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ORIGINAL RESEARCH PAPER

DEVELOPMENT OF AN EFFECTIVE METHOD FOR ISOLATING OF HYDROPHOBIN-TYPE PROTEINS FROM *TRICHODERMA VIRIDE* SUBMERGED CULTURE AND EVALUATION OF THEIR PROPERTIES

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Abstract: Hydrophobins are low-molecular proteins with very high surface activity and great potential for application in food and pharmaceutical industries. In this study an efficient method for isolation of hydrophobin-type proteins from *Trichoderma viride* mycelia biomass and native liquid of culture medium was elaborated. Extraction buffer and parameters of the extracting agents, such as buffer *p*H value and its molarity, as well as extraction temperature and duration time were selected. Obtained proteins were purified and their molecular weight was determined as 8 ± 1 kDa. Surface active and foam stabilizing activities of obtained proteins were also determined. Surface activity of isolated hydrophobin-type proteins was compatible to the activity of sodium dodecil sulfate, while foam stabilizing effect was higher and the action was much longer then when sodium-caseinate or Tween-80 were used.

Keywords: *biosurfactants, emulsifiers, extraction, foam-stabilization, multiple regression analysis, submerged cultivation of fungi*

INTRODUCTION

Hydrophobins are a class of small proteins, consisting of approximately 100 amino acids, produced by mycelial fungi. A peculiarity of these proteins is the presence of 8 cysteine molecules in the same position, forming 4 disulfide bridges. The surface of hydrophobin molecules is divided into hydrophilic and hydrophobic parts, which makes the protein molecule amphiphilic. The distribution of cysteine in protein molecules and the arrangement of hydrophobic and hydrophilic residues allow the hydrophobins to be divided into two classes, class I and class II. Class I hydrophobins are assembled in aggregates that are practically insoluble and start to brake down only at a temperature of 100 °C, and in the presence of high concentrations of strong organic acids, such as formic acid or trifluoroacetic acid. In contrast, aggregates formed by class II hydrophobins are less stable. They readily dissolve in the presence of 60 % ethanol or detergents [1 – 5].

Due to this amphiphilic structure, hydrophobins have properties of surface adsorption and surface activity. Due to their structure, hydrophobins can be assembled into strong amphipathic membranes at the phase interface, changing the nature of the surface from hydrophobic to hydrophilic, and vice versa, as well as organizing strong structures on various materials (Figure 1). In addition, hydrophobins are non-toxic and non-immunogenic.

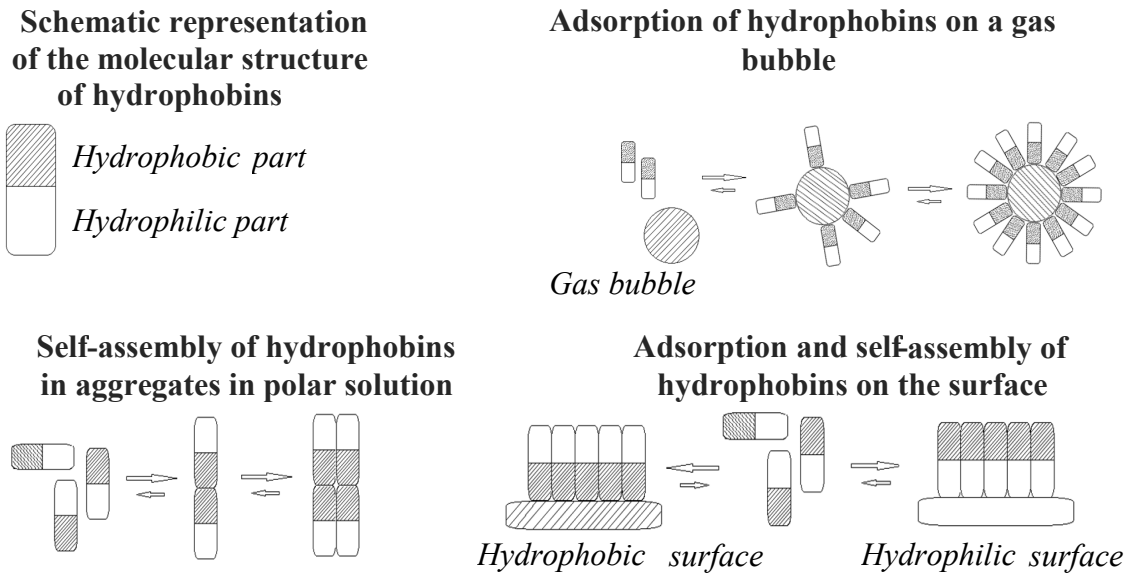

Figure 1. *Self-assembly of hydrophobins into aggregates*

Above listed properties open wide prospects of using hydrophobins in various industries. It is known that hydrophobins, even in small amounts, can form highly stable foams and emulsions. The effect caused by these proteins is much higher than that of all known natural foam stabilizers used in the food industry [6, 7]. At the same time emulsions formed by hydrophobins, in their consistency and taste, resemble fats. Up to 50 % of the fat base of a variety of products can be replaced with an emulsion containing these proteins [8]. In medicine, the use of hydrophobin-based implant coatings will prevent the adhesion of bacteria and prevent the formation of biofilms on the surface of the implant, as well as increase the biocompatibility between implant and

tissue [9 – 11]. In addition, hydrophobins can be used as an alternative to traditional surfactants in many other applications.
A wide range of hydrophobin applications necessitates research for the production and purification of these proteins. In this work, the mycelial fungus *Trichoderma viride* was used as the source of the hydrophobins. This fungus can accumulate hydrophobins in biomass and also to produce them into the culture liquid. *T. viride* can produce hydrophobins of class II. In this paper, the dynamics of the accumulation of hydrophobins and biomass by submerged cultivation of the fungus was studied, a selection of the extracting agent and extraction conditions of hydrophobins from biomass was carried out.

MATERIALS AND METHODS

Submerged cultivation of Trichoderma viride

The object of our study was the culture of fungus *Trichoderma viride* from the culture collection of St. Petersburg State Institute of Technology (Technical University).
Fungi was cultured under submerged conditions in flasks at a temperature of 28 °C on the rotary shaker (IR-1LT, Labtech, Moscow, Russia) in 750 mL Erlenmeyer flasks, containing 100 mL of semi-synthetic medium containing ($g \cdot L^{-1}$): glucose -10; peptone -2.5; KH_2P0_4 -0.6; K_2HPO_4 -0.4; $CaCl_2$ -0.05; NaCl -0.5; yeast extract -2.0; *p*H of the media before sterilization was 5.8-6.0.
Culture broth was separated into native liquid and mycelia biomass by centrifugation.

Extraction of hidrophobin-type proteins from biomass

Mycelia biomass obtained from submerged cultivation was subjected to successive freezing and defrosting with subsequent processing using ultrasonic disintegrator (Prointeh - Bio, Russia) in order to break the cell walls to achieve higher yield of the studied proteins.
Extraction of proteins from biomass of *Trichoderma viride*, including hydrophobins, was carried out using extracting agents consisting of buffers with different *p*H ranges and various molarities and 1 % SDS (sodium dodecyl sulfate), as well as at different periods of time and different temperatures. The volume of extracting agent was taken in a ratio of 3:1 per biomass weight. Extraction was carried out with stirring. After the extraction, the biomass was separated from the extract by centrifugation for 10 minutes at a rotation speed of 6000 min^{-1}. The SDS precipitation from the extract was performed by the addition of 2 M KCl, at a rate of 0.4 mL per 1 mL of extract at a temperature of 4 °C [12].
The residue of the SDS salt was separated from the extract by centrifugation for 10 minutes at a rotation speed of 6000 min^{-1} extract at a temperature of 4 °C. Class II hydrophobins in the solutions can form stable agglomerates, which are soluble in the organic solvents, for example in 60 % ethanol solution. Insoluble in ethanol high molecular proteins will precipitate, while hydrophobins will remain in ethanol solution. To dissolve hydrophobins obtained extract was treated with 96 % solution of ethanol, so, that the concentration of ethanol in the obtained solution will be 60 % of total volume. Further, obtained solution was incubated at 4 °C for 1.5 h and was separated

from the residue by centrifugation for 10 min at a rotation speed of 6000 min^{-1}. After that, ethanol was deleted by evaporation.
The resulting protein aqueous solution was freeze dried. The protein concentration in the resultant extract was determined by the Lowry method [13]. For the evaluation of surface activity, contact angles for the solutions of obtained protein preparations were determined.
To determine the optimal time range and temperature for extraction of hydrophobin-type proteins (Y), a method of regression analysis was used [14, 15]. In the experiment, the temperature (T, °C) and time (t, min) varied at three levels: minimum (-), mean (0), and maximum (+). The variation levels for these factors were selected based on economic feasibility, temperature stability of hydrophobins and analysis of preliminary studies. The experimental results were processed using statistical software package "STATISTICA".

Isolation of surface-active proteins from native liquid

The procedure for isolating and concentrating hydrophobins is based on their surface - active properties. Hydrophobin - type proteins will create persistent finely dispersed foam, when the solution is foamed. Foaming the native liquid of culture broth was carried out in a unit for foaming (Figure 2). 100 mL of native liquid was poured into vessel - 2 and then foaming was carried out with the aerator - 1. During the foaming process, fine foam, containing surface active proteins, was collected. The foaming process was carried out at a temperature of 4 °C, since at this temperature SDS, remaining in the solution, is not able to produce fine-dispersed resistant foam.
Obtained extract was treated with 96 % solution of ethanol, so, that the concentration of ethanol in the obtained solution was 60 % of total volume, to dissolve hydrophobins [3 – 5]. Further, obtained solution was incubated at 4 °C for 90 min and was separated from the residue by centrifugation for 10 min at a rotation speed of 6000 min^{-1}. After that, ethanol was evaporated under vacuum. The resulting protein aqueous solution was freeze dried. The protein concentration in the resultant extract was determined by the Lowry method. Contact angles for the solutions of obtained protein preparations were also determined to evaluate their surface activity [16].

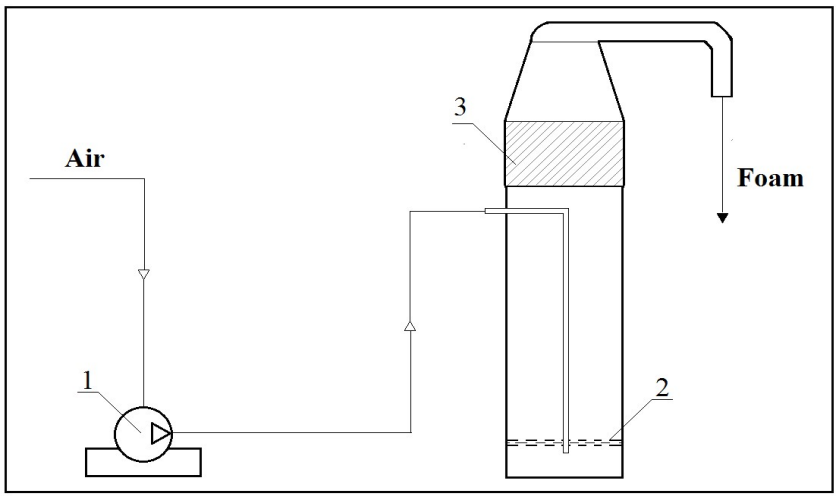

Figure 2. *Scheme of the unit for foaming the native liquid of the culture broth*
1 - compressor, 2 - vessel with a porous glass, 3 - section of fastening

Analysis of the extracts

Molecular weights of the obtained proteins were determined using electrophoresis method in polyacrylamide gel (PAGE) according to the method of Laemmli.

Electrophoresis was performed on BioRAD, Mini-Protean II Cell device, with PowerPack Basic, ("BioRad", USA). Proteins were separated in 15 % separating PAGE and 5 % concentrating PAGE. Tris-glycine buffer was used as the buffer. As a protein marker p7710s ("New England BioLabs", USA) was used. Electrophoresis separation was performed at an electric field voltage of 65 V. The visualization of the results was carried out by staining plates in a Coomassie solution of 0.22 %. Gels from the dye were washed in a solution of 7 % acetic acid [17]. The gels were scanned using ImageLab 5.0 on the Gel Doc EZ Imager ("BioRad", USA).

Estimation of foam stability properties of obtained proteins

To evaluate surface activity of obtained proteins we test their foam stabilizing properties. It is known that hydrophobins possess high foam stability, even at extremely low concentrations.

To determine the foam stabilizing properties of hydrophobins from *T. viride*, a solution containing 0.1 % of the resulting hydrophobin-type proteins was prepared. Xanthan gum was used as a solution thickener. Sodium benzoate was used as preservative.

The dried protein preparation was added to 10 mL of a solution containing 0.5 % xanthan gum and 0.01 % sodium benzoate. Sodium caseinate and Tween-80 at a concentration of 0.5 % were used for comparison [18].

Test and control samples were foamed in glass columns for 20 seconds using an aerator. Upon completion of aeration, columns with foam were placed in a refrigerator, where they were stored at 4 ºC. The resulting volume of foam was measured immediately after foaming and weekly for 2 months.

RESULTS AND DISCUSSION

Selection of extragent parameters for obtaining hydrophobin-type proteins from T. viride biomass

The *p*H and molarity of extraction buffers were selected to develop the efficient extraction procedure of hydrophobin-type proteins from *T. viride* biomass.

The biomass of the fungus *T. viride* was cultured for 30 h. After the extraction, concentration of the protein was determined by the Lowry method. The surface activity of the extracts was determined using the measurement of contact angles in the resulting solutions of hydrophobin-type proteins, containing 0.25 $mg \cdot mL^{-1}$ of protein. A pure buffer was used as a control. Surface activity was defined as the difference between the control and the sample in the contact angles of wetting of the Teflon surface.

The results for the selection of *p*H value of the extracting agents for the extraction of hydrophobins from *T.viride* biomass were summarized in the Table 1.

The highest yield of hydrophobin-type proteins from *T. viride* biomass was obtained, when an extracting agent consisting of Tris-HCl buffer (*p*H 9) and 1 % SDS.

Table 1. *The influence of the pH values of used* extracting agents *on isolation of hydrophobin-type proteins from biomass T. viride*

Buffer solution of extragent 0.25 M	***p*H of extragent solution**	**Protein concentration in dry biomass, [mg·g^{-1}]**	**The contact angle of wetting, [degree°] (degree of control - degree of sample)**
Citrate buffer	3	22.5 ± 3.0	9.1 ± 3.7
	4	31.1 ± 2.9	10.1 ± 5.3
	5	34.8 ± 3.3	9.2 ± 5.6
Phosphate buffer	6	36.8 ± 2.7	12.1 ± 5.8
	7	37.9 ± 5.6	14.5 ± 7.2
	8	39.2 ± 5.8	15.2 ± 7.1
Tris-HCl buffer	8	40.5 ± 4.9	21.4 ± 7.4
	9	41.3 ± 4.8	23.2 ± 6.8
Glycine-NaOH buffer	9	40.3 ± 5.1	21.7 ± 6.7
	10	39.4 ± 5.8	22.0 ± 8.2
	11	38.8 ± 4.9	21.9 ± 7.2
	12	38.7 ± 4.7	19.2 ± 7.4

After selecting the extracting agent buffer and the *p*H value, the buffer molarity was selected. Extraction of hydrophobin-type proteins from the biomass of *T. viride* was performed using Tris-HCl buffer (*p*H 9) with a different molar range, and 1 % SDS.
In the obtained solutions of hydrophobin-type proteins, the protein concentration was measured by Lowry's method and the surface activity was measured by the contact angle method. These operations were also performed with the control. Surface activity was defined as the difference in the contact angle of the Teflon surface wetting between the control and the sample. To measure the surface activity, solutions containing 1 mg·mL^{-1} of protein were prepared.
The results of the influence of the buffer molarity on the extraction of hydrophobins from *T. viride* biomass are summarized in Table 2.

Table 2. *The influence of the extracting agent's molarity (Tris-HCl buffer (pH-9)) on isolation of hydrophobin-type proteins from T. viride biomass*

The molarity of the extragent buffer, M	**Protein concentration in dry biomass, [mg·g^{-1}]**	**The contact angle of wetting, [degree °] (degree of control ° - degree of sample °)**
0.05	25.9 ± 3.9	20.1 ± 5.9
0.1	32.9 ± 6.7	19.4 ± 6.2
0.2	35.8 ± 4.5	23.6 ± 7.0
0.25	38.8 ± 3.9	26.5 ± 6.1
0.3	41.6 ± 6.4	28.2 ± 6.5
0.4	39.3 ± 5.8	26.6 ± 5.8
0.5	36.8 ±4.6	27.6 ± 5.1

The highest yields of hydrophobin-type proteins from *T. viride* biomass was obtained using an extragent consisting of 0.1-0.3 M Tris-HCl buffer (*p*H 9) and 1 % SDS.

Selection of extraction conditions for hydrophobin-type proteins from T. viride biomass

For the effective extraction of hydrophobin-type proteins from *T. viride* biomass, the influence of extraction temperature and period on the yield of extracted proteins were studied using multiple regression analysis method.

The extraction was performed using 0.1 M Tris-HCl (*p*H 9) buffer and 1 % SDS. In the solutions of obtained hydrophobin-type proteins, the concentration of the proteins was measured by Lowry's method. A pure buffer was used as a control.

Table 3 presents the planning and the results of an optimization experiment for the extracting of the hydrophobins–type protein in the extract from the biomass of *T. viride* at various time (t, min) and temperature (T, °C).

Table 3. *Experimental plan and the results describing dependence of the influence of time (t, min) and temperature (T, °C) on the yield and hydrophobin-type proteins during extraction from biomass T. viride*

№ of the experiment	**Factor variations levels**		**Absolute values of the factors temperature (T) and time (t)**		**Protein concentration (Y) in dry biomass, [mg·g^{-1}]**
	t, [min]	**T, [°C]**	**t, [min]**	**T, [°C]**	
1	-	-	45	30	37.3
2	-	0	45	40	41.8
3	-	+	45	50	40.1
4	0	-	90	30	40.4
5	0	0	90	40	39.8
6	0	+	90	50	40.2
7	+	-	135	30	39.8
8	+	0	135	40	40.3
9	+	+	135	50	42.4

Table 4 shows the parameters of the statistical characteristics of second-order polynomial models describing the dependence of the change magnitudes on the factors (T) and (t).

Table 4. *Statistical characteristics of the parameters for plotting values of (Y) from the factors time (t, min) and temperature (T, °C)*

Dependent variables	**Model parameters**	**Estimate**	**Standard error**	**T-Criterion**	**The level of significance, p**
Y	Constant	0.61	8.141	0.08	0.9420
	T	0.03	0.189	0.17	0.8700
	t	0.92	0.107	8.61	0.0001
	t^2	-0.01	0.001	-6.32	0.0001

Parameter T has P-value of 0.8700 (greater than 0.05) for independent variables. This parameter is not statistically significant at a confidence level of 95.0 % or more. Therefore, it was removed from the model. Table 5 shows the parameters of the

statistical characteristics of second-order polynomial models describing the dependence of the change magnitude on the factor (t).

Table 5. *Statistical characteristics of the parameters for plotting values of (Y) from the factor time (t, min)*

Dependent variables	Model parameters	Estimate	Standard error	T-Criterion	The level of significance, p
Y	Constant	1.89	2.843	0.67	0.5230
	t	0.92	0.102	9.11	0.0001
	t^2	-0.01	0.001	-6.69	0.0001

The statistical parameters of coefficients, represented in table 5 - are indicating, that their numerical values are statistically significant ($p<0.05$). This makes possible to use these coefficients to develop regression model describing the change of variables (Y) from the time (t, min) of the extractions from biomass *T. viride* in the form of equations (1).

$$Y = 1.889 + 0.924\,t - 0.005\,t^2 \quad (1)$$

Analysis of variance of model (Eqn. 1) shows high coefficients of determination (R^2=93.71), respectively, indicating their information capacity. Graphic interpretation of the equations 1 is represented in Figure 3.

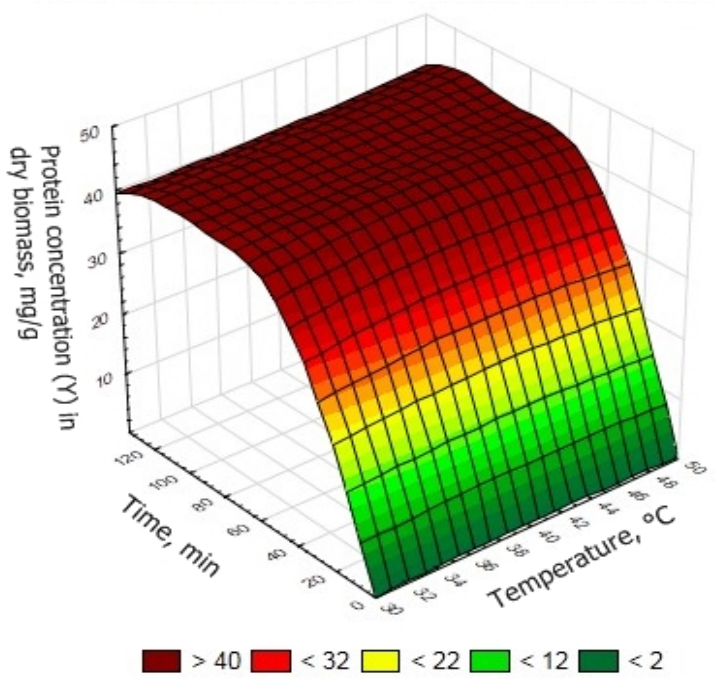

Figure 3. *A surface plot describing the time and temperature dependence on yield and hydrophobin-type proteins during extraction from biomass T. viride*

The results presented in Figure 3 show that under the experimental conditions the leading factor influencing the yield of protein from the biomass of *T. viride* during extraction is the extraction time. It is evident that in the case of extraction of

hydrophobin-type proteins from *T. viride* biomass, the temperature in the range from 30 to 50 °C practically does not influence the yield of the protein. Extraction in this case can be carried out at 30 °C. Extraction should be carried out for 60 min, since a further increase in time practically does not affect the yield of the protein.

Isolation of hydrophobins from the culture fluid of the fungus T. viride

As a source of hydrophobin, the native liquid of *T. viride* was also considered. The fungus was grown in flasks on a rotary shaker for 30 hours. The culture liquid was then separated into native liquid and biomass by filtration through a tissue filter.

The native liquid was used to isolate hydrophobin-s, with a protein concentration of 1.89 ± 0.20 $g \cdot L^{-1}$. The native liquid was foamed to concentrate the surface-active proteins in the foam. The foam was treated with a 96 % solution of ethanol, so that the concentration of ethanol in the solution became 60 %. The solution was separated from the precipitate and ethanol was removed by evaporation. The final protein concentration was 323.1 ± 3.9 $mg \cdot L^{-1}$. The contact angle of the evaporated solution on Teflon surface at a concentration of 0.5 $mg \cdot mL^{-1}$ was 50.2° ± 4.2°.

In the same way, hydrophobins were isolated from the biomass of the fungus *T. viride*. Hydrophobin-type proteins were extracted in three steps by consistently mixing of 90 g of biomass (moisture 90.6 %) with 270, 180 and 90 mL of extragent. At the end of each step, the biomass was separated from the extract and SDS was removed. The extragent consisted of 0.1 M Tris-HCl (*p*H 9) buffer and 1 % SDS.

The yield of dry protein extract from 1 g of biomass was 118.4 ± 4.2 $mg \cdot g^{-1}$, 20.9 ± 1.2 $mg \cdot g^{-1}$ and 2.2 ± 0.3 $mg \cdot g^{-1}$ after the 1st, 2nd and the 3rd steps of the extraction. The concentration of SDS after precipitation of 2 M KCl was 0.139 ± 0.02 $mg \cdot mL^{-1}$, this method allows removing 98.1 % SDS from the extract.

Further purification produced only the first extract. The extract was then dissolved in 96 % ethanol, so that the final ethanol concentration in the solution was 60 %. After incubation at 4 °C for 1.5 h, the ethanol was removed by evaporation.

The solution was further purified from low molecular weight impurities using dialysis membranes. The concentration of the protein was determined in dialyzed solution and the surface activity of 0.5 $mg \cdot mL^{-1}$ protein solution was determined by measuring the contact angle of wetting of Tephlon surface. The yield of the protein was 48.45 ± 3.2 $mg \cdot g^{-1}$ of initial dry biomass. The contact angle of wetting of the Teflon surface was 47.2° ± 6.2°. The contact angle of the SDS solution on Teflon surface at a concentration of 1 $mg \cdot mL^{-1}$ was 46.5° ± 5.3°. Surface activity of isolated hydrophobin-type proteins was compatible to the activity of sodium dodecil sulfate.

To determine the purity of obtained preparations and molecular weights of the proteins electrophoresis of the preparations was performed at different stages of purification. Electrophoregrams are shown in the Figure 4.

The proposed method of obtaining and purification of hydrophobin-type proteins allows obtaining low molecular weight and surface-active proteins. The molecular weight of hydrophobin-type proteins was 8.0±1.0 kDa, which corresponds to the molecular weight of hydrophobins.

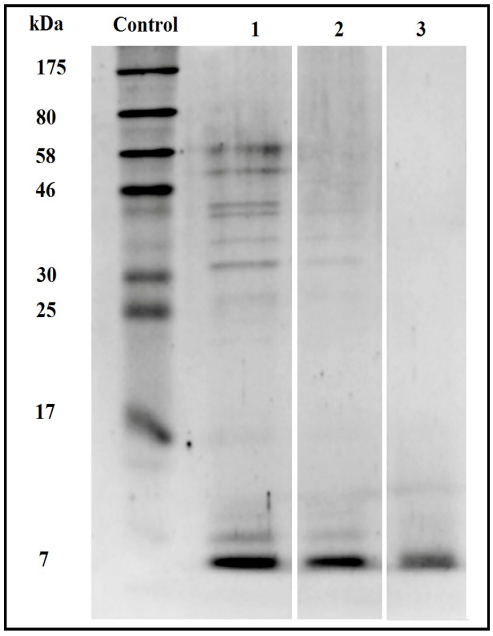

Figure 4. *Electrophoregram of protein fractions at different stages of purification of hydrophobin-type proteins obtained from the native liquid and biomass of T. viride 1 - proteins contained in the culture fluid; 2 - hydrophobin-type proteins obtained from the culture fluid; 3 - hydrophobin-type proteins obtained from the biomass*

Evaluation of the foaming and foam stability of the resulting hydrophobin-type proteins

Due to their properties, hydrophobins, even at low concentrations, have a high foaming and foam-stabilizing capacity. To assess the surface-active properties of hydrophobins, we determined their foam-forming and foam-stabilizing capabilities. To this end, 10 mL solutions were prepared from the extracts containing hydrophobins. Widespread food foam stabilizers 0.5 % Tween-80 and 0.5 % sodium caseinate were used. Immediately after preparation, solutions were foamed in a column, and the volume of foam was measured. After that, the volume of foam was measured, every day. The data on the change in the volume of the foam are presented in the form of a histogram, in Figure 5.

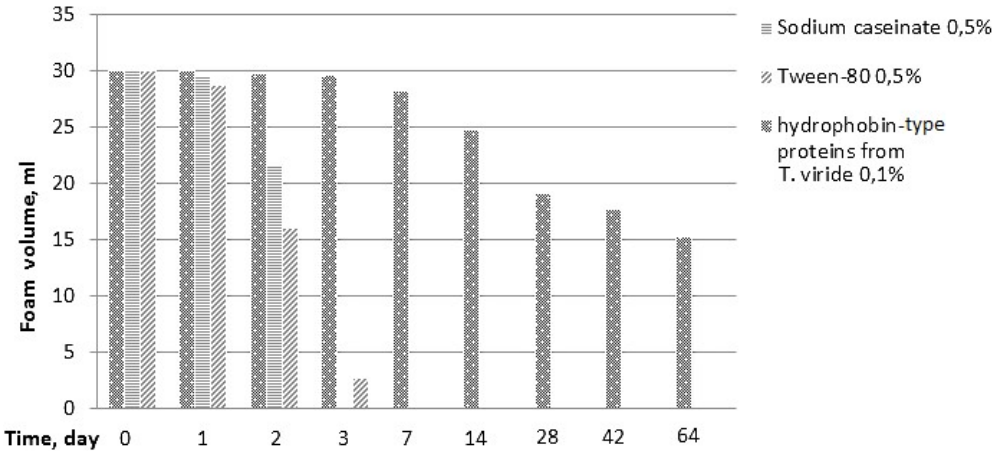

Figure 5. *The foam stabilizing ability of hydrophobin-type proteins and some food emulsifiers*

The resulting hydrophobin-type proteins allow the formation of persistent foams. The air phase losses in the test samples were about 50 % for 8 weeks for hydrophobin-type proteins while in the control samples a complete loss of the air phase was observed in a week. Foaming ability and foam-stabilizing properties of the obtained hydrophobin-type proteins proved to be much higher than in food emulsifiers.

CONCLUSIONS

The highest yield of hydrophobin-type proteins from *T.viride* biomass was obtained with 0.1 - 0.3 M Tris-HCl buffer (*p*H 9). Extraction period was estimated to be 60 minutes. Also, the temperature in the range 30 - 50 °C does not significantly affect the yield of the protein during extraction;
The molecular weight of the obtained hydrophobin-type proteins from the fungus *T. viride* was determined as approximately 8 kDa;
The foaming capacity and foam-stabilizing properties of the hydrophobin-type proteins obtained were significantly higher than for tested food emulsifiers. Surface activity of obtained proteins was compatible to the activity of SDS.

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