Analysis of unfrozen water in suspensions of permafrost bacteria with NMR technique at the temperature of permafrost occurrence

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Abstract. The data of investigation of microorganisms influence on water phase state in frozen suspensions of from permafrost and frozen solutions of their metabolites were obtained using nuclear magnetic resonance (NMR) relaxation spectroscopy. The measurements were taken at the temperature of permafrost occurrence. For the first time, NMR relaxation parameters were determined for suspension of bacteria extracted from permafrost and solution of their metabolites. Quantity of unfrozen water was compared and contrasted in frozen suspension of bacteria extracted from permafrost, frozen solution of their metabolites and frozen water solutions of high-molecular cryoprotectors. According to the given results, it was found out that the weight of unfrozen water might be much more than the weight of biomaterial contained in the suspensions of bacteria extracted from permafrost and solutions of their metabolites at the temperature of permafrost occurrence. It was shown that microorganism metabolites extracted from permafrost exerted higher influence on unfrozen water content than higher-molecular cryoprotectors.

1. Introduction
At the present time there is overwhelming evidence that some of microorganisms preserved in ice or permafrost can not only live but keep their functioning [1-4].

Mechanisms of this survival have been staying unknown up to the present day. One of the most important factors affected on germinative faculty of biological objects is water phase state (liquid or solid) of their habitat where nutritious matter and metabolism products are transported. At the temperatures of permafrost occurrence some quantity of intracellular and extracellular water might stay unfrozen for an unlimited period of time. However, the influence of the presence of biomaterial extracted from permafrost on water phase state has not been yet estimated.

Microorganism survival at negative temperatures is associated with an outflow of peculiar substance named a cryoprotector [5]. Presence of cryoprotectors let microorganisms keep functioning for a long time over a wide temperature range. However, the influence of metabolites of microorganisms extracted from permafrost on the quantity of unfrozen water in frozen bacterial suspensions has not studied yet.

This paper presents the first results of our study on the influence of microorganisms extracted from permafrost on the quantity of unfrozen water in frozen bacterial suspensions and frozen solutions of their metabolites. To determine water phase state the method of NMR relaxation spectroscopy was used.

Content of unfrozen water in frozen bacterial suspension, frozen solution of their metabolites and frozen water solution of high-molecular cryoprotector measured to compare and contrast the influence of microorganisms extracted from permafrost and their metabolisms on the quantity of unfrozen water.
2. Experimental section

Bacteria *Bacillus sp* (in accordance with the 16s RNA sequence analysis) strain MG8, soilborn sporogenous, gram-positive organisms were used. This bacteria strain was extracted from permafrost of late Neogene (Central Yakutia, Mammoth Mountain). A suspension of microorganism cells and solutions of metabolites were prepared by the staff of the Tyumen Scientific Center, SB, RAS using previously developed methods [6,7].

The proportion of solid residue (the ratio of the dry residue mass to the initial sample mass) suspensions of *Bacillus sp*. strain MG8 and the solutions of their metabolites (MG8) (further bacterial suspensions and metabolites solutions correspondingly) was 0.1 wt% and 0.015 wt% correspondingly.

Polyvinyl alcohol (PVA) was used as a higher-molecular cryoprotector [5]. Polyvinyl alcohol [-CH2-CH(OH)-] n – artificial, water-soluble, nontoxical, biocompatible polymer. In this work, it was used PVA 16/10 GOST 10779-78. The PVA concentration for PVA solution was 0.1 wt% in all experiments.

The samples of bacterial suspensions and PVA solutions were loaded into the refrigerating chamber. Then, the samples were held there at the temperature of ~20 °C no less than 24 hours.

Nuclear magnetic relaxation measurements were carried out by the Bruker Minispec mq NMR relaxometer operating at 20 MHz. The Carr-Purcell-Meiboom-Gill (CPMG) pulse sequences were used to determine the spin-spin relaxation time T2. The interval between pulses was set at 3.5 ms. During the CPMG pulse sequence realizing the first radio frequency deflected nuclear magnetization at an angle of 90°. Further 180° pulses led to the formation of spin echo [8,9]. Quantity of unfrozen water was determined by comparison of NMR signal of a calibration sample and NMR signal of the measured sample. Polyethylsiloxane liquid PES-5 was used as the calibration sample. PES-5 is polydiethylsiloxane polymer of linear structure.

As shown exponential approximation nuclear magnetic decay, one-exponential decay occurred for all measured samples:

\[ A = A_0 \exp (-t/T_2), \]  

(1)

where \( A_0 \) – the maximum amplitude of the nuclear magnetization.

3. Results and discussion

Sedimentation processes of biomaterial in suspensions may decrease their spatial homogeneity in time. Samples of initial just-prepared suspensions had approximately the volume of 100 ml. However, a sample of suspension that extracted for NMR relaxation measurements had the volume no more than 2 ml. Within this framework, spatial homogeneity might lead to great spread of NMR measurement results. Thus, to exclude the presence of spatial homogeneity in bacterial suspensions and the influence of the sedimentation of biomaterial on the NMR measurements, time T2 was determined for samples taken step by step at a height (top to bottom) with the 1 ms step in just-prepared suspensions. Obtained data of the relaxation time T2 was shown in table 1.

According to the received results, T2 for various samples collected from one initial just-prepared suspensions reproduced well (measurement errors are close to the instrument measurement error T2 of 5%). That indicated a lack of influence of the processes of biological material sedimentation to the results of NMR measurements for NMR measurements just-prepared samples of bacterial suspensions.

NMR measurements of T2 were started over with the same samples at the same conditions after two days. During these two days the sample were being stored at the constant temperature of 4 °C. The average relaxation time of T2 for just-prepared microbial suspensions did not differ (within the error) from the average relaxation time of T2 measured after two days of storage. Samples of suspensions intended for NMR measurements were not stored for more than two days.
Table 1. Spin-spin relaxation time $T_2$ at the temperature of 40 °C for suspensions of Bacillus sp. strain MG8.

| Sample number | $T_2$ (ms) | $[(T_2-T_{2av})100/T_{2av}]$ (%) |
|---------------|------------|----------------------------------|
| 1             | 1986       | 1                                |
| 2             | 2064       | 4                                |
| 3             | 1864       | 6                                |
| 4             | 1987       | 1                                |
| 5             | 2003       | 1                                |
| 6             | 1985       | 0                                |
| 7             | 1977       | 0                                |
| 8             | 1936       | 2                                |
| Average relaxation time | 1975 | 2 |

$^a$ $T_{2av}$ – arithmetic average of a relaxation time $T_2$.

The relaxation time $T_2$ of the samples of bacterial suspensions and metabolite solutions stored at the temperature of −20 °C was measured at the temperature of −5 °C. Likewise, the suspension samples that were measured at the temperature of 40 °C, all suspension samples that were measured at the temperature of −5 °C, the nuclear magnetization decay was mono-exponential. Experimental data of measured relaxation time $T_2$ is given in the table 2.

Table 2. Spin-spin relaxation time $T_2$ at the temperature of −5 °C for frozen bacterial suspensions and frozen metabolite solutions.

| Sample number | $T_2$ (ms) | $[(T_2-T_{2av})100/T_{2av}]$ (%) |
|---------------|------------|----------------------------------|
| Suspensions Bacillus sp. strain MG8 | | |
| 1             | 194        | 33                               |
| 2             | 188        | 29                               |
| 3             | 172        | 18                               |
| 4             | 109        | 25                               |
| 5             | 109        | 25                               |
| 6             | 102        | 30                               |
|                | 146        | 27                               |
| Metabolites MG8 | | |
| 1             | 90         | 8                                |
| 2             | 101        | 22                               |
| 3             | 74         | 11                               |
| 4             | 74         | 11                               |
| 5             | 66         | 20                               |
| 6             | 93         | 12                               |
|                | 83         | 14                               |

According to the data presented in the table 1 and table 2 it might be conclusion that the freezing process dramatically decreased relaxation time $T_2$.

The proton-containing components in the frozen bacterial suspensions were ice, bacteria cells, unfrozen water. It is known that the relaxation time $T_2$ of ice protons is no more than several
microseconds. Hence, amplitude of the nuclear spin echo was measured after the period of time of several milliseconds. For this time, the signal related to ice almost declined up to the zero. Thus, the ice proton contribution might be neglected for the samples of bacterial suspensions.

Intracellular water mass that remained unfrozen at the temperature lower than –20 °C was more than biomaterial mass. Coincidently, the specific content of protons in water was several times higher than in the biomaterial. Consequently, the contribution of biomaterial into the NMR signal at the temperate of –20 °C might be neglected as well.

Whence, NMR signal of the frozen bacterial suspensions at the temperature lower than –20 °C related only with the presence of unfrozen water in the samples.

For the relaxation time of the nuclear magnetization of liquid water protons in biological systems usually take place the following correlation [8]:

\[ T_2^{-1} = pT_{2s}^{-1} + (1 - p)T_{2v}^{-1}, \]  

(2)

where \( T_{2s} \) – spin-spin relaxation time of water connected with biomaterial, \( T_{2v} \) – spin-spin relaxation time of bulk water, \( p \) – portion of water connected with biomaterial. Relaxation time \( T_{2s} \) was much lower than relaxation time \( T_{2v} \).

![Figure 1. Changing relaxation time T_2 for the frozen metabolite suspensions of MG8.](image)

In figure 1, it was shown the data that demonstrated changes in relaxation time \( T_2 \) of metabolite suspensions with the temperature. With the temperature decrease from 0 to –15 °C the relaxation time \( T_2 \) decidedly decreased (figure 1). According to the expression (2), the portion of water connected with biomaterial enhanced. With the further temperature decrease the relaxation time \( T_2 \) did not significantly change. Ordinarily, it is a reflection of the fact that water is in a well-connected with biomaterial [8].

The data of unfrozen water quantity measurements for the frozen MG8 metabolite solution and frozen water solutions of high-molecular cryoprotector (0.1 wt % PVA solutions) was shown in figure 2. According to the presented results there might be seen that with temperature decrease up to –15 °C the quantity of unfrozen water significantly decreased for metabolites.

Further temperature decrease up to –25 °C showed far less changing of unfrozen water quantity. There was evidence that unfrozen water was inseparable from biomaterial at the temperature no lower than –15 °C. It is well coincided with the results of relaxation time measurements. As to frozen solutions of PVA, unfrozen water in it was detected only at the temperature no less than –5 °C.
Figure 2. Changing of unfrozen water quantity in frozen suspension of metabolites MG8 and water solution of PVA (0.1 wt %).

To estimate the influence of substances loaded into water on the presence of unfrozen water in it at the negative temperatures, parameter $D_m$ was used. $D_m$ meant the relation of unfrozen water mass to substances mass contained in samples. The parameter $D_m$ increasing was evidence the fact that biomaterial had an influence on the quantity of unfrozen water and vice versa. Dry solid mass of the samples was designated as biomaterial mass of the samples. The calculated values of the $D_m$ parameter for the studied systems are given in table 3.

Table 3. Parameter $D_m$ of frozen samples.

| System                                      | Parameter $D_m$ |
|---------------------------------------------|-----------------|
| Bacterial suspension Bacillus sp. strain MG8 |                 |
| Metabolite suspension MG8                   |                 |
| PVA water solution (0.1 wt %)               |                 |

According to the data presented in table 3, the unfrozen water mass in frozen suspensions of microorganisms extracted from permafrost and frozen suspensions of their metabolites might be much more than the mass of biomaterial contained in the samples.

4. Conclusion

1. It was established that the mass of unfrozen water connected with the microorganisms extracted from permafrost might be ten times higher than the mass of biomaterial containing in the samples at the temperatures of permafrost occurrence. Thereby, for frozen bacterial suspensions of Bacillus sp. strain MG8 extracted from permafrost the unfrozen water mass was 32 times higher than biomaterial mass at the temperature of $-5\, ^\circ C$. 

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2. It was shown that metabolites of microorganisms extracted from permafrost are capable of having great influence on water phase freezing. Moreover, this influence turned out to be much higher than the influence of high-molecular cryoprotector. For example, Dm relation of unfrozen water mass to metabolite mass (MG8) was 55 higher than Dm relation of unfrozen water mass to dry solid PVA mass at the temperature of –5°C.

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