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CONSERVATION OF LIQUID CULTURE, CONTAINING LAMINARINASE AND LICHENASE, PRODUCED FROM TRICHODERMA SP. 405 STRAIN M_7

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ABSTRACT

Trichoderma sp. 405 Strain M_7 produces laminarinase and lichenase, which pertain to the β-glucanases. The conservation of the liquid culture, containing both enzymes in a fridge (4-8°C) immediately after the cultivation was investigated. Both enzyme activities were determined after freezing of the liquid culture at -20 ÷ -25°C followed by unfreezing and conservation at 20°C . The laminarinase and lichenase activities were determined also by freezing of the liquid culture at -20 ÷ -25°C followed by unfreezing and preservation in a fridge (4-8°C). The conservation by lyophilization of the liquid culture, produced from Trichoderma sp. 405 Strain M_7 was investigated. It was established that both enzyme activities remain highest after conservation of the liquid culture by the process of lyophilization.

Keywords: conservation, laminarinase, lichenase, freezing, unfreezing, lyophilization.

INTRODUCTION

Among the microorganisms, synthesizing enzymes that catalyse the degradation of β-D-glucans (b-glucanases) are the strains of genus *Trichoderma- Trichoderma reesei* [1], *Trichoderma harzianum* [2], *Trichoderma viride* [3], *Trichoderma koningii T 199* [4], *Trichoderma sp. GXC* [5] and *Trichoderma asperellum* [6]. The β-glucanases belong to the group of hemicellulases, in which include also the laminarinase and the lichenase. The laminarinase [E.C. 3.2.1.39] hydrolyses 1,3-β-D-glycosidic bonds into 1,3-β-D-glucans. Its substrate is the polysaccharide laminarin [7]. The lichenase [E.C. 3.2.1.73] attacks β-D-glucans, containing 1,3- and 1,4-bonds. Its substrates are the lichenin and the β-D-glucans in the cereals. The contents of β-glucans in dufferent Bulgarian oats varieties ranges from 2,71 % to 4,26 % [8] and the contents of β-glucans in dufferent Bulgarian barley varieties is in the range of 3,15 %-6,08 % [9]. The level of β-glucans in traditional USA oats varietes is around 4,4 - 4,9 % and in some special selected varietes reaches to 7,5 -7,8 % [10].

Improvement of the organoleptic wine characteristics, its clarifying and filtering were determined when β-glucanases, produced from *Trichoderma*, were added in the production of wine. More effective degradation of glucans and viscosity decreasing of brewing wort and beer were established when enzymes with endo-β-1,3(4)-glucanase and xylanase activities were used in the production process of beer [10]. Adding β-glucanases in the animal food helps for its better conversion and absorption and for mass increase of the animals, respectively [11-13].

Different methods exist for preservation of the bio-objects and their vitality. New methods are looking for conserving the enzyme activities for a longer period of time. Some enzymes are successfully preserved in a fridge (4-8°C) for months. The enzymes conservation at 20°C leads to fast decreasing of their activity. Some enzymes preserve their activity stable after conservation in a frozen state at -20 or -50°C. The most effective method, which guarantees long term preservation of enzymes is lyophilization [14]. It is a process of taking away the water substance of the

solid matrix of moisture - containing materials by sublimation in vacuum [15].

The aim of this research is to investigate the conservation of liquid culture, containing laminarinase and lichenase in a fridge (4-8°C), its preservation in a frozen state and following unfreezing at 20°C and in a fridge (4-8°C), as well as its conservation by lyophilization.

EXPERIMENTAL

The *Trichoderma sp. 405 Strain* M_7 was bought from the National bank for industrial microorganisms and cell cultures - NBIMCC.

A spore inoculation product was received from the strain producer cultivated on potato-dextrose agar (PDA- Biokar Diagnostics) slants.

Mandel's sowing media contained: NH_4Cl- 1.00 g/l; Urea- 0.30 g/l; KH_2PO_4- 2.00 g/l; $(NH_4)_2SO_4$- 1.40 g/l; $MgSO_4.7H_2O$- 0.30 g/l; $CaCl_2.2H_2O$- 0.40 g/l; Corn extract- 10.00 ml/l; Glucose- 20.00 g/l.

100 ml Mandel's sowing media was inoculated with ~ 1 cm^2 agar block from the spore sowing material.

Mandel's fermentation media contained: NH_4Cl- 1.00 g/l; Urea- 0.30 g/l; KH_2PO_4- 2.00 g/l; $(NH_4)_2SO_4$- 1.40 g/l; $MgSO_4.7H_2O$- 0.30 g/l; $CaCl_2.2H_2O$- 0.40 g/l; Corn extract- 0.1 %; Glucose- 0.2 %; Wheat bran- 1 %.

50 ml Mandel's fermentation media was inoculated with 10 ml sowing material [16]. The cultivation of the Trichoderma strain was carried out by the method of deep fermentation in 500 ml flasks on a shaker "Inkubations - Schüttelschrank BS-4 B.Braun" (220 rpm) for 168 hours at 28°C. The liquid culture was filtered through filter paper and the laminarinase and lichenase activities were analysed every 24 hours.

The activity of both enzymes was obtained spectrophotometrically by measuring the reducing groups, which are released when hydrolyzing their substrates- laminarin and lichenin, respectively. An Unicam SP 1800 Ultraviolet spectrophotometer was used. For determination of enzyme activities was applied Somogyi-Nelson's method [17, 18]. One international unit (IU) of laminarinase activity is the amount of enzyme which catalyses the transformation of 1mmol of reducing sugars in 1 cm^3 per minute at 40 °C in a citric phosphate buffer of pH 5.00. One international unit (IU) of lichenase activity is the amount of enzyme which catalyses the transformation of 1mmol of reducing sugars in 1 cm^3 per minute at 40°C in an acetate buffer of pH 4.00.

The laminarinase and lichenase activities of the samples, taken immediately after the fermentation, were analysed after 35 days conservation in a fridge (4-8°C) by the above mentioned Somogyi-Nelson's method [17, 18].

Every 24 hours during the cultivation were taken samples, which were frozen at -20 ÷ -25°C. They were conserved at -20 ÷ -25°C for 12 months. After that, these samples were unfrozen at 20°C and in a fridge (4-8°C), and both enzyme activities were again determined by the above mentioned Somogyi-Nelson's method [17, 18]. The protein contents in the liquid culture in mg ml^{-1} was obtained by the spectrophotometric method (OD_{280}/OD_{260}) [19]. The lyophilization was carried out in a freeze-drying installation „HOCHVAKUUM" TG-16. The freezing process of the liquid culture was done at minus 35 ± 2°C, without addition of cryoprotectants. Lyophilization's duration was 28 hours. The cooling temperature of the desublimator was minus 75°C and the temperature of the plates was minus 35°C. The enzyme activities were recovered by adding 10 ml distilled water to the lyophilized liquid culture and then they were analysed by the above mentioned Somogyi-Nelson's method [17, 18].

RESULTS AND DISCUSSION

The conservation of the liquid culture, produced from *Trichoderma sp. 405 Strain* M_7 was investigated. The strains from genus *Trichoderma* are mainly producers of cellulases, but they also produce hemicellulases. It was determined that the enzyme activity of the β-glucanase enzymes produced from *Trichoderma sp. 405 Strain* M_7 is lower than the β-1,3-1,4-glucanase activity of a recombinant yeast, the activity of which, for example, reaches up to 45,1 IU/ml [20].

Protein contents in the liquid culture

The protein contents in the liquid culture was determined during the fermentation of *Trichoderma sp. 405 Strain* M_7. It was established that during the cultivation the protein contents was increased. At the 24th hour it was an average of 4,553 mg ml^{-1}. At the 120th hour the protein content reached 6,915 mg ml^{-1} and at the 144th hour of the fermentation, the protein contents is highest- 8,445 mg ml^{-1}.

Table 1. Laminarinase activity of the liquid culture, produced during cultivation of Trichoderma sp. 405 Strain M_7 and conserved in a fridge (4-8°C) immediately after taking the samples.

Sample taken at hour	Laminarinase activity IU ml^{-1}							
	During the cultivation		After 7 days conservation at 4-8°C		After 21 days conservation at 4-8°C		After 35 days conservation at 4-8°C	
24 th	0,127	0,017	0,124	0,008	0,122	0,011	0,121	0,008
48 th	0,248	0,016	0,241	0,012	0,239	0,013	0,235	0,010
72 nd	0,300	0,003	0,292	0,008	0,289	0,013	0,284	0,011
96 th	0,343	0,021	0,335	0,013	0,329	0,011	0,326	0,011
120 th	0,456	0,013	0,445	0,020	0,439	0,018	0,433	0,010
144 th	0,520	0,027	0,510	0,009	0,500	0,011	0,493	0,027
168 th	0,458	0,009	0,445	0,012	0,441	0,015	0,436	0,008

Total number of trials (n) = 5.

Table 2. Lichenase activity of the liquid culture, produced during cultivation of Trichoderma sp. 405 Strain M_7 and conserved in a fridge (4-8°C) immediately after taking the samples.

Sample taken at hour	Lichenase activity IU ml^{-1}							
	During the cultivation		After 7 days conservation at 4-8°C		After 21 days conservation at 4-8°C		After 35 days conservation at 4-8°C	
24 th	0,043	0,001	0,041	0,001	0,039	0,001	0,037	0,001
48 th	0,061	0,002	0,057	0,001	0,055	0,001	0,053	0,001
72 nd	0,070	0,002	0,066	0,001	0,064	0,001	0,061	0,001
96 th	0,085	0,002	0,080	0,001	0,077	0,001	0,073	0,001
120 th	0,096	0,002	0,091	0,002	0,087	0,002	0,083	0,001
144 th	0,107	0,003	0,101	0,001	0,096	0,001	0,092	0,001
168 th	0,095	0,001	0,090	0,001	0,086	0,001	0,083	0,001

Total number of trials (n) = 5.

Conservation of the liquid culture in a fridge (4-8°C)

The conservation in a fridge (4-8°C) of the liquid culture, produced during the cultivation of *Trichoderma sp. 405 Strain M_7* was investigated. The laminarinase and lichenase activities were determined during the preservation of the liquid culture for 35 days in a fridge (4-8°C). Table 1 presents the analysis for the laminarinase activity during the fermentation process and after the conservation of the liquid culture in a fridge (4-8°C). Table 2 summarizes the results for the lichenase activity during the cultivation and after the preservation of the liquid culture in a fridge (4-8°C). The analysis showed that after 35 days conservation of the liquid culture in a fridge (4-8°C) both enzyme activities were decreased. However, this method of preservation was accompanied with the appearance of secondary fermentation processes. This resulted into an unpleasant odour of the conserved samples, which would hinder putting them into food products.

Conservation of the liquid culture in a frozen state

Samples of the liquid culture were frozen every 24 hours at -20 ÷ -25°C during the process of cultivation of *Trichoderma sp. 405 Strain M_7*. They were conserved at -20 ÷ -25°C for 12 months after that they were

Table 3. Laminarinase and lichenase activities of the liquid culture, produced during cultivation of Trichoderma sp. 405 Strain M_7 after 12 months conservation in a frozen state at -20 ÷ -25°C and following unfreezing and conservation at 20°C.

Sample taken at hour	Laminarinase activity IU ml^{-1}						Lichenase activity IU ml^{-1}					
	During the cultivation		1 hour after unfreezing		7 days after conservation at 20°C		During the cultivation		1 hour after unfreezing		7 days after conservation at 20°C	
24 th	0,127	0,017	0,109	0,005	0,097	0,004	0,043	0,001	0,032	0,001	0,031	0,001
48 th	0,248	0,016	0,210	0,008	0,191	0,004	0,061	0,002	0,045	0,001	0,044	0,001
72 nd	0,300	0,003	0,259	0,006	0,230	0,004	0,070	0,002	0,052	0,001	0,050	0,001
96 th	0,343	0,021	0,292	0,006	0,265	0,006	0,085	0,002	0,063	0,001	0,060	0,001
120 th	0,456	0,013	0,390	0,005	0,354	0,006	0,096	0,002	0,071	0,001	0,069	0,001
144 th	0,520	0,027	0,446	0,018	0,400	0,005	0,107	0,003	0,080	0,002	0,076	0,001
168 th	0,458	0,009	0,394	0,011	0,348	0,005	0,095	0,001	0,070	0,001	0,066	0,002

Total number of trials (n) = 5.

Table 4. Preservation of laminarinase and lichenase activities in % of the liquid culture, produced during cultivation of Trichoderma sp. 405 Strain M_7 after 12 months conservation in a frozen state at -20 ÷ -25°C and following unfreezing and conservation at 4-8°C.

Sample taken at hour	% of laminarinase activity preservation			% of lichenase activity preservation		
	After conservation 7 days at 4-8°C	After conservation 30 days at 4-8°C	After conservation 55 days at 4-8°C	After conservation 7 days at 4-8°C	After conservation 30 days at 4-8°C	After conservation 55 days at 4-8°C
24 th	96,06	93,70	89,76	86,05	69,77	65,12
48 th	97,18	93,15	90,32	85,25	70,49	67,21
72 nd	97,33	92,67	90,33	85,71	70,00	67,14
96 th	96,21	92,71	89,50	85,88	69,41	67,06
120 th	97,59	92,54	91,01	84,38	70,83	66,67
144 th	96,73	93,08	90,19	84,11	70,09	67,29
168 th	96,94	93,01	90,17	85,26	70,53	67,37

Total number of trials (n) = 5.

unfrozen at 20°C and in a fridge (4-8°C) and the laminarinase and lichenase activities were determined. In Table 3 are presented the results for the enzyme activities during the fermentation and after the unfreezing of the liquid culture at 20°C. The analysis shows that both activities were fastly decreasing when the liquid culture was unfrozen and conserved at 20°C. The percentage of laminarinase and lichenase activities preservation during the conservation of liquid culture in a frozen state and its following unfreezing at 20°C was calculated. One hour after the unfreezing of the samples the laminarinase activity decreases on average to 84,68 % - 86,33 % and after 7 days conservation at 20°C it decreases to an average of 75,98 %-77,63 %. The lichenase activity one hour after the unfreezing of the liquid culture at 20°C decreases on average to 73,68 % -74,77 % and after 7 days preservation at 20°C it decreases to an average of 69,47 % - 72,13 %. The conservation at 20°C is also accompanied with the appearance of secondary fermentation processes and therefore is not suitable for enzyme preservation.

Table 4 shows the percentage of enzyme activities preservation during the conservation of the liquid culture in a frozen state and its following unfreezing and continuous preservation in a fridge (4-8°C). The laminarinase activity on the 7th day after the unfreezing

Table 5. Preservation of laminarinase and lichenase activities in % of the liquid culture after lyophilization.

Sample taken at hour	% of laminarinase activity preservation			% of lichenase activity preservation		
	Immediately after lyophilization	1 month after lyophilization	11 months after lyophilization	Immediately after lyophilization	1 month after lyophilization	11 months after lyophilization
24 th	98,43	98,43	91,34	97,67	97,67	88,37
48 th	98,79	98,39	91,94	98,36	96,72	88,52
72 nd	98,33	98,33	92,00	98,57	97,14	87,14
96 th	97,96	97,67	92,13	98,82	98,82	87,06
120 th	98,68	98,46	91,45	97,92	97,92	88,54
144 th	97,88	97,69	91,73	99,07	99,07	87,85
168 th	97,60	97,60	93,01	98,95	97,89	88,42

Total number of trials (n) = 5.

and conservation in a fridge (4-8°C) was preserved on average at 96,06 %-97,59 %, whereas the lichenase activity decreased to an average of 84,11 % -86,05 %. The laminarinase activity after unfreezing and conservation for 55 days in a fridge (4-8°C) was decreased to an average of 89,50 %-91,01 %, whereas the lichenase activity was decreased to an average of 65,12 %-67,37 %.

Conservation of the liquid culture by lyophilization

The conservation by lyophilization of the liquid culture, produced from *Trichoderma sp. 405 Strain M_7* was investigated. The enzyme activities were determined immediately after the lyophilization, 1 month, and 11 months after the lyophilization. The results show that both enzymes have highest activity levels at the 144th hour of cultivation. Table 5 presents the percentage of laminarinase and lichenase enzyme activities conservation of the liquid culture after the process of drying. The laminarinase activity at the 144th hour immediately after the lyophilization of the liquid culture was an average of 141,39 IU g^{-1}. One month after the process of drying it was preserved, and was on average 141,11 IU g^{-1}. The laminarinase activity 11 months after the conservation in a lyophilic state was an average of 132,50 IU g^{-1} i.e., it was preserved to an average of 91,73 %. The lichenase activity determined on samples at the 144th hour of the fermentation, immediately after lyophilization of the liquid culture, was an average of 29,44 IU g^{-1}. One month after conservation in a lyophilic state it was completely preserved at an average of 29,44 IU g^{-1}. 11 months after the process of drying it was an average of 26,11 IU g^{-1} and therefore it was conserved at an average of 87,85 % as compared to the lichenase activity determined during the cultivation of *Trichoderma sp. 405 Strain M_7*.

CONCLUSIONS

During the fermentation both enzymes have highest activity levels at the 144th hour of *Trichoderma sp. 405 Strain M_7* cultivation. The results from this research for laminarinase and lichenase activities show that both activities were preserved at highest degree after continuous conservation of the liquid culture in a lyophilic state. It was established that the laminarinase activity was preserved between 91,34 % and 93,01 % after conservation for 11 months in a lyophilic state in comparison to the activity determined during the fermentation. The lichenase activity 11 months after the lyophilization was preserved in the range of 87,06 % - 88,54 % as compared to the activity, determined immediately after the cultivation of the strain. Cryoprotectants were not used in the freeze-drying process which made it less expensive.

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