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FREE AND BOUND WATER INFLUENCE ON *SPIRULINA PLATENSIS* SURVIVAL

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The heat effects arising at heating of *Spirulina platensis* cell culture containing different quantity of water (from 98.2 to 10.5 mass% H_2O) have been studied. The hydration of *Sp. pl.* cells determined by the method of microcalorimetry at 25°C (Δn) was equal to 0.32±0.02 g H_2O/g of dry biomass. The heat ($-Q$) evolved by cells in the temperature range 5–52°C decreased exponentially at decrease of mass% H_2O and reached zero value at 30.5±3.0 mass% H_2O. The total heat of cell denaturation did not change in the range 98.2–40.5 mass% H_2O and it sharply dropped at lower values of water.

Keywords: *bound water, calorimeter, heat denaturation, heat production, Spirulina platensis*

Introduction

A great amount of works has been devoted to study of blue-green microalgae *Spirulina platensis* (*Sp. pl.*) physiology, biology and biotechnology for the last decades [1]. The mechanisms connected with photosynthesis and survival of these cells at various conditions of environment: temperature, irradiation [2], neutral salts [3], pH medium [4] have been studied in detail. The interest to *Sp. pl.* is mainly connected with the fact that *Sp. pl.* biomass is widely used not only in the food and pharmacological industries, but also in medicine. The biomass used is usually in the form of suspension, powders and pills. In out-door conditions this algae is usually in the form of suspension, but in extremal conditions due to loss of water, it can exist both in paste like and in film like states. Due to this, the knowledge of physico-chemical characteristics of this biomaterial being in various aggregative states has undoubtedly both fundamental and biotechnological importance. At present, as far as we know, there are no data concerning the influence of 'free' and 'bound' water on the energy of *Sp. pl.* cells in the literature. It should be noted that a role of 'free' and 'bound' water on biomacromolecules stability has been studied quite well [5–8].

A goal of a given work was to find out a contribution of water into *Sp. pl.* cell survival and energetics. The tasks of the work were to determine the cell heat production and to evaluate the stability of subcellular structures in the wide range of mass% H_2O.

Experimental

The measurements were carried out with a differential scanning microcalorimeter (DSC) created on the basis of works [9, 10]. The sensitivity of the DSC was 0.42 μW, the temperature range of measurements was 5–150°C, the measuring vessel volume was 0.3 cm^3 for suspension and 0.04–0.01 cm^3 for biomass with 85–10.5 mass% H_2O. The calorimeter resolution ratio, in accordance with thermal capacity change (i.e. significant deviation from the baseline), was 10^{-5} J $°C^{-1}$. The accuracy of absolute temperature measurement was not less than 0.05°C.

The new in this DSC in comparison with our previous calorimeters is the following:

- the loading of measuring and comparative vessels is carried out through 6 mm^2 thin-walled stainless pipes. The vessels have forms of truncated cone. They are inserted into doubled copper block having two holes in the form of cone. This provides a good contact between block and vessels and, consequently, a stable baseline in all range of measurements. The blocks are connected by a thermobattery containing 150 chromel-constantan thermocouples.

It works on line with Pentium 3. The measurements were carried out at a sampling frequency of 1 point per 1 s, at a heating rate of 0.6°C min^{-1}, which gave a temperature resolution of 0.02°C.

The baseline was straight at slow heating rates because of absence of heat capacity denaturation increment (at a given sensitivity we do not observe it); it

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was obtained by joining of points corresponding to the beginning and the end of the process (Fig. 1) [4, 10].

The baseline deflected at heating rate 0.42°C min^{-1} and above because of origin of temperature gradient in the measuring vessel. In this case a special program, developed by us, was used. It gave us the possibility to draw a baseline on the basis of coordinate values of the beginning and the end of the observed process by the calculation of the third degree polynom coefficients by the method of least squares. It should be noted that denaturation parameters, T_d, Q_d, are insignificantly changed in the range of scanning from 1 to 0.1°C min^{-1} and they practically are not changed in the range of scanning lower than 0.1°C min^{-1}, that gives us the possibility to carry out the deconvolution of dependence curve $dQ/dT=f(T)$ (Fig. 7).

The calculation of heating parameters $-Q$, Q_d, T_d and ΔT_d was also carried out by the program developed by us and the deconvolution of curve – by the software program Origin 6.0.

The errors in determination of heat evolution ($-Q$) and heat absorption (Q_d) of *Sp. pl.* cells were not more than 12%. T_d of deconvulation peaks was determined with the exactness ±1°C and ΔT_d – ~0.5°C.

The Institute of Plant Physiology, Russian Academy of Sciences kindly granted as the strain IPPA B 256. Details about cultivation, technicology, determination of dry mass, ash, data of Polyacrylamide gel electrophoresis, growth inhibition and morphology of *Sp. pl.* cells see in [4, 11].

The determination of 'bound' water was carried out with the help of modified DSC [10] working in the regime close to isothermic one. The volume of measuring vessel was 0.005–0.02 cm^3. The operational temperature range of instrument can be varied between 0–60°C. The change of temperature was not more than 0.1° during 300 min, the sensitivity – 2.0 μW.

The determination of 'bound' water was carried out on the basis of evaporated water enthalpy measured in *Sp. pl.* sample at atmospheric pressure and constant temperature. The evaporation was realized at simultaneous opening of the measuring vessel and the vessel of comparison, which contained inert mass of the same heat capacity as the preparation.

Results and discussion

The microcalorimetric curves observed in the process of *Sp. pl.* heating in the temperature range 2–52°C and 2–125°C are presented in Figs 1 and 2. As it is seen from Fig. 1, the intensity and profile of exotherm are significantly depended on the content of water (mass% H_2O) in the samples. In particular, the total value of heat produced by *Sp. pl.* cells ($-Q$) sharply decreased from 330±30 to 130±13 J g^{-1} of dry biomass in the temperature range 5–52°C (Figs 1 and 3) when the water content decreased from 98.2 to 96.8 mass%.

$-Q$ value was calculated from the area under the exotherm (Fig. 1). Figure 1 also shows that the temperature 48.1±0.5°C corresponding to maximum of exotherm does not change in this range of mass% H_2O. The decrease of water in the samples from 96.8 to 46.5 mass% leads to further decrease of $-Q$ from 130 to 3.6±0.4 J g^{-1} of dry biomass.

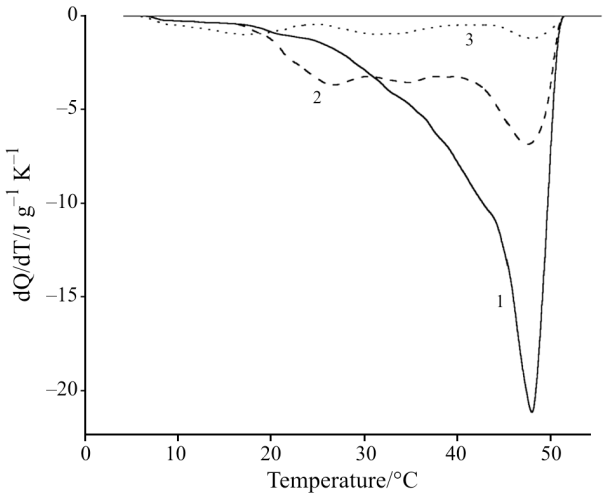

Fig. 1 Heat evolution curves as a function of temperature (dQ/dT/J g^{-1} K^{-1}) of *Sp. pl.* cells in Zarrouk's medium in stationary state, in the dark and in a closed vessel (pH 9.95). Water content in the samples: 1 – 98.2, 2 – 96.8 and 3 – 84.5 mass%. The scanning rate: 0.167°C min^{-1}. The operational temperature range: 0–60°C. Dry biomass content: 1 – 2.45, 2 – 3.1 and 3 – 2.5 mg

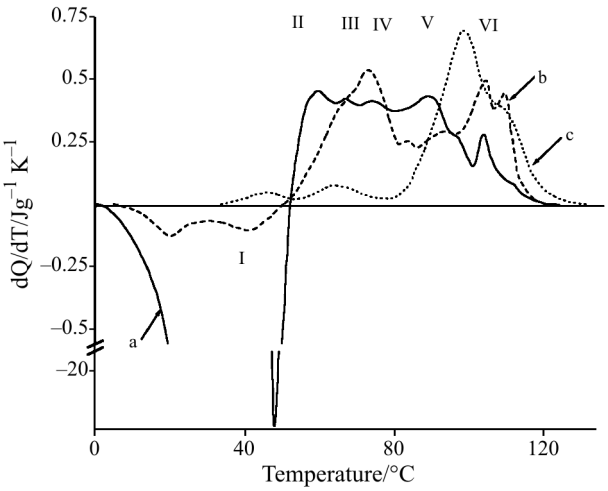

Fig. 2 Heat absorption curves as a function of temperature (dQ/dT/J g^{-1} K^{-1}) of *Sp. pl.* cells (conditions as in Fig. 1) in the temperature range 2–125°C. Water content in the samples: a – 98.8, b – 46.5 and c – 10.5 mass%. The scanning rate: 0.11°C min^{-1}. Dry biomass content: a – 7.10, b – 6.84 and c – 15.2 mg

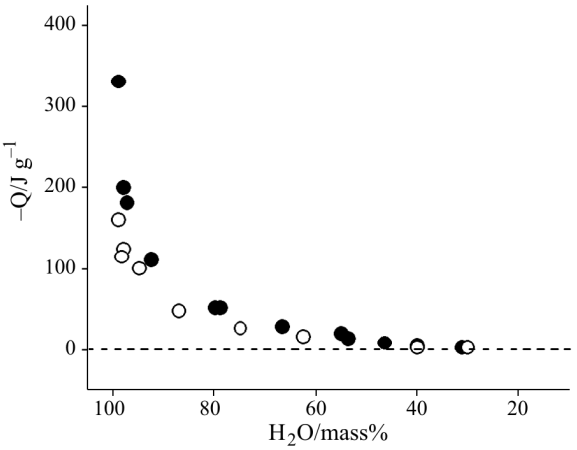

Fig. 3 The dependence of *Sp. pl.* cell heat production (–*Q*) at various water content (conditions as in Fig. 1); the scanning rate: ● – 0.11 and ○ – 0.167°C min^{-1}

In this range of mass% H_2O, a total value of heat absorption, connected with subcellular structure denaturation (Q_{total}) (Figs 2 and 4), was calculated from the areas under endotherms covering the temperature range 52–125°C. Q_{total} did not change and was equal to 24.8±2.5 J g^{-1} of dry biomass. The redistribution of heat between endotherms II–IV, their shift up the temperature scale and the appearance of a sharp peak (Fig. 2, curve b) instead of weakly expressed shoulder about 112°C at 98.8 mass% H_2O (curve a) were observed in this range of mass% H_2O.

The endotherm with $\Delta C_{max}(dQ/dT) = 0.055$ J g^{-1} K^{-1} observed in the temperature range 30–50°C with T_d≈44°C (curve c) is connected with membrane protein denaturation [11] but it was not found out because of powerful exothermic peak at high content of water in the samples. In the range lower than 30 mass% H_2O no exothermic peak on the heat capacity curve was observed (Fig. 2, curve c). The endotherms connected with denaturation of proteins and their complex in the temperature range 30–80°C are weakly expressed. Q_{total} determined at 10.5 mass% H_2O was equal 10±1.0 J g^{-1} of dry biomass (Figs 2 and 4).

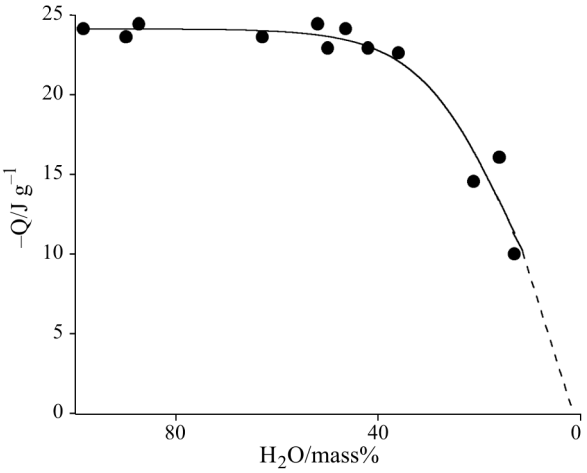

Fig. 4 The dependence of denaturation heat of *Sp. pl.* cells (conditions as in Fig. 1) on mass% H_2O

It was interesting to find out if an observed sharp decrease of Q_{total} lower than 40 mass% H_2O is connected with damage of hydratic shells of membrane proteins and subcellular structures of *Sp. pl*. cells. It should be noted that similar decrease of Q_d in dependence on mass% H_2O was early observed for thymus DNA [12], poly d(A–T) poly d(A–T) [13] and collagen [14].

The microcalorimetric records of bidistilled water evaporation process and water from *Sp. pl.* biomass in the regime close to isothermic one are presented in Figs 5 and 6, accordingly. The enthalpy of evaporated water (H_{free}) was calculated from the area located between the dependence curve $dQ/dt=f(t)$ and the baseline, and hydration of *Sp. pl* cells – from the ratio [15]:

$$\Delta n(H_2O) = \Delta H_{bound}/H(T)M_{dry} \quad (1)$$

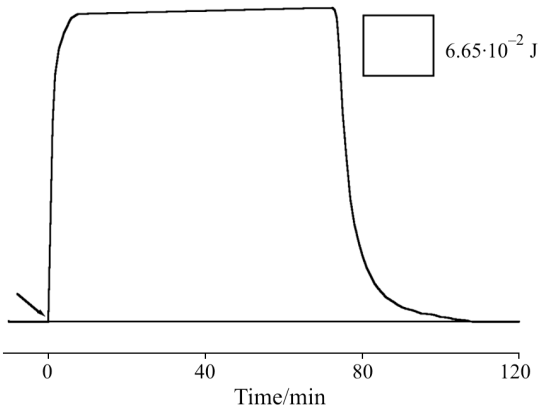

Fig. 5 Microcalorimetric record of bidistilled water evaporation process. The measuring vessel contained 0.485 mg H_2O. The moment of vessel opening (T=25.05°C) is indicated by an arrow. The heat calculated from the area under $dQ/dt=f(t)$ curve and the baseline was equal to 2438.15 J g^{-1}. $H_{pure\ water}$=2441.80 J g^{-1} at 25.0°C [16]

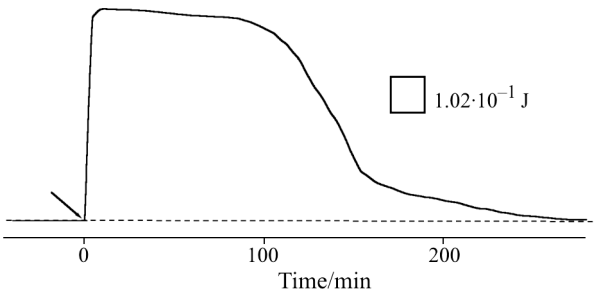

Fig. 6 Microcalorimetric record of water evaporation process from biomass of *Sp. pl.* cells. The measuring vessel contained 2.740 mg of *Sp. pl.* cells, M_{dry}=0.875 mg. The moment of vessel opening (T=25.05°C) is indicated by an arrow

where $\Delta H_{bound}=H_{expected}-H_{free}$, $H(T)$ – enthalpy of evaporation of 1 g of water at a given temperature (Table in [16]), M_{dry} – dry mass of *Sp. pl.* biomass.

Calculations showed that the heat absorption at evaporation of water from *Sp. pl.* biomass (2.740 mg) (H_{free}) was equal to 3.871 J (Fig. 6), $H_{expected}$ calculated from the total content of water in the biomass was equal to 4.565 J. We got the value of *Sp. pl.* cell hydration at 25.05°C equal to 0.32±0.02 g H_2O/g of dry biomass substituting the obtained values in the equation.

The total heat [11] evolved in the temperature range 5–52°C at heating rate less than 0.083°C min^{-1} equal 0.380 kJ g^{-1} of dry biomass, was the heat of vital capacity maintenance (it includes respiration, mobility, osmotic work of cells, opposition to inhibition influences, etc.) of *Sp. pl.* cells which were in the stationary state, in the dark and in 'non-aerobic' conditions.

This value coincides with early obtained values for proteins measured by different methods [5, 8]. In the given case under 'bound' water we suppose the water, which is not evaporated from the preparation at atmospheric pressure and at a given temperature.

In the well-known work it was established [17] that there is a direct connection between the quantities of used O_2, full value of heat production and heat evolution of curve profile for many bacteria and yeast growing in non-aerobic and aerobic conditions. In the case of *Sp. pl.* being in the dark, when the contribution of photosynthesis is minimum, the cell respiration is mainly determined by the rate of oxygen absorption [2].

In the presented experiments the influence of photosynthesis on thermal characteristics of *Sp. pl.* cells was minimum. This is connected with the specificity of DSC measurements. Cell suspension in the process of heating is in a closed titan vessel; therefore, we think that total heat ($-Q$) evolved by *Sp. pl*. cells in the temperature range 5–52°C is mainly determined by the rate of oxygen absorption. As for the sharp decrease of $-Q$ value from 330±30 to 130±13 J g^{-1} of dry biomass, it is connected with spontaneous formation of aggregates from *Sp. pl.* threads in the range 98.8–96.8 mass% H_2O (Figs 1 and 3) and this involves a sharp decrease of oxygen absorption rate. It is also not excluded that a certain contribution to the decrease of $-Q$ occurs because of decrease of *Sp. pl.* individual trichome mobility due to its aggregation. The further decrease of mass% H_2O causes a weaker decrease of $-Q$ value, which equals to 3.6 J g^{-1} of dry biomass at 46.5 mass% H_2O. The registration of $-Q$ is difficult because of small thermal effect at further decrease of mass% H_2O. $-Q=f$(mass% H_2O) dependence extrapolation to zero value of $-Q_{total}$ gives value 30.5±3.0 w% H_2O (Fig. 3). The hydration of *Sp. pl.* cells (Δn) in Zarrouk's medium, calculated from the Eq. (1), is equal to 0.32±0.02 g H_2O/g of dry biomass. Consequently, it may be affirmed that the biochemical reactions proceeding in *Sp. pl.* cells in the process of their function cease when there is not 'free' water and all water is in 'bound' state.

The decrease of mass% H_2O also influences on the parameters of subcellular structure denaturation. As it is seen from data presented in Fig. 4, the total value of denaturation heat (Q_{total}) was not practically changed in the range 98.8–46.5 H_2O, it changed weakly in the range 46.5–35 mass% H_2O and it decreased sharply in the range 35–10.5 mass% H_2O (Figs 2 and 4). According to work [11] and data presented in Fig. 2 (curve a), one powerful exotherm, five endotherms and two shoulders were observed in the temperature range 5–125°C in the case of *Sp. pl.* suspension. The exotherm was connected with biochemical processes proceeding in a living cell, endotherms II–V – with denaturation of C-phycocyanin, phycobilisome complex and other cytoplasmic structures, endotherm VI and a weakly expressed shoulder at 111.5°C were attributed to the denaturation of nucleoproteid DNA [11].

The observed sharp changes of heat absorption curve profile (Fig. 2, curve b) connected with significant shift to high temperatures of endotherms II–V and the unification of endotherms II–IV to one endotherm, a sharp increase of weak heat absorption at 111.5°C and the location immutability of endotherm VI without change of Q_{total} at decrease of water content from 98.8 to 46.5 mass% H_2O (Fig. 4) demanded explanations. Therefore, we decided to carry out the deconvolution of dependence curve $dQ/dT=f(T)$ for *Sp. pl.* containing 46.5% H_2O (Fig. 7). In work [18] the model of death rate for bacteria, heated at a constant rate, was proposed.

It was shown that the temperature, corresponding to maximal death rate of bacteria (T_{dmax}), which is directly connected with kinetic denaturation of subcellular structure, decreased only by 1.2±0.1°C at decrease of heating rate from 0.6 to 0.1°C min^{-1}. These data with good agreement correspond to our results (Experimental).

Small change of T^{max}~1°C of endotherm at low values of heating rates from 0.1 to 0.03°C min^{-1} gave us the possibility to carry out the deconvolution of dependence $dQ/dT=f(T)$ on Gaussian constituents.

The deconvolution of $dQ/dT=f(T)$ dependence curve (46.5 mass% H_2O) (Fig. 2, curve b) on elementary Gaussian constituents gave ten peaks (Fig. 7).

Let's now consider in detail the nature of deconvulation peaks VIII and X. Endotherm IX is so small that we think it is a result of some inaccuracies at deconvolution. Taking into consideration that the area under endotherm VIII with T_d=106±1°C is equal to

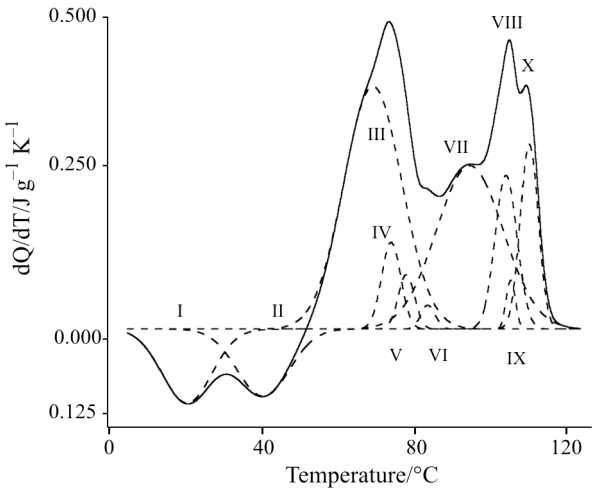

Fig. 7 Deconvolution of dQ/dT=f(T) dependence at 46.5 mass% H_2O in *Sp. pl.* (dash line) (conditions as in Fig. 1)

5.82% from total area of deconvulation peaks II–X, and DNA content in *Sp. pl*., according to literature data, is equal to ~1.0 % from the total dry mass, then we got the value 92.5±12 J g^{-1} for denaturation heat of nucleoproteid DNA calculated from endotherm VIII area.

This value coincides, in the limits of experimental error, with the value of DNA chromatin denaturation enthalpy ($\Delta H_d(Q_d)$) determined early by DSC measurements of tissues and cells of C3HA and BALB/c mice [19] and hepatocyte nuclei of mice and various human pathological tissues [20]. The obtained value was by 31.5 J g^{-1} more than melting enthalpy (ΔH_d) of DNA dilute solutions at high molarity [21, 22] of neutral salts when all negatively charged phosphate groups were neutralized by anti-ions and T_d of DNA becomes equal to ~105°C. Consequently, this 34% rest of ΔH_d comes to the unfolding of superhelical DNA of *Sp. pl*. and to the melting of proteins taking part in NP complex stabilization. This supposition coincides with data of works [19, 20].

Therefore, we conclude that nucleoproteid DNA in *Sp. pl.* has one stage of denaturation with parameters of transition Q_d=92.5±12 J g^{-1}; T_d=106±1.0°C, ΔT_d=4.95°C. As for the origin of endotherm X with T_d=111.5±1.5°C and ΔT_d=3.1°C, it is clear (after the mentioned above data) that it is not connected with nucleoproteid DNA denaturation. We suppose that this endotherm corresponds to the melting of protein having a high-organized structure. This suggestion is also confirmed by DSC measurements of 9 different bacteria, including Gram-negative, Gram-positive and spore forms [18]. In all cases the clear peaks connected with denaturation of protein complexes were observed in the temperature range from 110 to 120°C.

It should be noted that a sharp decrease of Q_{total} lower than 35 mass% H_2O (Fig. 4) is mainly connected with denaturation of proteins and their complexes due to cell dehydration, because only 5.8% of Q_{total} comes to nucleoproteid DNA denaturation. As for the constancy of $Q_{d\ total}(\Delta H_{d\ total})$ and quite significant shift of endotherm II–IV to high temperatures, when the water content decreases from 98.2 to 46.5 mass% H_2O, it means that entropy, connected with protein denaturation, decreases as 'free water' is lost ($\Delta S_d=\Delta H_d/T$).

Conclusions

At present we cannot give a more clear explanation of so strong increase of bulk proteins thermostability T_d=99 and 111.5°C (Fig. 2, curve c). But we know that phycobiliproteins, composing the main bulk of protein in *Sp. pl.* and playing a great role in phycobilisome formation, can form crystalline compounds, melting temperature of which is higher than T_m of individual molecules.

So, we came to the following conclusions:

- Structural energetics of *Sp. pl.* cells. does not change till 'free water' is in the system but it sharply decreases as the system is dehydrated.
- The hydration of *Sp. pl.* in Zarrouk's medium (pH 9.85) at 25°C is equal to 0.32±0.02 g H_2O/g of dry biomass. It is suggested that all biochemical reactions proceeding in *Sp. pl.* are inhibited in case of 'free' water loss by *Sp. pl.* cells.
- The nucleoproteid DNA in situ has one-stage denaturation with transition parameters: Q_d=92.5±12 J g^{-1} and T_d = 106±10°C and does not depend on the content of 98.2–40.5 mas% H_2O.
- The thermostability of proteins in situ is sharply increased as the water is removed from a sample and it is equal to 99 and 111.5°C at mass% H_2O.

Acknowledgements

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