OBTAINING NUCLEIC ACID PREPARATIONS AND THEIR HYDROLYSATES FROM BIOMASS OF METHANE-OXIDIZING BACTERIA

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ABSTRACT

Due to the unfavourable environmental, social and economic situation, the need for the treatment of oncological diseases and diseases associated with impaired activity of the immune system is increasing. A lot of these drugs are made on the basis of nucleic acid components, the industrial production of which is practically non-existent in Russia. Therefore, a task of current interest is to develop the basis of the technology for obtaining components of nucleic acids, which can be widely used in medicine as immunomodulatory, wound-healing, antiviral, and diagnostic medicine, as well as for cancer treatment.

Most of the described in literature methods of isolating nucleic acid components from plant, animal and microbial raw materials are based on the use of toxic and expensive organic solvents, that's why it is impossible to apply these methods outside of laboratory conditions. The most promising source of raw materials for nucleic acids is the biomass of microorganisms (yeast and bacteria) from biomass, since the use of such source makes it possible to quickly obtain a large enough amount of biomass, and, consequently, a larger amount of nucleic acids. This allows obtaining DNA in addition to RNA. RNA and DNA substances can be used to obtain nucleosides and nitrogenous bases, which are also widely used in medicine.

The purpose of these studies was to select the conditions for the extraction of RNA and DNA from the biomass of methane-oxidizing bacteria in one technological cycle, as well as to compare the efficiency of alkaline and acid hydrolysis of microbial RNA and DNA. The need for a two-stage extraction of nucleic acids from the biomass of methane-oxidizing bacteria in order to separately extract RNA and DNA was Substantiated. It was ascertained that at the first stage of extraction at a temperature of 90 ° C, pH 9.0 for 90 min, at least 85% of RNA is extracted. After the separation of the extract by centrifugation, the partially denuclearized biomass must be re-processed under the same conditions in order to extract DNA by at least 83%. The modes of concentration of RNA and DNA solutions by ultrafiltration were selected. It was found that in order to achieve effective deposition of nucleic acids at the isoelectric point, the concentration of the RNA solution must be carried out on the UPM-10 membrane at the concentration degree of 7, and the DNA solution on the UPM-100 membrane at the concentration degree 6. The dynamics of decomposition of nucleic-protein complexes in the medium of monoammonium phosphate was investigated. It was shown that the

transition of NA into solution by at least 80% is achieved at a monoammonium phosphate concentration of 1.7 M, a temperature of 55 ° C for 90 min. The use of 5-fold washing of oligonucleotide substances with acidified water (pH 2.0) to remove excess mineral impurities was substantiated. A comparative assessment of acid and alkaline hydrolysis of RNA and DNA was carried out in order to obtain derivatives of nucleic acids.

Keywords: *methane-oxidizing bacteria, RNA, DNA, acid hydrolysis, alkaline hydrolysis*

INTRODUCTION

Due to the unfavourable environmental, social and economic situation, the need for the treatment of oncological diseases and diseases associated with impaired activity of the immune system is increasing. A lot of these drugs are made on the basis of nucleic acid components. DNA and RNA preparations of various origins, as well as their derivatives are promising therapeutic and immunomodulatory agents and can be used in food, cosmetic and other industries [1], [2].

In the cell nucleic acids are in a complex with proteins and lipoproteins. Therefore, the complexity of the isolation of nucleic acids lies in separating them from the above impurities, as well as their high degree of destruction under the influence of external factors. In this regard, the choice of reagents for DNA isolation, which inhibit or inactivate cellular nucleases, but leave the native structure of nucleic acids intact, is of great importance [3], [4].

A common method for the isolation of nucleic acids is the destruction of cells of microorganisms and animal tissues. In laboratory conditions, cell disintegration is carried out using liquid nitrogen or mechanical grinding with silicon oxide (or aluminum oxide). This step can be realized directly in a lysis buffer containing nucleases and proteases [5].

After lysis, there are two possible approaches to purifying the target DNA. The first involves processing a DNA solution by extraction with an organic solvent (phenol, chloroform), followed by precipitation of DNA with alcohols and dissolution in water and Tris-EDTA buffer. According to the second approach, differential sorption of DNA is carried out on a solid support (most often, silica gel), after which the sorbent is washed with organic solvents, and then the DNA is washed off with water or Tris-EDTA buffer.

Each of these approaches has advantages and disadvantages. In the first case, it is possible to obtain high molecular weight DNA (more than 15000 nucleotide pairs), however, significant DNA losses are possible, and the resulting preparations contain a significant amount of impurities. In the second case, it is possible to obtain highly purified preparations, however, the isolated DNA turns out to be of low molecular weight.

Known methods for producing RNA from yeast allow the isolation of low-polymer RNA, for example [6], which is used in medicine in the treatment of a wide range of diseases: from viral infections to memory disorders [7].

Work [8] describes a method for obtaining high-polymer RNA from yeast by suspending it in an aqueous 0.3-1.2 M solution of 2-ethylhexanoic acid containing 0.1-0.5 M NaCl at 92-98°C. The disadvantage of this method is the use of a synthetic, expensive and highly toxic lytic agent - 2-ethylhexanoic acid, which requires deep purification of the final product, as a result of which this method is not economical and practically not scalable.

In the article [9], an attempt was made to replace 2-ethylhexanoic acid with a large-tonnage food reagent, which significantly reduces the cost of the process since it makes it practically waste-free and easily scalable.

In the research [10] it is proposed to incubate biological material with pre-treated 3.5-7% solution of hydrofluoric acid for 2-6 hours with finely dispersed meshed glass in a buffer solution containing chaotropic agent, which makes it possible to reduce process duration, increase RNA yield and ensure isolation of both low-molecular and high-molecular RNA fractions.

In a number of works it is proposed to isolate RNA from the blood by sorption on ion-exchange resins in quaternary ammonium form [11], cationite in H^+-form [12], from cells of microorganisms, plant and animal tissues by treatment with acid reagents, sorption on cationite in combination with treatment with proteases, desorption of nucleic acids with ionites with buffer solutions [13].

Most of the described in literature methods of isolating nucleic acid components from plant, animal and microbial raw materials are based on the use of toxic and expensive organic solvents, that's why it is impossible to apply these methods outside of laboratory conditions.

The most promising source of raw materials for nucleic acids is the biomass of microorganisms (yeast and bacteria) from biomass, since the use of such source makes it possible to quickly obtain a large enough amount of biomass, and, consequently, a larger amount of nucleic acids. This allows obtaining DNA in addition to RNA. RNA and DNA substances can be used to obtain nucleosides and nitrogenous bases, which are also widely used in medicine.

The purpose of these studies was to select the conditions for the isolation of RNA and DNA from the biomass of methane-oxidizing bacteria in one technological cycle, as well as to compare the effectiveness of alkaline and acidic hydrolysis of microbial RNA and DNA.

MATERIALS AND METHODS

The object of the study was condensed biomass of methanoacidating bacteria Methylococcus capsulatus, containing 20% dry substances, 10% nucleic acids (including 13.5% DNA), 65% raw protein. The determination of total nitrogen was carried out by the Kjeldahl micrometode. The phosphate ion concentration was determined by the Fiske-Subarrow method. The concentration of nucleic components was determined by Spirin and the concentration of DNA by Dische.

The process of concentrating nucleic acids solutions was carried out on a laboratory ultrafiltration apparatus with a volume of 1000 ml, using membranes of the UPM type (polysulfonamide membranes).

In the analysis of the nucleic acids hydrolysates, the high molecular weight fraction was separated by precipitation with 50% trichloroacetic acid. The low molecular weight fraction was analyzed by the Spirin method.

RESULTS AND DISCUSSION

Several methods of extracting nucleic components from the biomass of bacteria are known from the literature, but they are often multistage [3] and involve the use of organic solvents, for example, phenol and chloroform.

The most promising is the use of an aqueous alkali solution as an extractant. In these studies, it was proposed to extract nucleic acids from a 20% suspension of biomass of Methylococcus capsulatus bacteria at a pH of 9 and a temperature of 90 ° C. Extraction was carried out for 4 hours. At certain time intervals, suspension samples were taken, biomass was separated by centrifugation at 6000 rpm, and the concentration of total nucleic components in extracts was determined by the Spirin method, DNA by the Dishe method. On the basis of the obtained data, the degree of extraction of total nucleic acids and DNA was determined. The obtained results are shown in Fig. 1, from which it follows that the extraction time providing the extraction of total nucleic acids by not less than 94% should not exceed 180 minutes (3 hours). It should be noted that primarily RNA having a lower molecular weight is extracted from microbial cells, and DNA extraction begins only in the 90th minute of the process.

Thus, it was found that in order to achieve at least 94% recovery of nucleic acids in the form of protein-nucleic complexes, extraction should be carried out at a temperature of 90°C, pH 9.0, extraction time - 3 hours. At the same time, the degree of DNA extraction is 97.6%, and RNA is 94.1%.

According to previous studies, these concentrations of nucleic acids do not allow their effective precipitation at the isoelectric point, therefore, the preliminary concentration of the obtained extract by ultrafiltration is required. To separate the mixture of DNA-protein and RNA-protein complexes, the effectiveness of the ultrafiltration method was evaluated using membranes with cutoff molecular weights of 100 and 10 kDa.

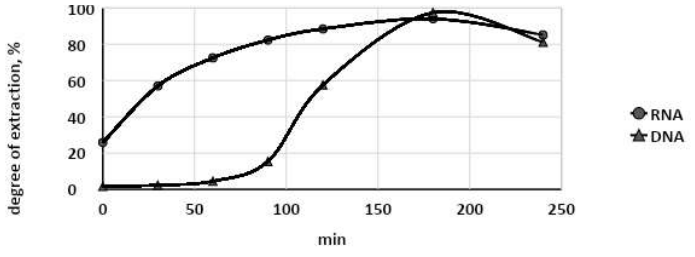

Fig. 1. *Dynamics of extraction of total nucleic acids and DNA from biomass of methanoxic bacteria Methylococcus capsulatus*

Initially, the nucleic extract was concentrated on the membrane UPM-100 to concentrate the DNA-protein complex and reduce the viscosity of the resulting filtrate. To evaluate the effectiveness of the ultrafiltration process, differential and integral selectivity and specific productivity were determined. Figure 2 shows the dependence of the specific productivity of the UPM-100 membrane on the concentration of nucleic acids in the concentrate.

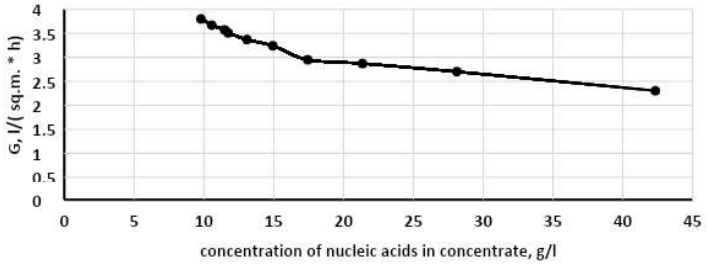

Fig. 2. *Dependence of specific productivity of UPM-100 membrane on the concentration of nucleic acids in concentrate*

From the above data, it follows that a decrease in specific productivity of 1.5 times is observed only at a concentration of total nucleic acids above 30 g/l, which is quite sufficient for subsequent effective precipitation of nucleic acids.

Figure 3 shows the differential selectivity relationships of membrane UPM-100 for nucleic components (2) and for DNA (1). From the data obtained, it can be seen that the values of selectivity for nucleic acids and DNA are close to each other, that is, there is no enrichment of the DNA concentrate. Therefore, a single-step extraction is not feasible, and a 2-step extraction option was studied in the next stage of the studies.

Sequential extraction of nucleic acids from the biomass of methane-oxidizing bacteria was carried out as follows. At the first stage carried out extraction of RNA at a temperature of 90 wasps within 90 min. On the expiration of the specified time biomass was separated centrifugation at 6000 rpm within 30 min. The received extract was directed to an ultraconcentration stage on UPM-10 membrane. The partially denucleinized biomass was suspended in distilled water to obtain a 10% suspension. DNA extraction was carried out at 90 ° C, pH 9.0 for 90 minutes, taking suspension samples at predetermined intervals at which the DNA concentration was determined by Dishe's method. The dynamics of DNA extraction are shown in Figure 4.

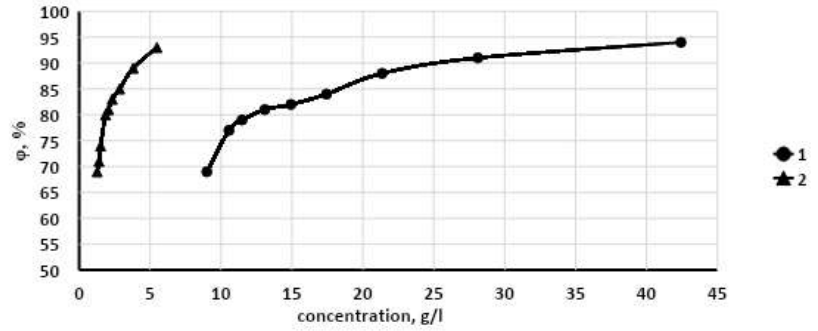

Fig. 3. *Dependence of differential selectivity of the membrane UPM-100 on DNA (1) and nucleic acids (2) on the concentration of nucleic acids and DNA in the concentrate.*

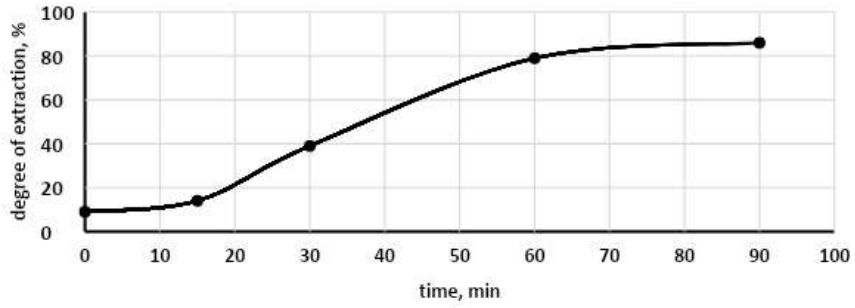

Fig. 4. *Dynamics of DNA extraction from biomass of methane-oxidizing bacteria in 2-stage extraction*

It follows from the figure that in 90 minutes of extraction, a DNA extraction rate of at least 85% is achieved. A further increase in extraction time is not advisable, as this leads to DNA destruction.

Thus, an RNA-containing extract (RNA concentration 8.0 g/l) and a DNA-containing extract (DNA concentration 1.4 g/l) were obtained. The extracts obtained were further concentrated by ultrafiltration on membranes UPM-10 and UPM-100, respectively.

The concentration of the RNA-containing extract was carried out on the membrane of the UPM-10, and the DNA-containing extract on the membrane of the UPM-100.

Similar to the ultra concentration studies described above, specific performance, integral and differential selectivity were determined for the concentration process in both cases. Figures 5 and 6 show the dependencies of specific performance and differential selectivity on the concentration of nucleic acids in the concentrate. From the presented data, it can be seen that a significant decrease in the performance of the ultrafiltration plant is observed at an RNA

concentration in the concentrate of more than 23 g/l, which allows concentrating the RNA-containing extract by at least 6 times. The RNA integral selectivity is not less than 72%.

These data indicate that the DNA concentration process is characterized by high differential selectivity (above 86%), as well as a decrease in the performance of the ultrafiltration device by no more than 1.5 times, which allows concentrating the DNA extract by no less than 6 times.

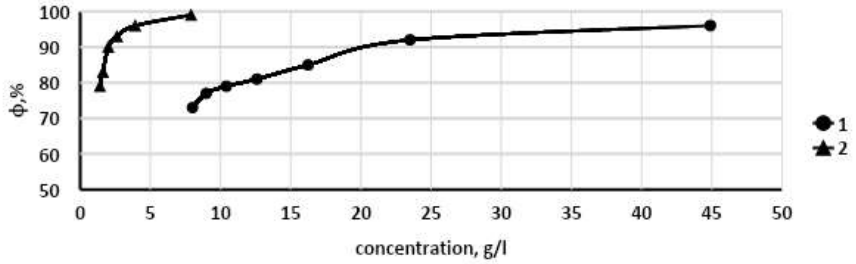

Fig. 5. *Dependence of differential selectivity of membrane UPM-100 on the concentration of DNA (1) and membrane UPM-10 on the concentration of RNA (2) in concentrate*

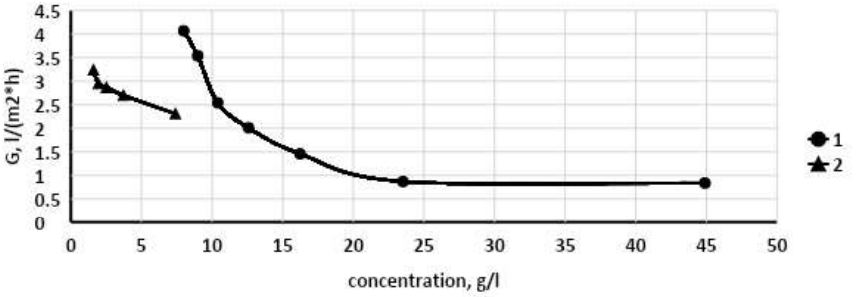

Fig. 6. *Dependence of specific productivity of membrane UPM-100 on the concentration of DNA (1) and membrane UPM-10 on the concentration of RNA (2) in concentrate*

As a result of the ultra-concentration step, an RNA-containing concentrate and a DNA-containing concentrate were obtained, from which the RNA-protein and DNA-protein complexes were precipitated at an isoelectric point.

To this end, the resulting concentrates were cooled to 4-6 ° C, after which the pH of these solutions was set to 1.8-2.0 using a concentrated hydrochloric acid solution. The precipitated DNA-protein and RNA-protein complexes were separated by centrifugation at 6000 rpm for 15 minutes. From the obtained data, the degree of precipitation of nucleoproteins was calculated, the values of which were: for the DNA-protein complex - 80%; for the RNA-protein complex - 83%.

To decompose the protein-nucleic complex, a solution of diammonium phosphate was used, which interacts with the protein of the intracellular protein-nucleic complex and allows the release of nucleic acids in free form. In this case, the protein-phosphate complex precipitates, and nucleic acids pass into solution. The process was carried out at a temperature of 60 ° C, concentration of mmonium phosphate 2.0 mol/l, pH of medium 6.0-6.5. Samples of the suspension were collected periodically, the precipitate was separated by centrifugation at 6000 rpm, and the concentration of RNA in the supernatant was determined by Spirin and DNA by Dishe's method. From the obtained data, the proportion of dissolved nucleic acids from their content in the initial protein-nucleic complexes was determined (Figure 7).

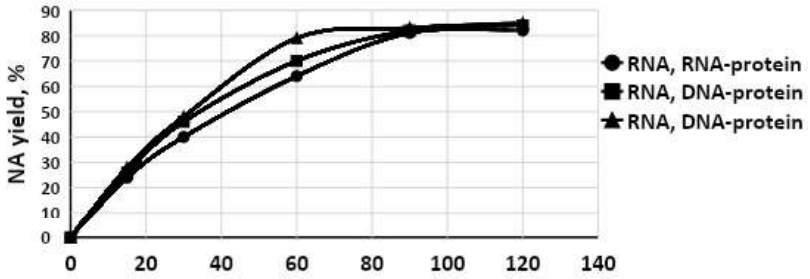

Fig. 7. *Dynamics of nucleic acid dissolution in monoammonium phosphate solution*

From Figure 7 it follows that for both protein-nucleic complexes in 90 minutes, nucleic acid yield to the solution at the level of 80-85% is achieved. Therefore, the optimal processing time of the protein nucleic complexes was assumed to be 90 minutes.

Further, the precipitate of the protein-phosphate complex was separated by filtration, and RNA and DNA were precipitated from the obtained solutions in the same manner as described above. To purify the RNA and DNA precipitates from phosphate ions, they were washed with water acidified to pH 2.0, treated with 96% ethyl alcohol solution and dried at 50 ° C. The RNA preparation contained at least 85% of the main substance, and the DNA preparation contained at least 76%.

As previously noted, RNA and DNA preparations are generally the starting materials for the production of nucleosides and nitrogenous bases. Acid and alkaline hydrolysis are used for their production. Therefore, at the next stage of work, the effectiveness of these hydrolysis methods was compared. from which it follows that both in the case of RNA and DNA, alcaline hydrolysis proceeds with a higher yield under milder conditions.

Table 2. *Effectiveness of acid and alkaline hydrolysis of RNA and DNA preparations*

Preparation	Hydrolysis conditions			Hydrolysis products yield, %
	Temperature, °C	Hydrolyzing agent	Concentration of hydrolyzing agent, %	
RNA	90	Sodium hydroxide	10	14,4
RNA	115	Sodium hydroxide	10	20,6
RNA	90	Sodium hydroxide	20	20,6
RNA	115	Sodium hydroxide	20	13,4
RNA	90	Hydrochloric acid	36	9,5
RNA	115	Hydrochloric acid	36	11,6
DNA	90	Sodium hydroxide	10	27,8
DNA	115	Sodium hydroxide	10	28,0
DNA	90	Sodium hydroxide	20	27,3
DNA	115	Sodium hydroxide	20	25,4
DNA	90	Hydrochloric acid	36	27,9
DNA	115	Hydrochloric acid	36	25,2

CONCLUSION

The need for two-step extraction of nucleic acids from the biomass of methane-oxidizing bacteria in order to separately extract RNA and DNA is justified. It was found that at the first stage of extraction at a temperature of 90°C, pH 9.0 during 90 minutes, at least 85% RNA is recovered. When partially denucleinized biomass is reprocessed under the same conditions, DNA is extracted by at least 83%.

It has been found that in order to achieve efficient precipitation of nucleic acids at an isoelectric point, concentration of the RNA solution must be carried out on the membrane UPM-10 at a concentration degree of 7, and the DNA solution on the membrane UPM-100 at a concentration degree of 6.

A study of the degradation dynamics of protein-nucleic complexes in the medium of monoammonium phosphate showed that the transition of nucleic acids into a solution of at least 80% is achieved at a concentration of monoammonium phosphate of 1.7 M, a temperature of 55°C for 90 minutes.

It has been established that the hydrolysis of both RNA and DNA is expediently carried out using sodium hedroxide with a concentration of 10% at a temperature of 120° C.

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