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SHORT COMMUNICATION

TECHNOLOGY OF OBTAINING MILK-CLOTTING ENZYME FROM FUNGAL CULTURE *FUNALIA* SP. FOR APPLICATION IN CHEESE PRODUCTION

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Abstract

Cheese-making is a process known since ancient times. Traditionally, cheese preparation was based on coagulation of milk using rennet. Due to the high cost of rennet, an important task of the cheese industry is finding its alternatives. An important requirement for milk-clotting enzymes is low non-specific total proteolytic activity. One of the promising sources of milk-clotting enzymes are basidial fungi. Earlier, we found a high milk-clotting activity (MCA) of the fungus *Funalia* sp. The aim of our research was to find the optimal cultivation conditions for the fungus *Funalia* sp., which ensure the maximum yield of the milk-clotting enzyme. The fungus was submerge-cultured on a glucose-peptone nutrient medium for 7 days. The MCA determination of the native solution was carried out using the Kawai-Mukai method. To optimize the composition of the nutrient medium the method of full factorial experiment was used. According to the results of the study, a nutrient medium with a concentration of glucose and peptone of 15.5 and 3.6 g L^{-1} was selected. For further concentration and purification of the enzyme the method of ultrafiltration was used. As a result, an enzyme preparation with a high level of MCA (333.5 U mg^{-1}) and a low level of proteolytic activity (0.096 U mg^{-1}) was obtained. According to its characteristics, the enzyme is not inferior to commercial rennet and is promising for use in the food industry.

Keywords: milk-clotting, fungi, cheese making, submerge cultivation

Introduction

For hundreds of years rennet was used for clotting of milk in process of cheese making. Rennet is enzyme obtained from the stomach of young ruminants. Currently, due to the shortage of rennet and its high cost, it is widely practiced to use enzyme substitutes that are close in their action to the rennet. One of the common problems of rennet substitutes is high proteolytic activity. Too high proteolytic activity leads to the formation of a large number of low molecular weight peptides, which causes the appearance of bitterness in the cheese and reduces the yield of the final product. In this regard, enzymes with high proteolytic activity cannot be used to produce high-quality cheeses (Teply, 1980; Gudkov, 2004; Raposo, Domingos, 2008; He et al., 2011). The search for inexpensive and effective rennet enzyme substitutes is an important task in food biotechnology.

Fungi are a promising source of milk-clotting enzymes. Modern methods of submerge cultivation of fungi can significantly reduce the process of obtaining enzymes, as well as increase the yield of the final product.

The aim of our research was to find the optimal cultivation conditions for the fungus *Funalia* sp., which ensure the maximum yield of the milk-clotting enzyme.

Materials and methods

The object of our study was the culture of basidiomycete *Funalia sp*. This fungus was chosen as a result of screening a number of fungal cultures for milk-clotting activity. The fungus was submerge-cultured in Erlenmeyer flasks on a rotary shaker at a temperature of 28–30 °C. For the inoculum growing and submerge cultivation glucose-peptone nutrient medium was used. The duration of cultivation was 7 days. After cultivation, the native liquid solution was separated from the biomass by centrifugation with 6000 rpm (Centurion K240R, Centurion Scientific, USA). In the native solution the level of milk-clotting activity (MCA) and protein concentration (Lowry et al., 1951) were determined. To determine the milk-clotting activity, the Kawai-Mukai method was used (Kawai, Mukai, 1970). This method is based on determining the time of formation of the milk clot under the action of the enzyme. Calculation of MCA was conducted according to the following equation (Gagaoua, 2017):

$$MCA = \frac{2400 \times V}{t} \times v \qquad (1)$$

where:

MCA – milk-clotting activity, U mL^{-1},
t – time of milk clot formation, s;
V – the volume of milk, mL
v – amount of enzyme preparation, mL.

Protein biosynthesis is greatly influenced by the concentration of carbon and nitrogen sources in the medium, as well as their ratio. In this regard, the effect of the composition of the nutrient medium on the milk-clotting activity was studied. Method of multiple regression analysis was used to optimize the composition of the culture medium. Data processing was performed using the software package Statistica 10 of the company "Statsoft" (USA).

The concentrations of glucose (X_1) and peptone (X_2) were as variation factors. The parameter of optimization was the level of the milk-clotting activity of the native solution of the culture liquid (Y).

The optimization of the medium was carried out in the following intervals of variation of the main sources:

$$10 \text{ mg mL}^{-1} < C_{glucose} < 20 \text{ mg mL}^{-1}$$
$$2.5 \text{ mg mL}^{-1} < C_{peptone} < 5.5 \text{ mg mL}^{-1}.$$

These intervals were selected based on previously conducted experiments. The glucose and peptone concentrations in the nutrient medium were varied at three levels: a minimum (-), mean (0) and maximum (+). The remaining components of the medium were taken in the following concentrations (g L^{-1}): KH_2P0_4 – 0.6; K_2HP0_4 – 0.4; $MgSO_4$ – 0.05; NaCl – 0.5; yeast extract – 2.0.

The degree of aeration of the cultivation medium has a great influence on the biosynthesis of enzymes (Wang et al., 2005). We studied the effect of oxygen concentration on the synthesis of a milk-clotting enzyme when cultivated in 5 different modes of aeration of the medium. Fungus was cultured in 750 mL Erlenmeyer flasks with different quantity of liquid medium – 50, 75, 100, 125, 150 and 200 mL. The dissolution rate of oxygen rate was determined by the sulphite method (Yegorov, 1976).

Ultrafiltration was used to purify and concentrate the enzyme. For this the ultrafiltration cell with membrane "MIFIL-PA-20" (MIFIL, Belarus) was used. In the permeate the level of milk-clotting and total proteolytic activities (GOST 20264.2-88, 1988), as well as the protein concentration by Lowry method (Lowry et al., 1951), were determined. Obtained enzyme preparation was compared with commercial preparation of a rennet enzyme (Institute of Butter and Cheese making, Uglich city, Russia) by milk-clotting and total proteolytic activities.

Results and Discussion

In order to increase the milk-clotting activity of the fungus *Funalia* sp., we selected conditions of submerged cultivation.

We carried out the selection of concentrations of glucose and peptone in the medium to determine their effect on the level of the milk-clotting activity. The planning and the results of an experiment are shown in Table1.

Table 1

The influence of the concentration of glucose and peptone in the medium on the MCA level

No	Factor variations levels		Absolute values of glucose and peptone concentrations		MCA, U mL^{-1}
	X_1	X_2	$C_{glucose}$, g L^{-1}	$C_{peptone}$, g L^{-1}	
1	–	–	10	2.5	34.8±1.2
2	–	0	10	4.0	34.8±1.0
3	–	+	10	5.5	0
4	0	–	15	2.5	115.8±1.7
5	0	0	15	4.0	177.8±0,6
6	0	+	15	5.5	34.0±0.3
7	+	–	20	2.5	66.8±0,4
8	+	0	20	4.0	80.0±0.92
9	+	+	20	5.5	53.6±1.0

MCA – milk-clotting activity

According to the results of the experiment, a regression equation escribing the dependence of the MCA on the concentration of carbon and nitrogen sources in the medium was compiled:

$$Y = -733 + 78.44 \cdot X_1 + 140.48 \cdot X_2 - 2.56 \cdot X_1^2 - 20.68 \cdot X_2^2 + 0.72 \cdot X_1 \cdot X_2$$

Graphic dependence of milk-clotting activity on the composition of the medium is shown in Figure 1.

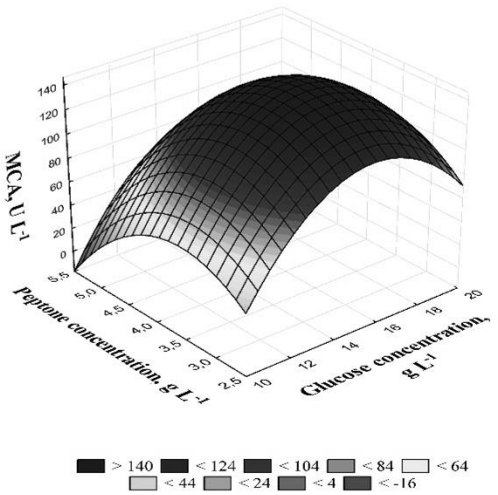

Figure 1. Dependence of the milk-clotting activity level of the fungus *Funalia* sp. on the concentration of glucose and peptone in the medium

Based on the results, the medium with a glucose concentration of 15.5 g L^{-1} and peptone concentration 3.6 g L^{-1} was selected for cultivating of the fungus *Funalia* sp.

We have studied the biosynthesis of the milk-clotting enzyme during the cultivation of the fungus in 5 different modes of aeration of the medium. The results are shown in Table 2.

Table 2

The influence of oxygen concentration in the medium on the synthesis of a milk-clotting enzyme

Nutrient medium volume, mL	Oxygen dissolution rate, g L^{-1} h^{-1}	MCA, U mL^{-1}
50	2.74	65.3±2.4
75	2.69	65.4±1.0
100	1.77	98.0±1.7
125	1.41	163.3±3.5
150	1.34	150.1±1.4
200	0.91	93.6±2.1

MCA – milk-clotting activity

From the results it can be seen that the highest level of MCA was observed during aeration, ensuring the solubility of oxygen at a rate of 1.41 g L^{-1} h^{-1}.

To study the dynamics of enzyme accumulation in the medium, we measured the MCA from 3 to 7 days of cultivation of the fungus. The results are shown in Figure 2.

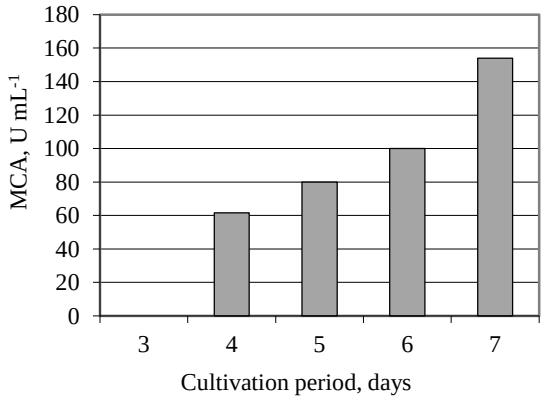

Figure 2. Dynamics of changes in milk-clotting activity (MCA) of the fungal culture *Funalia* sp.

The highest level of milk-coagulating activity was observed in the native solution of the culture liquid of the fungus on the 7th day of cultivation.

For further purification and concentration of the enzyme the ultrafiltration method was used. The comparative characteristics of our preparation and the standard rennet enzyme preparation is shown in Table 3.

Table 3

Comparative characteristics of the milk-clotting enzyme from the fungal culture *Funalia* sp. and commercial rennet preparation

Preparation	Characteristics		
	Specific milk-clotting activity, U mg^{-1}	Total proteolytic activity, U mg^{-1}	Ratio MCA:PA
Preparation from the fungal culture *Funalia sp.*	333.5±1.0	0.096±0.018	3473:1
Standard rennet preparation*	291.2±0.4	0.082±0.020	3551:1

MCA : PA – the ratio of specific milk-clotting activity and total proteolytic activity.

* Institute of Butter and Cheese making, Uglich city, Russia; GOST 9225-84.

From the results it can be seen that the milk-clotting enzyme preparation obtained from the fungal culture *Funalia* sp. has a high milk-clotting activity and a low total proteolytic activity. According to its specific milk-clotting and total proteolytic activities, it is not inferior to commercial rennet preparation. The level of milk-clotting activity is comparable to the level of activity of other coagulants used in the cheese-making industry (de Silva et al. 2013).

Conclusions

As a result of the study, the cultivation conditions of the fungus *Funalia* sp., providing the highest yield of the milk-coagulating enzyme, were selected (cultivation on the medium with glucose concentration of 15.5 g L^{-1} and peptone concentration 3.6 g L^{-1}, with oxygen dissolution rate of 1.41 g L^{-1} h^{-1} during 7 days). The enzyme preparation, which has a high milk-clotting activity and low proteolytic activity, and is not inferior in its characteristics to commercial enzyme was obtained. This preparation is promising for use in the food industry.

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