacteria with abnormally low content of nucleic acids [36], ammonium-oxidizing *Crenarchaeota* [38], *Verrucomicrobia* and *Acidobacteria* from soil environments [39, 40], etc. Application of diluted media with low content of mineral salts led to successful cultivation of the previously unknown acidophilic methanotrophic bacteria [41, 42].

It should be noted that the nutrient concentration even in diluted traditional media is not always comparable with their concentration in the solutions of natural microbial habitats. For example, agarized R2A medium, which had been initially proposed for determination of the total number of heterotrophic bacteria in drinking water, is often used for isolation of microorganisms from natural ecosystems [43]. Even in 100-fold diluted R2A, the final concentration of easily digested organic substances is of the order of tens of mg/L. At the same time, according to the current knowledge, the concentration of biologically available organic carbon in solutions of aquatic ecosystems is 10–100 μg/L, and the values of half-saturation of the transport systems in oligotrophic organisms are mostly from several to several hundred μg/L [44, 45]. Therefore, already at a concentration of organic compounds of several mg/L the rates of transport processes (and probably of all major metabolic processes) may reach

the values providing for specific growth rates close to maximum; thus, these concentrations can barely be classified as low. In our opinion, in order to cultivate microorganisms at low concentration of substrate(s) it is reasonable in somecases not to dilute the medium, but to choose the methods of cultivation in which actual concentrations of the growth-limiting substance in the growing culture remain sufficiently low during the whole period of growth.

Choice of the Method and Mode of Cultivation

One of the most important stages in the isolation of target NM from environmental objects is the choice of the most appropriate method(s) and mode(s) of cultivation.

The totality of the conditions of laboratory cultivation of a microbial culture may be divided into three stages:

(1) Starting (initial) cultivation conditions.

(2) Conditions during the main growth period.

(3) Conditions for termination of cultivation.

We use the term starting conditions as the conditions during inoculation, including the methods of inoculation, and initial growth of the culture. It should be noted that qualitative and quantitative parameters of the medium composition and conditions of growth in the starting period of cultivation may differ considerably from the conditions of the main growth period. For example, upon continuous dialysis cultivation, the main period of growth occurs at extremely low concentrations of a growth-limiting substrate diffusing to the culture through a semipermeable membrane, while during the starting period the culture may grow in a batch mode, that is, at high initial nutrient concentrations. Both characteristics of the starting growth medium and the size of the inoculum (sample volume) may be also considered the starting conditions. Application of the inoculum containing a minimum number of cells in combination with cultivation in poor media were to a considerable extent responsible for the progress in isolation from natural sources of many of the slow-growing microorganisms which had previously been considered non-culturable [46–48]. Obviously, evaluation of cell number in the starting sample becomes important to determine the required cell concentration in the inoculum. Preliminary determination of the total cell number in a sample by fluorescence microscopy or flow cytometry has been used successfully for this purpose [49].

Conditions of the main period of growth are defined by the type (method) and mode of cultivation, medium composition, and physical conditions of growth.

The main parameter of the final stage of cultivation is the duration of cultivation, determined first of all by the doubling time of the cultivated microorganisms. A number of cultures of slowly growing microorganisms were obtained upon a considerable increase of the incubation time to many weeks and months [47, 50]. To grow, for example, bacteria performing anaerobic oxidation of ammonium or anaerobic oxidation of methane, months and even years of cultivation are needed (Fig. 1) [51–53]. An example in Fig. 1 illustrates another aspect of the importance of prolonged incubation for stable growth of the target culture. In the interval from day 0 to day 200 of the experiment, regular bursts of activity of ammonium consumption (at days 40, 80, 120, and 160) were observed; they were followed by activity drops probably associated with short-term development of some strains of microorganisms and later replaced with a more competitive strain of anammox bacteria.

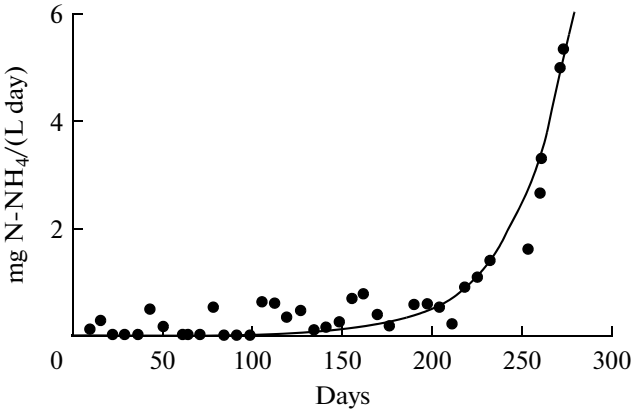

Fig. 1. Dynamics of specific rate of ammonium oxidation in an enrichment reactor for production of a microbial community enriched with anammox bacteria. The rate of accumulation of ammonium nitrogen reflects the abundance of anammox bacteria in the bioreactor (calculated according to [52]).

The methods of cultivation of microorganisms are typically divided into two groups: continuous and batch cultivation. Continuous cultivation deals with open or partially open systems, that is, systems components of which (such as biomass, substrate, and metabolism products) may enter the system or leave it. Batch cultures belong to closed systems, which cannot exchange components with the environment [54]. Despite the existence of a wide diversity of cultivation techniques, they may be arranged into groups in the following way (Fig. 2).

Continuous cultures are characterized by a constant supply of nutrients and removal of metabolism products from the system. Flow cultures, such as chemostat, turbidostat, pH-stat, and culture of complete substitution are continuous ones.

In the case of traditional batch cultivation, no substrate is supplied to the system in the course of growth, metabolism products are not removed, and the physi-

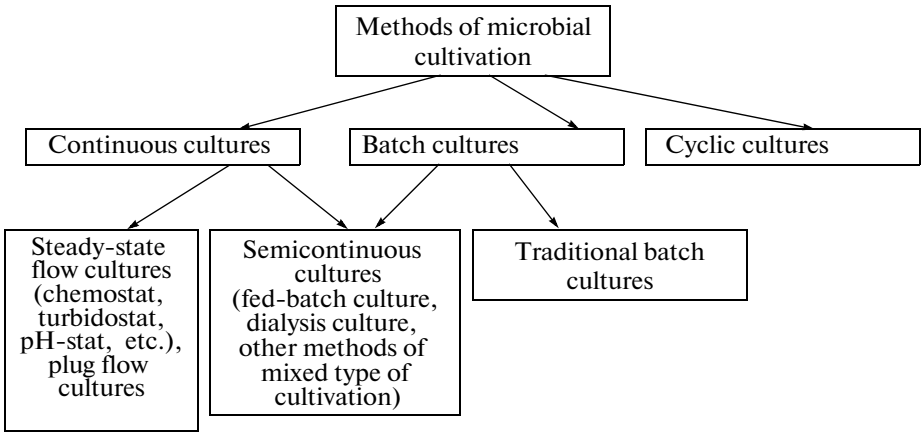

Fig. 2. The main methods of cultivation of microorganisms.

cochemical parameters (for example, temperature and oxygen concentration) or are either maintained at a constant level are not controlled. Many intermediate techniques comprising the elements of both continuous and batch cultures may be identified, for example, as fed-batch or dialysis cultures. The former ones are characterized by continuous supply of a feeding solution to the system. In a continuous dialysis system, nutrients are supplied to the system and metabolism products are removed from it via diffusion across a semipermeable membrane separating the volume with microbial cells from the nutrient solution. Such variants of batch cultivation as fed-batch culture may also be viewed as mixed ones or a hemicontinuous culture.

Depending on the chosen method of cultivation, quantitative characteristics of the medium may change considerably in the course of cultivation or, on the contrary, may remain constant. The latter variant is typical of the steady-state continuous cultures, while in the case of batch cultivation the concentration of nutrients in the course of cultivation decreases progressively.

As a rule, batch cultures are used for NM cultivation, although there are examples of successful application of continuous cultivation for this purpose as well [35, 55]. Batch cultivation is not free from considerable limitations. One of its disadvantages in the case of low-concentration media is the problem of accumulation of sufficient amounts of target cell biomass. Obviously, it is difficult to obtain a dense enrichment culture (homogeneous or in colonies) under conventional batch growth conditions at low starting concentrations of the substrates. For example, in the case of growth in the medium containing organic substances at the initial concentration of 100 μg/L, the number of grown cells of the volume of 1 μm^3 is approximately 50000 cells/mL, which is comparable to the concentration of bacteria in many natural water bodies. To overcome this disadvantage, the method of progressive transfer from low nutrient concentrations to "normal" ones may be successful; it was used to isolate, for example, pure cultures of ultramicrobacteria belonging to the *Actinobacteria*, which are common in freshwater environments [56].

The most common way to solve the problem of cultivation at extremely low concentrations of nutrients is periodic or continuous supply of new portions of nutrients and restriction of the biomass of growing cells: cell growth in the attached form on insoluble (dense) surfaces, application of semipermeable membranes, sedimentation of a microbial suspension, etc. These growth approaches are widespread in natural habitats. For example, a technique of flow growth of the microcolonies of difficult-to-cultivate bacteria pre-encapsulated in gel microparticles was developed. Microparticles are placed in a chromatography column, through which a solution of nutrients close in composition to the natural one is passed for 5 weeks or more. Diffusion of the substances from solution to gel particles provides for continuous cell growth with simultaneous removal of metabolism products and provides for intercellular metabolite exchange. After the development of microcultures, gel microparitcles are analyzed by flow cytometry [55].

Apart from the traditional continuous and batch cultures and variants thereof, the experience of microbial cultivation is being spread to a relatively new field of enriched NM cultures, which are growth under cyclically changing conditions with duration of a single cycle below the period of microbial culture generation.

CULTIVATION UNDER CYCLING CONDITIONS WITH CYCLE DURATION NOT EXCEEDING THE DOUBLING TIME OF A MICROBIAL CULTURE

In contrast to batch culture, cultivation conditions change repeatedly in the case of cyclic cultivation. Each cycle includes a progressive change of several phases (periods) of growth with different growth conditions. This sequence of growth phases provides for the competitiveness and accumulation of the target microorganism biomass. Duration of the period of biomass doubling should be lower than the duration of one cycle of growth, that is, at least one cycle of cultivation should be performed to achieve balanced cell growth. In the broad sense of the word, cyclic culture may include the elements of both continuous and batch cultivation, alternating in the course of a single cultivation cycle in a certain order. Any physicochemical parameter—for example, temperature, oxygen concentration, content and composition of organic compounds—may be the cycling factor.

Initially, the main aim of cultivation under cycling conditions was optimization of the yield of the target metabolic products in technological processes employing known cultivated microorganisms. It was found that cycling conditions of cultivation changed the physiological and morphological characteristics of microbial cells, could lead to synchronization of cell cycle, and stabilized certain physiological properties of the cultures [57]. For instance, cycling changes of temperature during *Escherichia coli* cultivation lead to irreversible stabilization of fatty acid composition in the cell membrane, corresponding to low temperature physiology [58]. However, in recent years, it was found experimentally that cycling conditions might promote preferential growth of certain physiological groups of microorganisms. For example, successive alteration at a certain frequency of the anaerobic growth phase with high concentration of easily available carbon and energy sources and the aerobic phase with high content of phosphate ions in the medium was successful in creating competitive advantages for phosphate-accumulating bacteria over other microorganisms [59].

It is a rather simple task to create a consecutive exchange of repeating physical parameters—such as temperature, pressure, and illumination—in the laboratory settings. Rapid change of the chemical composition and nutrient concentrations of the medium is generally more complicated. It may be achieved, for example, by pulse introduction of concentrated solutions, fast transfer of the biomass from one bioreactor to another, or by retention of the biomass in a reactor during the replacement of used medium with a fresh one. The latter variant seems the best elaborated and is performed using several methods: application of membranes separating the culture and the nutrient source to sustain the biomass; growth in the form of precipitating or floating (extractable) suspension; growth in the form of a biofilm on the surface of an insoluble material (media). All these methods of cultivation are actively used in various biotechnological processes, for example, in the course of wastewater treatment [60]. The method of biomass separation from waste medium by sedimentation of a suspension gained the widest spreading. In biotechnology of wastewater treatment, such suspension is termed activated sludge; it is removed in the end of the process and is returned back to the head of the process [61].

For cyclic cultivation of microorganisms, the so-called sequencing batch reactor (SBR) gained the widest application; in this reactor a culture is passed through a series of phases, the duration and sequence of which are preset by the researcher and are predetermined by the aim of the cultivation within a single cultivation cycle in a single bioreactor vessel. A cultivation cycle in an SBR begins with reactor fill-up and ends with the removal of the waste medium, similarly to the common batch culture. Growth conditions during each phase of the cycle may differ considerably: phases of starvation and growth, with aerobic and anaerobic conditions, periods characterized by different concentrations of organic substances or mineral components, temperature, acidity, etc. may be present in a cycle. The main condition for SBR cultivation is the ability of growing biomass for sedimentation (in a broad sense, for a sufficiently fast separation from the liquid phase); therefore the biomass is grown in the form of rapidly precipitating sludge or on the surface of packing. The main cycle of a classic SBR process is presented on Fig. 3. After the period of filling (1), bioreactor moves to the main stage (2), which in turn may proceed in several phases differing by physicochemical conditions. The main stage is followed by the period of sedimentation (3), waste medium removal (4), and, if needed, removal of a part of the sediment (so-called excess activated sludge) (5). Then the cycle is repeated.

The technology employing SBR has been well-developed and is presently widely used for wastewater treatment at industrial sites. Under laboratory conditions, reactors of this type are used to perform scientific research aimed at optimization of the wastewater treatment equipment [62]. Despite the applied technological nature of the works with SBR, application of these reactors made it possible to obtain the cultures enriched with phosphate-accumulating bacteria, the main representatives of which have not been isolated in pure cultures and are traditionally referred to NMs [63]. An example of SBR cultivation of phosphate-accumulating bacteria is presented on Fig. 4. The phase of reactor filling with fresh medium is followed by an anaerobic phase (absence of aeration) characterized by a rather low concentration of oxygen, which is necessary for the major biochemical processes of

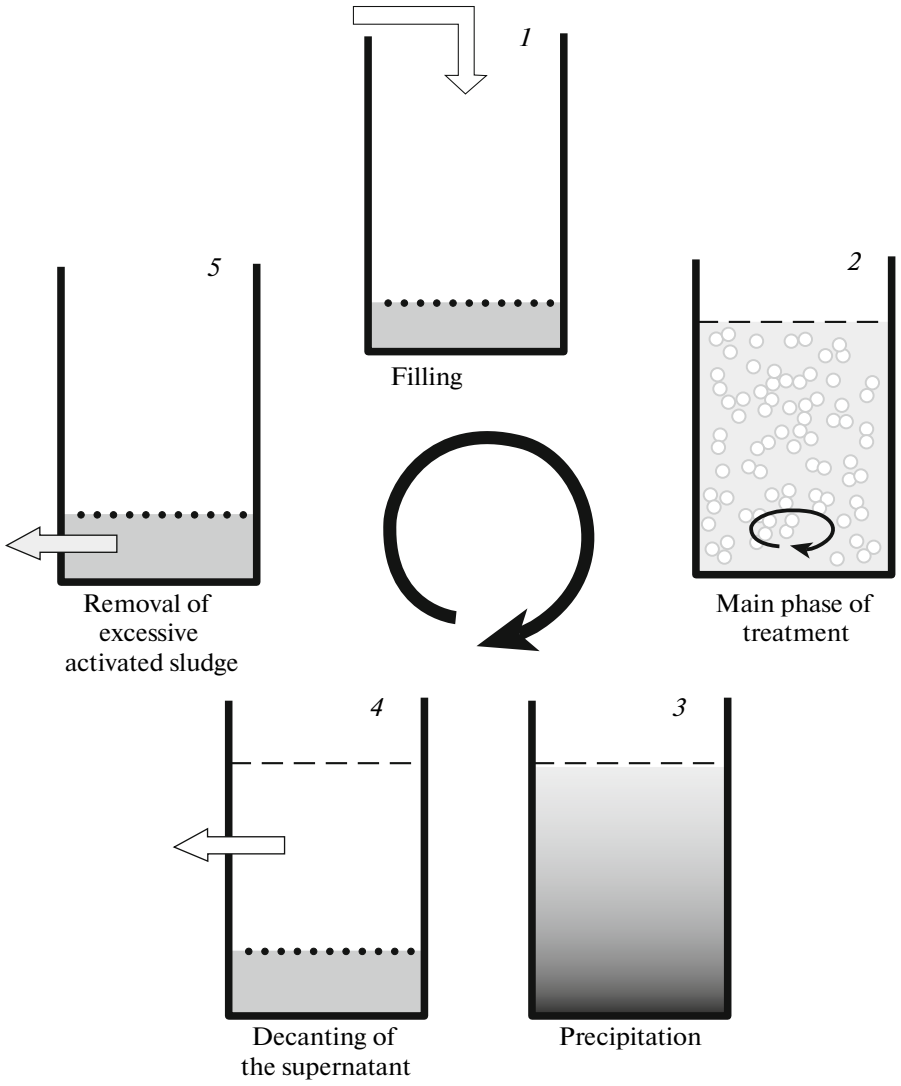

Fig. 3. An example of the main cycle of an SBR process.

phosphate removal (Fig. 6). Then, aeration is forced, oxygen concentration increases, and the culture is transferred into the aerobic cultivation phase. After the aeration system and the mixing system are turned off, the suspended fraction of the microbial community is precipitated, and a portion of the supernatant is removed. Then, the cultivation cycle is repeated.

Cyclic growth conditions are created to a certain extent in other (not SBR) technologies of wastewater treatment. Among the large-scale systems, most common are the processes of wastewater treatment with activated sludge in reactors of various configurations where, particularly, the population of phosphate-accumulating bacteria is maintained at the level sufficient for removal of most of the phosphates of wastewater (typically at concentrations of several mg/L).

The reason for accumulation of biomass of noncultured phosphate-accumulating bacteria in a cyclic culture becomes evident upon analysis of the physiological features of these microorganisms.

According to the modern concepts, if electron acceptors (oxygen or nitrates) and orthophosphate ions are present in the medium and readily oxidized storage compounds (for example, polyhydroxybutyrate) are present inside the cells of phosphate-accumulating bacteria, they consume phosphate and accumulate it in the form of polyphosphates [64]. The general scheme of polyphosphate formation is presented on Fig. 5. An opposite picture is observed when polyphosphate-containing bacterial cells are transferred into the medium with easily digested organic substrate (for example, acetate) under anaerobic conditions.

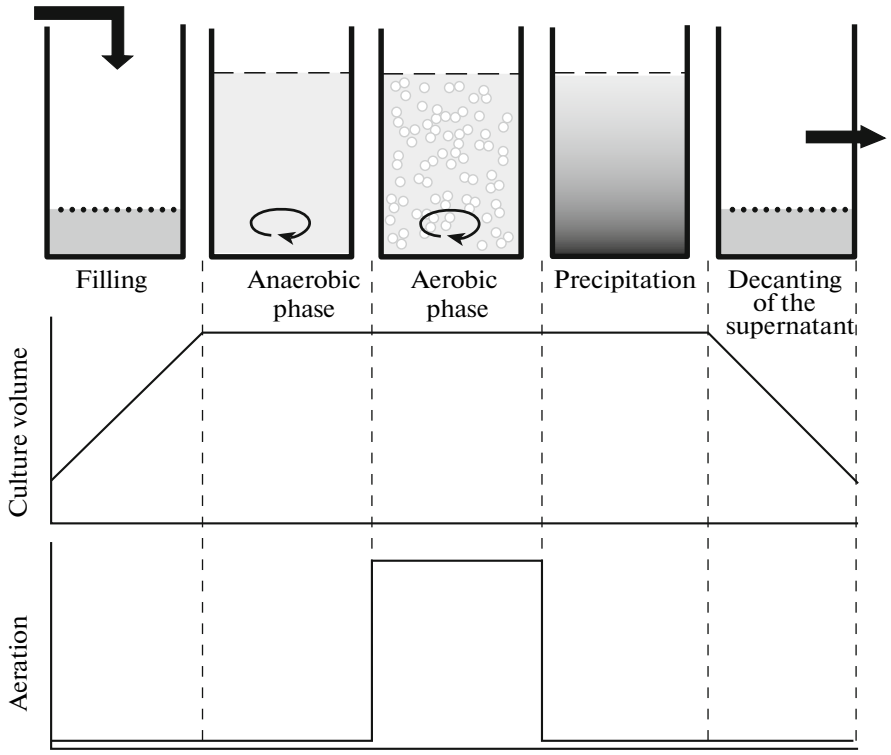

Fig. 4. Sequence of events in SBR operation during the cultivation of phosphate-accumulating bacteria. The stage of removal of excessive activated sludge (biomass gain) is not shown.

Acetate is consumed, converted to polyhydroxybutyrate, and stored in this form. Since polyhydroxybutyrate is a more reduced compound than acetate, a reducing agent in the form of NADH, formed in the cells upon glycogen conversion to polyhydroxybutyrate, is required. The required energy in the form of ATP is produced upon polyphosphate hydrolysis. Soluble orthophosphates are secreted into the environment.

Figure 6 schematically presents the dynamics of concentrations of the major extra- and intracellular metabolic components upon change of anaerobic conditions to aerobic ones in the culture of phosphate-accumulated bacteria in the presence of extracellular orthophosphate and volatile fatty acids (easily absorbed organic substrate) in the beginning of the anaerobic period.

As a result of consecutive passing through anaerobic and aerobic periods, the biomass of phosphate-accumulating microorganisms increases and the final concentration of orthophosphates in the medium decreases. The process forms the basis for the technology of biological removal of phosphorus from wastewater, and the SBR-type reactor provides the technical facilities for realization of a cyclic culture of phosphate-accumulating bacteria.

Multiple experimental data obtained with cyclic mixed cultures of the organisms of activated sludge reliably evidence the increase of the fraction of phosphate-accumulating bacteria in the sludge, up to 80–90% of the total cell biomass. In most works, accumulation of *Candidatus* Accumulibacter phosphatis, the major candidate for the leading role in the group of microorganisms capable of accumulation of intracellular polyphosphate, was noted [65–68].

Although the application of cyclic cultures led to successes mainly in the development of wastewater treatment technologies, in our opinion cyclic changes in growth conditions in the course of cultivation may turn out to be a significant factor increasing the efficiency of cultivation of some groups of microorganisms, including the NMs.

Multiple examples of cyclic changes (with frequency of about one day or less) of physicochemical parameters in the life of microbial populations, typical for natural habitats of the microorganisms, indirectly evidence the importance of regular changes of growth conditions. For example, in soils the examples of cyclic changes include periodic bursts of easily absorbed organic substances as root secretes in the rhizosphere of plants, which is associated with photosynthetic cycling [69], daily variations in the concentration of inorganic ions, as well as periodic and aperi-

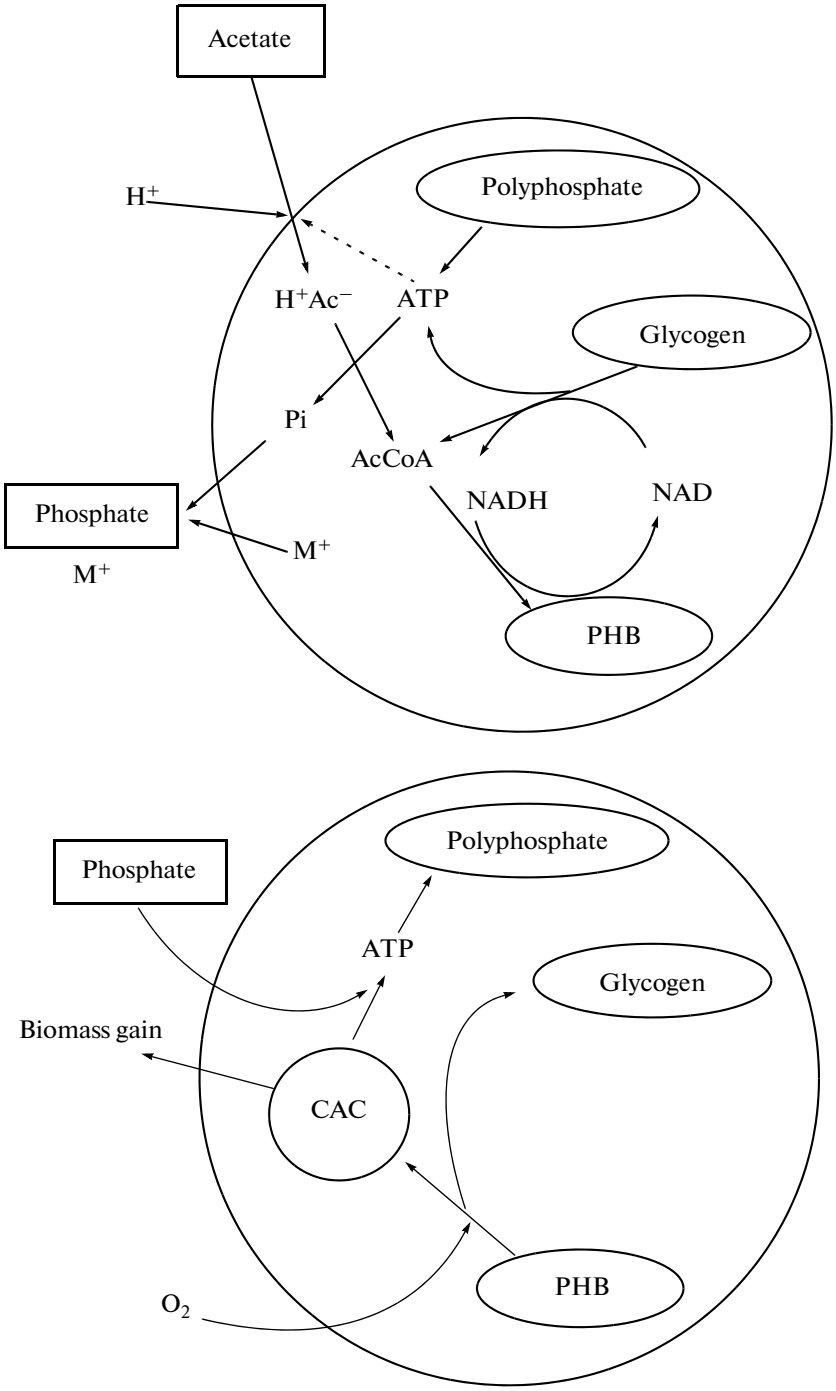

Fig. 5. The scheme of intracellular polyphosphate accumulation by phosphate-accumulating bacteria in the course of the change from anaerobic conditions (top) to aerobic (bottom). The cells are depicted as circles. PHB, polyhydroxybutyrate; M^+, metal cations (adopted from [64]).

odic changes in temperature, humidity, and gaseous composition of the upper soil horizons [70]. Daily cycle of phytoplankton activity and intensity of exudate excretion is typical of surface water ecosystems [71]. A close relation between the varying physiological parameters of higher organisms, including humans, and the microbiome development has been elucidated [72]. There are other ecosystems characterized by regular cycling conditions caused by, for example, periodic bursts of thermal water, high and low tides (in the littoral zone), etc.

Thus, the cyclic method of microbial cultivation may be of interest not only for optimization of technological processes, but also for the preparation of

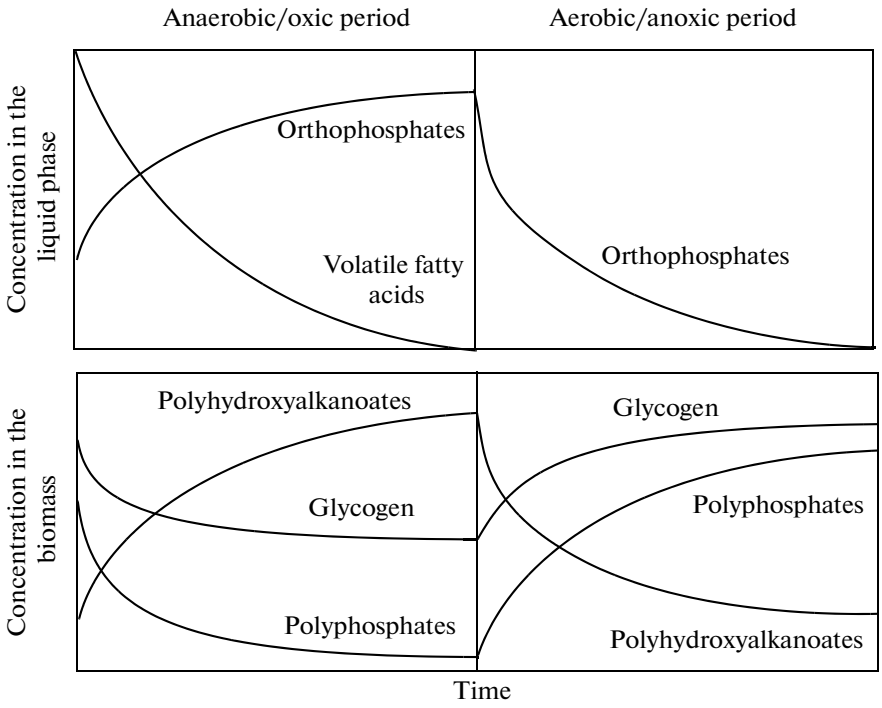

Fig. 6. Dynamics of the major processes in the course of biological removal of phosphorus (adopted from [59]).

enrichment cultures and isolation of new microorganisms from natural habitats.

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