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OBTAINING OF HYDROPHOBINS FROM SUBMERGED CULTURED *TRICHODERMA VIRIDE*

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Abstract

The search for new efficient emulsifiers for the food industry nowadays is an actual task. One of such promising emulsifiers are hydrophobins. They are low molecular structural proteins of fungi. They have very high surface activity and can be used like emulsifiers and foam stabilizer in food products. The foam-stabilizing effect caused by hydrophobins is much higher than for all known food emulsifiers. The aim of the study was to select the conditions for cultivation of the fungus to increase the yield of hydrophobin-type proteins.

The object of the study was the culture of the fungus *Trichoderma viride* selected as a producer of hydrophobin-type proteins. Submerged cultivation was performed using glucose-peptone nutrient medium with different concentrations of glucose and peptone. To study the effect of oxygen concentration on the biosynthesis of hydrophobin-type proteins, cultivation was carried out in different aeration regimes. By the end of the cultivation the native liquid was foamed and treated with 70% ethanol for extraction of hydrophobin-type proteins. Hydrophobin-type proteins from biomass were extracted with 2% sodium dodecyl sulfate (SDS) solution. The protein concentration in the extracts was determined by the Lowry method. Surface activity of the extracts were evaluated by measurement of the contact angle of their solutions.

Concentrations of glucose and peptone in the medium and aeration mode provided maximum yield of hydrophobin-type proteins were selected based on the results of the studies. Obtained extracts showed high surface activity. The foam stabilizing effect of the extracts significantly exceeds the effect of food industrial emulsifiers. The losses of the air phase in the samples stabilized with the extract amounted to about 50% in 8 weeks.

Selected conditions of cultivation ensure high yield of hydrophobin-type proteins which can be used in food industry.

Key words: *Hydrophobins, Submerge cultivation, Fungi, Emulsifiers, Surfactants, Nutrient medium*.

1. Introduction

Hydrophobins are low-molecular-weight (6-20 kDa) surface-active proteins produced by fungi. The molecules of hydrophobins are amphiphilic and can self-assemble into amphipathic monolayers at the interface. Hydrophobins have a large number of hydrophobic and hydrophilic amino acid (Wessels *et al.*, [1]; de Vries *et al.*, [2]).

Depending on the structural features and distribution of hydrophobic and hydrophilic amino acids hydrophobins are divided into 2 classes - I and II (Sunde *et al.*, [3]). The aggregates formed by these two classes differ in their solubility and morphology. Aggregates formed by class I hydrophobins are slightly soluble and can only dissociate under the action of concentrated strong acids (such as formic acid or pure trifluoroacetic acid). Complexes formed class II hydrophobins are less stable and can be easily dissolved in 60% ethanol solution or 2% sodium dodecyl sulfate (SDS). Because of the difference in the solubility of hydrophobins of different classes, the methods for their isolation are very different (de Vries *et al.*, [2], Askolin *et al.*, [4], Wessels [5]).

Due to their surface active properties, hydrophobins can be used in various areas. One of the most promising applications of hydrophobins is their use in the

food industry as stabilizers for foams and emulsions. The effect caused by hydrophobins is much higher than in all known emulsifiers and foam stabilizers used in the food industry. A special feature of natural emulsions formed by hydrophobins is that, in consistency and taste, they resemble edible fats.

The use of hydrophobins in the food industry opens up new opportunities for the production of food products with improved functionality, for developing new textures and for reducing the caloric content of products (Green *et al.*, [6], Wang *et al.*, [7], Cox *et al.*, [8]).

The level of aeration, the concentration of carbon and nitrogen sources in the nutrient medium, as well as their ratio, have a great effect on protein production by fungi. In this regard, the aim of our work was the selection of the composition of the nutrient medium and conditions for cultivation of the fungus *T. viride* providing the highest yield of hydrophobins.

2. Materials and Methods

2.1 Submerge cultivation

The object of our study was the culture of fungi *Trichoderma viride* from the collection of fungi of St. Petersburg State Institute of Technology (Technical University). Initial cultures were submerge cultured in flasks at a temperature of 28 ^{0}C for 72 hours on the rotary shaker (IR-1LT, Labtech, Moscow, Russia) using semi-synthetic medium containing (g/L): 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; pH of the media before sterilization - 5.8 - 6.0. The influence of different levels of glucose (from 10 to 20 g/L) and peptone (from 1 to 4 g/L) on the production of hydrophobin-type proteins were studied.

A method of multiple regression analysis (Draper and Smith, [9], Richard and Dean, [10]) was used to determine the optimal ratios of glucose and peptone in nutritive media for *T. viride* fungi to increase the syntheses of hydrophobin-type proteins in biomass (Y_1, mg/mL) and in native liquid (Y_2, mg/mL). In the experiment, the glucose (Cg mg/mL) and peptone (Cp mg/mL) concentrations in the culture medium were varied at three levels: a minimum (-), mean (0) and maximum (+). The levels of variation for these factors were selected on the basis of economic feasibility and analysis of preliminary studies. The experimental results were processed using a statistical software package «Statistica».

After separation of mycelium by filtration from culture broth, mycelium and native liquid were used for extraction of hydrophobins.

The concentration of oxygen in the medium has a great effect on the biosynthesis of proteins by fungi (Wang *et al.*, [11]). Therefore, synthesis of hydrophobins can depend on a degree of aeration of the medium. To determine the effect of aeration of the medium on the synthesis of surfactant proteins, mushroom was cultured in 750 ml Erlenmeyer flasks with different quantity of liquid medium - 50, 100 and 150 mL.

Aeration rate was determined by the sulfite method (Yegorov, [12]). The dissolution rate of oxygen in the flasks was: for the 50 mL volume of medium - 4.4 g/(L·h), for 100 mL of medium - 3.0 g/(L·h), and for 150 mL of medium - 1.8 g/(L·h).

2.2 Extraction of hydrophobin-type proteins

The biomass was subjected to successive freezing and thawing with the aim of destroying the cell wall of the fungus and the most complete release of hydrophobins. Hydrophobins from biomass were extracted with 2% SDS solution at room temperature for one hour. After this, SDS was precipitated with 2M KCl and removed by centrifugation. To isolate the hydrophobins from the native solution, it was foamed using an aerator, after which the foam was mechanically separated. After that the liquid was foamed using an aerator, then the foam was mechanically separated and dissolved in a 70% solution of ethanol. Insoluble in ethanol precipitate was separated by centrifugation at 4100 g for 15 minutes. The ethanol from the solution was removed by evaporation on a vacuum rotary evaporator at 40 ^{0}C. The resulting aqueous solution was freeze-dried. The protein content of the extracts was determined by the Lowry method.

2.3 Estimation of foam stability properties of obtained proteins

Hydrophobines are strong foam stabilizers and emulsifiers. We evaluated the foam-stabilizing ability of the obtained hydrophobin-type protein (the solution for measurement contained 0.1% protein). As a comparison, a 0.5% solution of Tween 80 and a 0.5% solution of sodium caseinate was used. As a thickener, xanthan gum was used in a concentration of 0.5%. To preserve the protein solution, sodium benzoate was added at a concentration of 0.1%.

20 mL solutions were foamed in a column with a porous glass, using an aerator, and the foam volume was measured immediately after foaming. Further, the volume of the formed foam was measured for two months.

3. Results and Discussion

3.1 Selection of nutrient medium composition

We carried out the selection of media composition to ensure the high level of biosynthesis of surfactant proteins. The biosynthesis of proteins is greatly influenced

by the concentration of carbon and nitrogen sources in the medium, as well as by their ratio. Therefore, we studied the effect of their content in culture medium on the biosynthesis of surfactant proteins.

Table 1 presents the planning and the results of an optimization experiment for the accumulation of the protein in the extract from the biomass (Y_1) and from the native liquid (Y_2) of *T. viride* at various concentrations of glucose (Cg) and peptone (Cp).

Table 2 shows the parameters of the statistical characteristics of second-order polynomial models describing the dependence of the change magnitudes of (Y_1) and (Y_2) on the factors Cg and Cp.

The statistical parameters of coefficients, represented in table 2 are indicating, that their numerical values are statistically significant ($p < 0.05$). This makes possible to use these coefficients for building regression models describing the change of variables (Y_1) and (Y_2) from the glucose (Cg and peptone (Cp)) concentrations in the nutrition medium for *T. viride* in the form of equations 1 and 2:

$$Y_1 = -348.30 + 44.56\,X_1 - 1.25X_1^2 + 56.52X_2 - 8.46X_2^2 \quad (1)$$

$$Y_2 = -161.59 + 29,.80X_1 - 0.78X_1^2 + 15.89\,X_2 - 0.71X_1\,X_2 \quad (2)$$

Analysis of variance of models (equations 2 and 3) shows high coefficients of determination ($R_1^2 = 98.86$; $R_2^2 = 95.22$), respectively, indicating their information capacity.

Equations 2 and 3 also have statistical significance ($p < 0.05$), as the value of Fisher criterion (F) for the equation 2 is F=145.17 > F cr. = 5.41 and level of significance ($p = 0.0000$), and for Equation 3 - F = 59.82 > F cr. = 5.14 and level of significance ($p = 0.0001$) [F cr. – Critical value of Fisher criterion]. Graphic interpretations of the equations 1 and 2 are represented in Figures 1 and 2 respectively.

Results presented in Figures 1 and 2 show, that in the conditions of the experiment, a leading factor influencing the accumulation of the protein concentration in the extract from the biomass and from the native liquid of *T. viride*, is the concentration of glucose in the nutrition medium. The optimum concentration of the parameter at which the maximum values of the dependent variable Y_1 were observed are in the range from 17.5 to 20.0 g/L. The value of the dependent variable of Y_2 increases with the concentration of glucose and prevents clearly pronounced peaks.

Table 1. Experimental plan and the results describing the accumulation of the protein in the extract from the biomass (Y1) and from the native liquid (Y2) of *T. viride* for different factor values (Cg, Cp)

No of the experiment	Factor variation levels		Absolute values of the factors, g/L		Protein concentration Y_1 (biomass), mg/L	Protein concentration Y_2 (native liquid), mg/L
	X_1	X_2	Cg	Cp		
1	0	+	15.00	4.00	135.2	127.3
2	+	+	20.00	4.00	138.2	131.2
3	+	0	20.00	2.50	126.4	124.3
4	0	0	15.00	2.50	125.9	121.2
5	-	+	10.00	4.00	56.2	95.4
6	-	-	10.00	1.00	18.9	65.3
7	-	0	10.00	2.50	70.4	79.9
8	+	-	20.00	1.00	96.9	122.5
9	*0*	-	15.00	1.00	85.8	119.4

Table 2. Statistical characteristics of the parameters for plotting values of (Y_1,Y_2) from the factors (Cg, Cp)

Dependent variables	Model parameters	Estimate	Standart error	T-Criterion	The level of significance, p
Y_1	Constant	-348.304	48.711	-7.150	0.002
	Cg	44.560	6.647	6.704	0.003
	Cp	56.519	12.435	4.545	0.011
	Cg^2	-1.245	0.221	-5.647	0.005
	Cp^2	-8.459	2.451	-3.452	0.026
Y_2	Constant	-161.589	23.573	-6.855	0.002
	Cg	29.803	3.121	9.550	0.001
	Cp	15.889	3.724	4.267	0.013
	Cg^2	-0.781	0.102	-7.689	0.002
	Cg x Cp	-0.713	0.239	-2.978	0.041

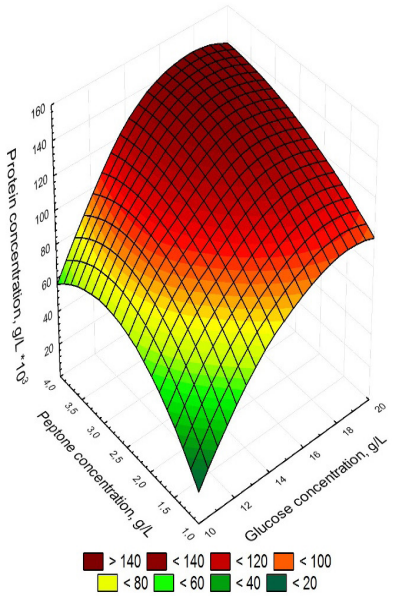

Figure 1. Relation of the concentration of protein in the extract from the mycelium biomass of *T. viride* from the concentration of glucose and peptone in the nutrition medium

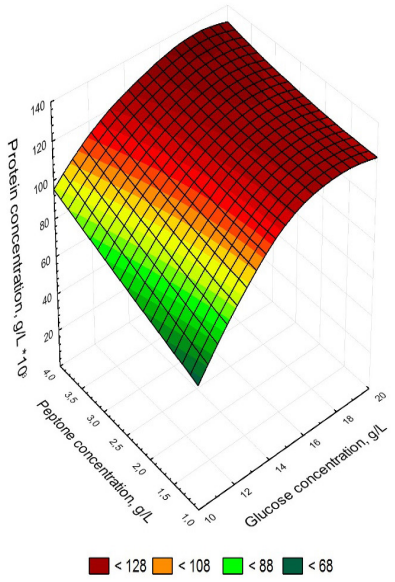

Figure 2. Relation of the concentration of protein in the extract from the native liquid of *T. viride* from the concentration of glucose and peptone in the nutrition medium

Increasing the concentration of the peptone in the medium contributes to an increase in the concentration of synthesized protein in the native liquid (Y_1), but starting from a certain point reduces the protein content in biomass (Y_2). Accordingly, for further culturing of the producer the medium with optimal concentration of glucose (17.5 - 20 g/L) was and peptone (4 g/L) was selected.

3.2 Effect of aeration of the culture medium on protein biosynthesis

A great influence on the biosynthesis of hydrophobins can be provided by the degree of aeration of the culture medium. We studied three aeration regime in which the rate of oxygen dissolution in the medium was 4.4 g/(L·h), 3.0 g/(L·h) and 1.8 g/(L·h). Extraction was carried out from the fungus culture on days 2, 3 and 4 of growth. The results are shown in Tables 3 - 5.

Table 3. Yield of surfactant proteins at culturing of *T. viride* at oxygen dissolution rate of 4.4 g/(L·h)

Parameters	2 day	3 day	4 day
Biomass, g/L	5.3	9.0	-
$C_{protein}$, mg/g of dry biomass	5.9	15.5	-
$C_{protein}$, mg/L of native liquid	47.2	110.6	220.3
$C_{protein}$, mg/L of culture liquid	78.5	250.1	220.3

Table 4. Yield of surfactant proteins at culturing of *T. viride* at oxygen dissolution rate of 3.0 g/(L·h)

Parameters	2 day	3 day	4 day
Biomass, g/L	4.5	8.3	-
$C_{protein}$, mg/g of dry biomass	6.2	16.1	-
$C_{protein}$, mg/L of native liquid	43.7	113.9	218.2
$C_{protein}$, mg/L of culture liquid	71.6	247.5	218.2

Table 5. Yield of surfactant proteins at culturing of *T. viride* at oxygen dissolution rate of 1.8 g/(L·h)

Parameters	2 day	3 day	4 day
Biomass, g/L	3.1	5.3	4.1
$C_{protein}$, mg/g of dry biomass	0.9	6.9	7.3
$C_{protein}$, mg/L of native liquid	10.6	40.7	50.7
$C_{protein}$, mg/L of culture liquid	13.4	77.3	80.7

Based on the results, the highest protein concentration is observed in extracts from a culture liquid grown for 3 days at dissolution rate of oxygen 3.0 g/(L·h). As can be seen from the results, an increase in the aeration level from 3 to 4.4 g/(L·h) does not significantly affect either the accumulation of biomass or the amount of extracted proteins, but with a decrease in the aeration level, both values are reduced. Further work was carried out with a culture grown for 3 days at oxygen dissolution rate of 3.0 g/(L·h).

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

Hydrophobins can form stable foams and emulsions even at very low concentrations in solution. To evaluate the surface-active properties of hydrophobins, we determined their foam-forming and foam-stabilizing properties. As can be seen from the graph in Figure 3, the extract from *T. viride* allows to obtain stable foams. The losses of the air phase in the samples stabilized with the extract amounted to about 50% in 8 weeks. In the control samples the complete loss of the air phase was observed in a week. The ability of foaming and foam stabilizing properties of the hydrophobin-type proteins proved to be much higher than in food emulsifiers.

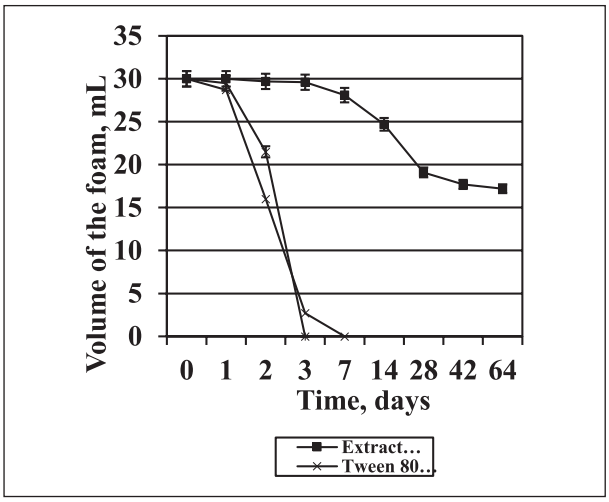

Figure 3. Foam-stabilizing ability of the obtained hydrophobin-type proteins and food foam stabilizers

4. Conclusions

- During the study cultivation conditions of fungus *T. viride* were selected to ensure the highest yield of hydrophobins. The composition of the nutrient medium was optimized and the aeration mode was selected.

- The extracts obtained from the biomass and from the native solution have a high surface activity. They allow to obtain resistant foams. Their foam stabilizing effect significantly exceeds the effect of food industrial emulsifiers.

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