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THE ANALYSIS OF VIABILITY EVALUATION METHOD FOR MICROORGANISM CELLULAR FORMS AFTER CRYOPRESERVATION

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SUMMARY

The review of existing evaluation methods of viability of microorganisms is presented. The feasibility to apply these methods for the estimation of viability of microorganisms after cryopreservation was analysed.

KEY WORDS: microorganism, cryopreservation, viability evaluation method

To develop immune biological preparations, methods of diagnostics of the infectional diseases, infections of alive microorganisms in the environment, conducting of epidemiological studies and providing the works of microbiological productions, the collections of different microorganisms [17] are used. Cryopreservation is one of the most reliable and widely applied ways of a long-term storage of microorganisms [4,13]. When creating the efficient regimens of microorganism cryopreservation it is necessary to imply the most distinct and adequate assessment methods for microorganism viability state after a low temperature effect. More frequently the cellular forms of microorganisms are used by researchers. These forms are by a cell structure divided into two groups: eucaryotes (algae, fungi, protozoa) and procaryotes (bacteria) [8,9,14].

The state of microorganisms is usually characterized by viability and survival. The term "viability" is an integral characteristics of living systems, including growth and ability to self-generation, anatomic integrity, coordinated structural and metabolic organization [5]. The "survival" or viability index is the ratio between cell viability and a total number of cells, that the sample under study comprises [5].

Now applied methods for the estimation of viability of microorganism cellular forms are divided into direct and indirect. Direct methods are based on the determination of cell reproductive cell capabilities, indirect ones - on registration of different manifestations of their vital activity.

Direct methods consist of micro- and macrocutural ones. Macrocultural methods are based either on accounting the population forming cells - classic method of the most probable number [25], or on the determination of colony-forming units -Koch's dish method [23]. Different modifications of these methods have also been developed.

The most probable number method consists in the fact, that some series of consecutive dilutions are prepared of the material under study. Certain volume of each dilution is placed into vials with a liquid nutritive medium. After incubation the number of vials with a turbid medium with grown microbe cell populations and one with a transparent medium with no living cells are considered.

Then according to special tables [5], developed on the base of the methods of variational statistics, the probable number of viable cells in the sample under study is calculated. There are some modifications of this method, registering different manifestations of vital activity of growing and dividing cells. Among them the most well-known are as follows:

- Clark method [18], in the base of which the registration of releasing gases or changes in the color of growing medium due to indicator dyes, introduced into a nutritive broth as well as this method modification on microcases is laid;
- material inoculation from the series of dilutions into a synthetic medium containing radio-labelled ^{14}C substrates with following registration of the amount of $^{14}CO_2$ being formed during the decay of labelled ^{14}C-substrates by microbic cells [26].

Dish Koch's method differs the method of the most probable number by the fact that the material inoculation from the series of dilutions is accomplished to the surface of dense nutritive media with the account that colonies formed by single microbic cells were at a some distance from each other. After colonies calculation the number of colony-forming cells in 1 ml of studied sample are calculated. Modifications of the plate method are various microcultural methods. The matter of them is that the samples of microorganisms are introduced in microvolumes into the holes, special microchambers, on plates and different means, containing nutritive media with agar or gelatin. After not long-term culturing the microcolonies are calculated by means of microscope. To enhance the accuracy of the calculations of microcolonies indicators, responsing to the products of vital activity of microbic cells,

luminescent dyes, fluorescent antibodies are added into nutritive media [7,16,19,21,22,30].

Cytophysiological methods can also be referred as direct ones. Their techniques are similar to microcultural analysis.

Different inhibitors blocking cell division, but not preventing their growth are introduced into nutritive media. Shape and size of dead cells in this case do not change. Alive cells as a rule transform into thread-like forms, that are fixed by means of light or electrone microscope. The most common inhibitors used are urea, mitomycin C, nalidixic acid, penicillin, chlorbiocin, acryl amide (the latter - only for gram-negative bacteria) [12, 29,31].

Indirect methods can be conditionally divided into cytophysiological methods of metabolic activity registration and ones for the estimation of cellular permeability barrier state.

Since cellular division is possible only when some enzymic reactions go, the determination of several key reactions or metabolism products can serve as the criterion for viability estimation. The most common ways to estimate viability are the determination of the activity for process of synthesis of DNA, RNA, proteins on the inclusion of labelled precursors [1,4,5,6,13]; respiratory activity on oxygen consumption and CO_2 release; ATP content, NAD H_2, NADPh H_2, pyruvic and lactic acids [4,5,13,14].

Electrochemical methods for defining different metabolites [5] in growth medium, determination of the ability of microbic cells, having an active electrone-transport system, to recover dyes [15,20], some biophysical and physicochemical methods - gas-liquid chromato-graphy, electrical impedance, chemiluminescent method, changes in electrical conductivity or electrical orientated effect can be also referred to cytophysiological ones [5,14].

Differential staining of alive and dead cells by vital dyes [32] luminescent microscopic, colorimetric, photometrical method [16] registration of the releasing of labelled and other macromolecules out of a cell [5] are considered as the method for the controlling of the viability on the state of cellular permeability barrier.

The selection of corresponding method to estimate viability is defined by peculiarities of microorganism and the effect. In the process of cryopreservation microorganisms as well as other biological objects are subjected to the effect of some physical and chemical factors [1,2,6,13,15]. There are 4 effects of the factors on microbic cells. The first one is death of cells at freeze-thawing stages. The second one - the cells keep the ability to normal reproduction after reparation of non-lethal impairments. The third one is the cells keep the ability to a limited number of divisions. The fourth- the cells lose the ability of growing and dividing, but within some period of time metabolic processes proceed in them.

In this connection there is the only practical and theoretical interest in respect of accumulated experience on the evaluation of viability for various cellular forms of microorganisms after cryopreservation.

The analysis of literature data devoted to the problems of microorganism cryopreservation shows that the most common direct method for estimation of viability is the method of calculating the colony-forming units [4,13]. Using this method only the cells, kept the ability to reproduce, are considered, i.e. completely referred as viable ones. The method is convenient when working with the majority of monocellular forms of microorganisms. It adequately records the change in the number of viable cells after freezing on various regimens.

However when cryopreserving streptococci it has been established that microcolonies form not separate cocci, but chains when freezing was conducted with the rates resulted in significant damages of sptreptococci, the rupture of chains occurred. As a result the number of macrocolonies increased that led to exceeding viability indices [15].

The question about application of direct methods of viability evaluation has remained unsolved when one cryopreserves actinomycetes and cyanobacteria. Actinomycetes on their structure are similar to fungi. They have substrate and air mycelium, reproduce by means of spores and partially fragments of substrate mycelium [8,9,14]. When evaluating viability of cryopreserved actinomycetes, colony-forming units (CFU) were used [1]. For spore forms this way is objective one.

When freezing vegetative forms the fragmentation of substrate myeclium is inevitable and CFU counting from our point of view shows the number of mycelium fragments that kept viability, but not initial integrity of mycelium. The authors of the research added to the estimation of viability on counting CFU by indirect method - study of culture capability of streptomycetes to produce antibiotics.

More complicated evaluation of viability is one for cyanobacteria. Both monocellular types and thread-like multicellular microorganisms, morphological unit of those is trichome, consisting of vegetative cells, heterocystes and spores comprise this class [14]. Since cyanobacteria are grown only in liquid media a direct method for estimation of their viability occurred to be impossible. Complex evaluation of viability was performed by two indirect methods: on the rate of biomass accumulation and on differences in chemiluminescence of "alive" and "dead" cells

[6].

Besides already mentioned auxiliary methods other indirect ones were used. The analysis of publications devoted to this question shows that more objective are the methods of estimation of the state of cellular permeability barrier. In particular non-phelometric recording of bacteria plasmolysis in 2 M NaCl solution [2], penetration of large molecules of luminescenting dyes into damage cells with following registration of luminescenting cells [10], registration of the change of electroconductivity of condensed cellular suspension due to the releasing of electrolytes [11] out of cells, determination of the change in electrical orientational effect of damaged cells [3] etc. were used. The disadvantage of these methods is first of all the necessity of preliminary building-up of calibration curves for each of the regimens of freeze-thawing using direct methods of viability investigation. Secondly, the conditions of growing (content of growth culture, culturing temperature, aeration, culture age) and content of cryoprotective medium can considerably affect the state of barrier functions of cellular membranes.

Cytophysiological methods of registration of metabolic activity from our point of view are less appropriate for the estimation of microorganism viability after cryopreservation.

These indices testify to the state of these or those cell systems after warming up to the time of cell division. Thus the study of the synthesis processes of nucleic acids, protein in E.coli bacteria, yeasts S.cerevisiae and in cyanobacteria demonstrated that in the first minutes after thawing the number of included into cell RNA and proteins labelled precursors reduced lower than the control indices. Then the activity of the synthesis of RNA and proteins increased and significantly exceeded an initial level [4,13]. Such deviations in the activity of biosynthetic processes are explained by the presence of non-lethal damages of cellular structures and activisation of regenerative processes.

Additional contribution to the total increase in the number of included precursors can be presented by the cells kept the ability to a limited number of divisions or cells that lost the capability to divide.

Indices of respiratory activity of cells and other processes of bioenergetics after cryopreservation as a rule are reduced in comparison with initial ones and do not coincide with the parameters of viability [13]. They in greater extent characterize the state of plasmatic membrane permeability and structures providing bioenergetical processes, than the ability to proliferate.

Then according to special tables [5], developed on the base of the methods of variational statistics, the probable number of viable cells in the sample under study is calculated. There are some modifications of this method, registering different manifestations of vital activity of growing and dividing cells. Among them the most well-known are as follows:

- Clark method [18], in the base of which the registration of releasing gases or changes in the colour of growing medium due to indicator dyes, introduced into a nutritive broth as well as this method modification on microplates is laid;
- material inoculation from the series of dilutions into a synthetic medium containing radio-labelled ^{14}C substrates with following registration of the amount of $^{14}CO_2$ being formed during the decay of labelled ^{14}C-substrates by microbic cells [26].

Summarizing the presented data one can conclude as follows. The most distinct methods for estimation of viability for microorganism cellular forms are direct ones, determining reproductive properties. At the second place on the degree of objectiveness are indirect ones, estimating the state of the barrier of cellular permeability. Cytophysiological methods of registration of metabolic activity have an auxiliary value. They are expedient to be used in combination of several methods, when correlative dependencies with preliminary results of viability estimation by direct methods were defined.

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АНАЛІЗ МЕТОДІВ ОЦІНКИ ЖИТТЄЗДАТНОСТІ КЛІТИННИХ ФОРМ МІКРОБІВ ПІСЛЯ КРІОКОНСЕРВУВАННЯ

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РЕЗЮМЕ

Представлено огляд існуючих методів оцінки життєздатності різних клітинних форм мікробів та проаналізовано їх придатність для оцінки збереження мікроорганізмів після кріоконсервування.

КЛЮЧОВІ СЛОВА: мікроорганізм, кріоконсервування, методи оцінки життєздатності

АНАЛИЗ МЕТОДОВ ОЦЕНКИ ЖИЗНЕДЕЯТЕЛЬНОСТИ КЛЕТОЧНЫХ ФОРМ МИКРОБОВ ПОСЛЕ КРИО-КОНСЕРВИРОВАНИЯ

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РЕЗЮМЕ

Представлен обзор существующих методов оценки жизнедеятельности разных клеточных форм микробов и проанализирована их пригодность для оценки сохранения микроорганизмов после криоконсервирования.

КЛЮЧЕВЫЕ СЛОВА: микроорганизм, криоконсервирование, методы оценки жизнедеятельности