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CHARACTERISATION OF COLLAGENOLITYC ACTIVITY OF *Coprinus* spp.

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Abstract

In this research the screening of several higher fungi - basidiomycetes cultures for the presence of collagenolytic activity was carried out. The highest collagenase activity was detected in submerged culture of higher fungus Coprinus lagopides. The enzyme preparation of collagenase was isolated from culture liquid of this producer. The optimum temperature and pH of the collagenolytic activity of the enzyme preparation were determined. The selection of nitrogen and carbon sources and ratios of carbon and nitrogen sources in the composition of nutrient mediums was carried out in order to increase the collagenase activity of fungus submerged culture. In addition to the collagenase activity of producers culture the amount of accumulated biomass, pH and protein concentration in the culture liquid of basidiomycete were also determined.

Key words*: submerged cultivation, basidiomycetes, Coprinus lagopides, collagenase, collagenolytic activity.*

INTRODUCTION

Proteolytic enzymes today make up about half of the production of enzyme preparations in the world market. Their efficiency surpasses the synthetic catalysts considerably. They are highly specific against their substrates and accelerate rigorously defined chemical reaction without of byproducts formation. These characteristics of proteolytic enzymes explain their widespread use in the pharmaceutical industry, food industry and other industries (De Souza et al., 2015).

As is well known, one of the major problems nowadays is the development of waste-free technologies. In the food industry it can be solved by maximizing the involvement of protein secondary resources and wastes of meat industry. Ensuring of strong growth in this sphere could be achieved by the development of new technologies of production of highly efficient enzymes preparations.

Practical application of enzymes preparations for the treatment of meat industry wastes shows that not all of enzymes which have a high proteolytic activity give the required effect (Anzani et al., 2017). This is due to the presence of proteins in such wastes which are difficult to cleave by digestive enzymes. One of the main of such hardly cleavable proteins is collagen. One of the most effective ways to solve this problem is the use of collagenase - unique enzyme which can hydrolyze the specific peptide bonds in collagen that are resistant to hydrolysis by other enzymes (Anzani et al., 2017).

It should be noted that the field of application of collagenolytic enzymes is not limited to the meat industry. Due to its ability to cleave collagen they may be used in the medical industry, as well as in cosmetology (Alipour et al., 2016). They are part of many drugs intended for wound healing, prevention and treatment of post-surgical, traumatic and post-burn scars and adhesions.

Collagenases which are used nowadays have some significant drawbacks. The most well-known producer of collagenase - the bacterium *Clostridium hystoliticum* is the causative agent of gas gangrene. Therefore, the increased requirements are made to the safety at all stages of production and sales of this enzyme (Demina, 1996). Another well-known enzyme with collagenolytic activity is the crab collagenase which is isolated from the king crab hepatopancreas (Rudenskaya, 2003). It is safe for humans, but its production has seasonal nature and its enzyme preparations vary significantly in the degree of purity and level of activity.

Thus, the actual problem is to find the collagenase producers which wouldn't have the disadvantages referred above.

To date higher fungi - basidiomycetes are the promising objects of biotechnology. It is known that basidiomycetes are producers of a number of biologically active substances such as amino acids, enzymes, polysaccharides, etc. (Peralta et al., 2017). The method of deep cultivation of fungi is used in modern biotechnology production. It gives the opportunity to culture producers under controlled conditions and environments which leads to a relative standardization of products and the possibility of enzyme production in commercial scale.

The main aim of this work was to study the collagenolytic activity of several higher fungi - basidiomycetes.

MATERIALS AND METHODS

The objects of study were basidiomycetes strains from the collection of Microbiological Synthesis Technology department of St. Petersburg State Institute of Technology (SPbSIT). Fruiting bodies of these fungi were collected in the North-West of Russia. They were characterized and isolated in pure cultures. The preliminary screening for the production of collagenolytic enzyme was conducted among 35 cultures of basidiomycetes belonging to the genus *Bjerkandera*, *Ceriporiopsis*, *Cerrena*, *Coprinus*, *Coriolus*, *Corticium*, *Flammulina*, *Funalia*, *Fomes*, *Fomitopsis*, *Ganoderma*, *Grifola*, *Hapalopilus*, *Hypsiziyns*, *Irpex*, *Lentinus*, *Panus*, *Phellinus*, *Pleurotus*, *Pyptoporus* and *Trametes*.

Chemicals and reagents

Anhydrous glucose, molasses, dairy whey, wheat flour, beer wort, dry fermented peptone for bacteriological purposes, urea, ammonium nitrate and ammonium citrate, sodium chloride, potassium dihydrogen phosphate, potassium hydrogen phosphate, magnesium sulfate, calcium chloride and calcium carbonate, iron sulfate heptahydrate, zinc sulfate heptahydrate and fodder yeast extract (all from JSC "Reactive", St. Petersburg, Russia) were used for the preparation of nutrient mediums.

Hydroxide, sodium citrate, sodium carbonate, copper sulfate pentahydrate, bovine serum albumin and Folin-Ciocalteu reagent were used in determining of protein in the culture liquid.

Disodium hydrogen phosphate dihydrate, sodium chloride, calcium chloride hexahydrate, isopropanol (2-Propanol), ninhydrin, L - leucine and collagen were used to determine collagenolytic activity.

In the preparation of the phosphate buffer solutions potassium phosphate monobasic, sodium phosphate dibasic, sodium hydroxide and hydrochloric acid were used.

Submerged cultivation

Cultivation of basidiomycetes was carried out on the mediums with different ratios of carbon and nitrogen sources in nutrient medium composition (Table 1).

Table 1. The ratios of carbon and nitrogen sources in nutrient medium composition

Component of nutrient medium	Ratio of carbon and nitrogen sources								
	1.2:1	1.5:1	3:1	4:1	5:1	6:1	7:1	10:1	15:1
Carbon source, g/l	10	10	10	10	10	10	10	10	10
Nitrogen source, g/l	8.33	6.67	3.33	2.5	2	1.66	1.43	1	0.67

The mineral composition of mediums, g/l: NaCl - 0.5, KH_2PO_4 - 0.6, K_2HPO_4 - 0.4, $MgSO_4$ - 0.5, $CaCl_2$ - 0.05, $FeSO_4 \times 7H_2O$ - 0.005, $ZnSO_4 \times 7H_2O$ - 0.001. Fodder yeast extract was added as the growth factor to all mediums in the amount of 2 gram per 1 liter of medium.

At the stage of screening of collagenolytic cultivation of enzyme producers was carried out on the glucose-peptone nutrient mediums.

Cultures of higher fungus were grown for 7 days. Samples of culture liquid were taken on the 3, 4, 5, 6 and 7th days of cultivation. pH and the amount of accumulated biomass were determined. The resulting (native) solution was used for further study and determination of protein concentration and the total collagenolytic activity.

In the next stage of our study it was investigated the effect of different nitrogen and carbon sources and their ratios (Table 1) on collagenase activity of submerged culture of higher fungus which had the highest level of collagenolytic activity according to the results

of screening. The study was conducted in two steps:

1) culturing of the macromycete in mediums containing various nitrogen sources and ratios of carbon and nitrogen sources in the composition of nutrient medium, wherein the carbon source was glucose;

2) growing of the basidiomycete in mediums containing different carbon sources and ratios of carbon and nitrogen sources in the composition of the nutrient medium, wherein the nitrogen source was the one which was determined on the first step and which provide the highest level of collagenase activity of the fungus culture.

We used peptone, urea, ammonium nitrate and ammonium citrate as the nitrogen sources in the composition of the nutrient mediums for the basidiomycete cultivation.

Carbon sources were molasses, dairy whey, wheat flour and beer wort.

Determination of the amount of accumulated biomass

The culture liquid was separated into the wet biomass and native solution by filtration through a paper filter. The wet biomass was dried at 50°C. Native solution subsequently used to determine the pH, protein concentration and collagenolytic activity of macromycete submerged culture.

Determination of the protein concentration in the native solution

To determine the amount of protein formed during submerged cultivation required for the subsequent calculation of the specific activity of the enzyme the method of Lowry was used (Lowry, 1951).

Determination of the collagenolytic activity of native solution

Collagenase activity was determined by ninhydrin method (Rosen, 1957). The method of the activity measuring is based on the ability of the enzyme to break down the collagen with the release and transfer to a solution the products of hydrolysis. Concentration of released products is determined spectrophotometrically. The amount of enzyme (in micrograms) which being exposed to collagen for 1 hour obtains the hydrolysis products equivalent to 1 μg of L-leucine under standard test conditions was adopted as the unit of collagenolytic activity (UCA). Collagenolytic activity in 1 ml of native solution $A_к$ was calculated by the equation (1):

$$A_к = a \times V \times 2.5, \quad (1)$$

where: a is the difference in concentration of hydrolysis products in the test Ci and control Co solutions (μg/ml) and V is the volume of the test solution (ml).

Specific collagenase activity was calculated as the ratio of total collagenolytic activity to the protein concentration in the producer culture liquid.

Enzyme preparation

The enzyme preparation was obtained from the deep culture of basidiomycete which had a maximum level of collagenolytic activity according to the results of screening.

The culture liquid of basidiomycete was separated into native solution and wet biomass by filtration through a paper filter.

Concentration and purification of native solution from the low molecular weight impurities were carried out by ultrafiltration. The process was led on the ultrafiltration apparatus of non-flowing type FK 02-200 having a volume of 200 ml with the membrane "MIFIL PA - 20" (Minsk, Belarus) at an operating pressure of 0.15 MPa. The resulting concentrate was dried by lyophilization.

Then the pH-optimum and temperature optimum of collagenase activity of the obtained enzyme preparation were determined.

Determination of the optimum pH of the enzyme preparation

To determine the optimum pH of collagenase activity the 1% preparation solutions in phosphate buffer solutions with pH value ranges from 4.8 to 8.0 were prepared. Then collagenolytic activity was determined for the resulting solutions.

Determination of optimum temperature of collagenase activity of the preparation

When determining the temperature optimum of the enzymatic activity 1% solution of the preparation in the buffer with optimum value of pH was prepared. Then collagenolytic activity was measured at temperatures ranging from 20 to 45℃.

Statistical analysis

Statistical analysis of the results was carried out by determination of the confidence intervals of the obtained values using the

Student's coefficient in the Microsoft Office Excel 2007. Deviations from the mean values considered statistically significant if the confidence level was greater than or equal to 95%. Number of replicates in all dimensions was varied (from 4 or more). The experimental results were expressed as mean value ± standard deviation.

RESULTS AND DISCUSSIONS

The screening of collagenolytic enzyme producers was conducted among 35 cultures of basidiomycetes from the collection of Microbiological Synthesis Technology department of SPbSIT. It has shown that the highest level of collagenase activity has a deep culture of higher fungus *Coprinus* spp. (*Coprinus lagopides*) on the 7th day of cultivation in the glucose-peptone nutrient medium with a ratio of carbon and nitrogen sources 1.5:1. The literature reports as highest collagenase producers the fungal gender belonging to *Aspergillus, Cladosporium, Penicillium* and *Alternaria* (Wanderley et al., 2017). Few reports on collagenase are reported in relation to the basidiomycetaes, and the most studied gender was *Pleurotus* (Hu et al., 2017). Regarding *Coprinus* spp., for the enzymatic activity, mainly it was reported for its amylolytic activity (Frantz et al., 2019). Also, some milk-clotting properties, due to its protelotyic activity was reported (Shamtsyan et al., 2014).

The culture liquid of basidiomycete *Coprinus* spp. was concentrated in 2.5 times by ultrafiltration. The level of purification was 2 times. The concentrate was dried by lyophilization. The specific activity of obtained enzyme preparation of collagenase is 1242 UCA/μg of protein.

For the obtained preparation the optimum pH and the temperature of the enzymatic activity were determined as folows: the optimum pH is in the range of pH 7.5-7.6 (Figure 1), optimum temperature lies in the range of 35-38℃ (Figure 2).

On the first step of study of the effect of different carbon and nitrogen sources and their ratios on collagenase activity of deep culture of higher fungus *Coprinus* spp. was performed the cultivation of macromycete on the mediums containing different nitrogen sources and ratios of carbon and nitrogen sources in the composition of the nutrient medium, wherein the source of carbon was glucose.

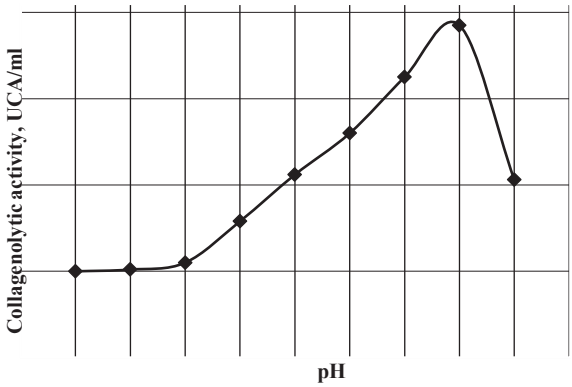

Figure 1. Dependence of collagenolytic activity on the pH of the preparation solution

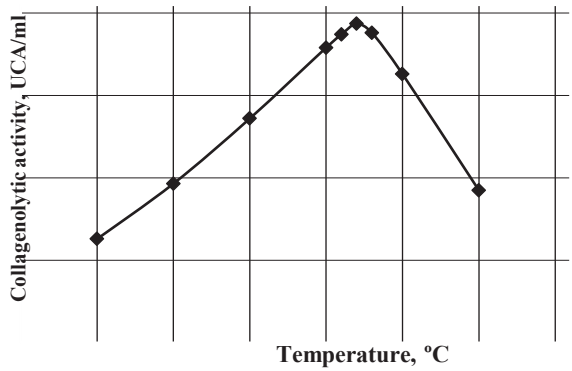

Figure 2. Dependence of collagenolytic activity on the temperature of preparation solution

According to the data obtained in the first step of study it can be concluded that culturing on the mediums with urea is accomplished with strong alkalization while the use of ammonium nitrate as a nitrogen source leads to strong acidification of the mediums. pH of submerge culture obtained in the medium containing ammonium citrate was slightly acidic. Cultivation of fungi in glucose-peptone nutrient mediums accompanied by a transition pH from a weakly acidic to weakly alkaline.

The greatest amount of fungal biomass accumulates on the 4th day of cultivation in glucose-peptone medium with the ratio of carbon and nitrogen sources 1.5:1 while the smallest amount of biomass accumulates on the mediums with urea.

The results of the quantitative determination of collagenolytic activity of macromycetes

submerged culture in the first step of the study are represented on the Figures 3-6.

Figures 3-6 shows that the basidiomycete deep culture produces the highest level of total collagenolytic activity on the 7th day of cultivation in the medium where urea is used as nitrogen source at a ratio of carbon and nitrogen sources 14:1 and 15:1.

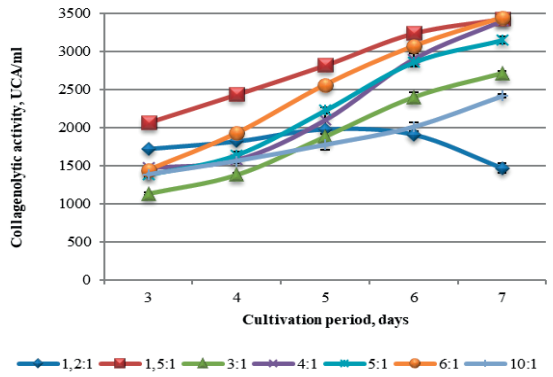

Figure 3. Collagenolytic activity level of basidiomycete *Coprinus* sp. deep culture in case of using glucose-peptone nutrient mediums

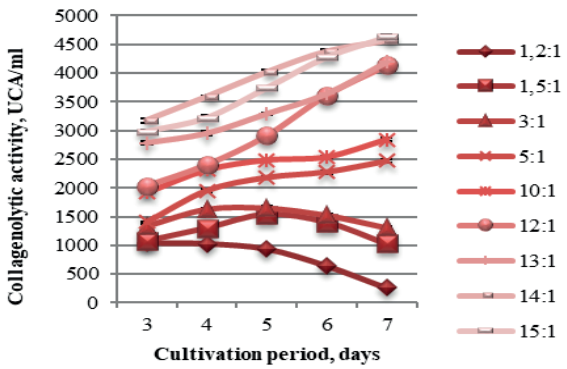

Figure 4. Collagenolytic activity level of macromycete deep culture in case of growth in the mediums with urea

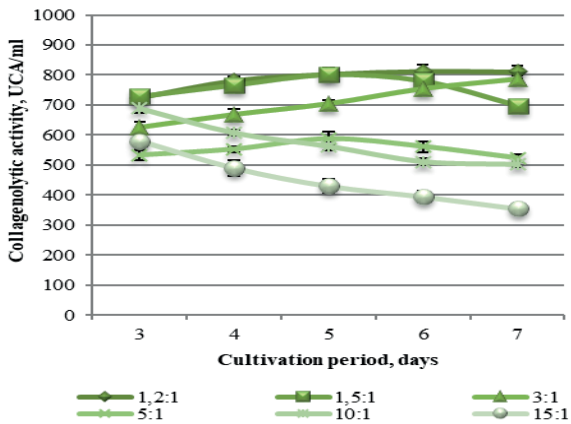

Figure 5. Collagenolytic activity level of producer culture in case of using mediums with ammonium nitrate

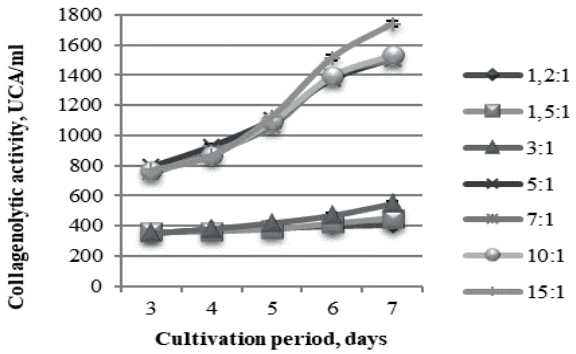

Figure 6. Collagenolytic activity level of basidiomycete deep culture in case of growth in the nutrient mediums with ammonium citrate

The maximum level of specific collagenolytic activity of basidiomycete *Coprinus* spp. culture was observed on the 7th day of cultivation in the medium with glucose and urea at a ratio of carbon and nitrogen sources 15:1.

Thus, for the subsequent study of the influence of different carbon sources in nutrient mediums composition on the enzymatic activity of producer deep culture urea was used as a nitrogen source.

According to the results obtained in the second step of the study the cultivation of macromycete in all carbon sources was accompanied by a strong medium alkalization, however, a lesser alkalization was registered in the mediums with beer wort.

The highest amount of accumulated biomass the fungus culture produces on the 4-5th days of growth in medium with beer wort at a ratio of carbon and nitrogen sources 1.5:1, but it is considerably lower than in the case of using glucose-peptone medium. The smallest amount of biomass the fungus culture accumulates on the 3-5th days of cultivation in medium with molasses at the ratios of carbon and nitrogen sources 10:1 and 15:1.

The results of the quantitative determination of collagenolytic activity of fungus submerged culture in the second step of the study are shown on the Figures 7-10.

Figures 7-10 shows that the highest total collagenolytic activity of basidiomycete *Coprinus* sp. deep culture is registered in case of using the nutrient medium where dairy whey is used as the carbon source.

In order to increase the collagenase activity of producer culture the pH of dairy whey mediums was changed by: (1) the adjustment

of mediums pH to 6.5 by 0.1M NaOH; (2) addition of calcium carbonate in the amount of 10 g/l.

The results of the quantitative determination of collagenolytic activity of the fungus deep culture in case of pH updating of dairy whey mediums are shown on the Figures 11 and 12.

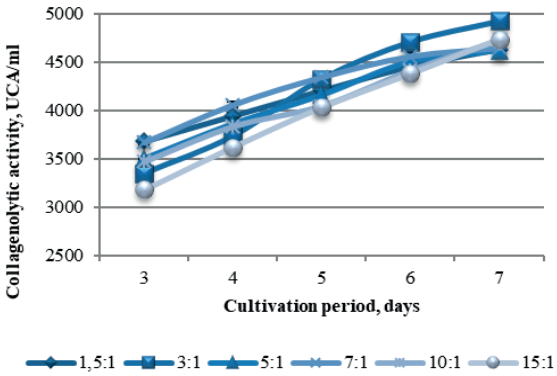

Figure 7. Collagenolytic activity level of basidiomycete culture in case of growth in the mediums with molasses

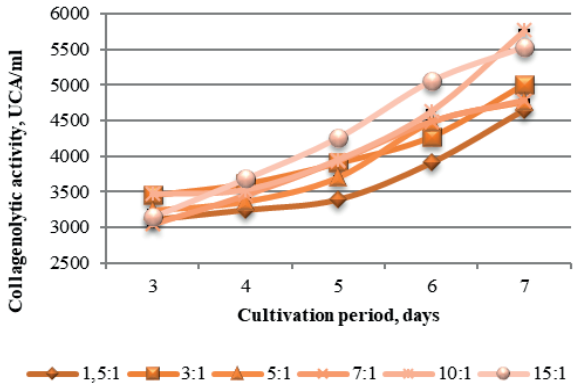

Figure 8. Collagenolytic activity level of the fungus culture in the mediums containing dairy whey

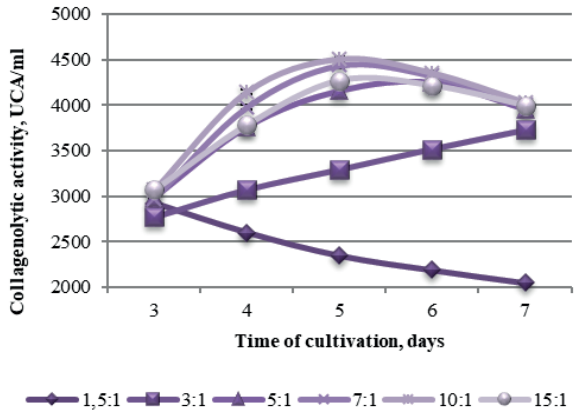

Figure 9. Collagenolytic activity level of *Coprinus* spp. deep culture in case of using the mediums with wheat flour

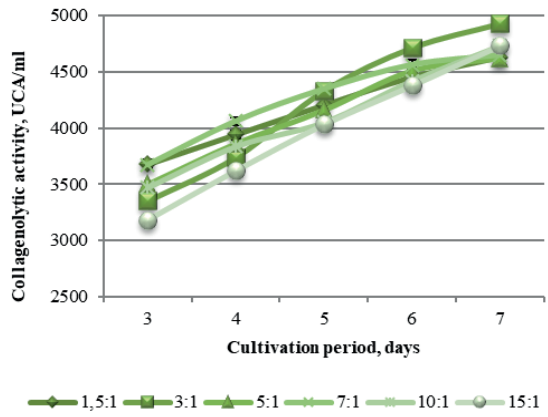

Figure 10. Collagenolytic activity level of producer culture in the mediums with beer wort

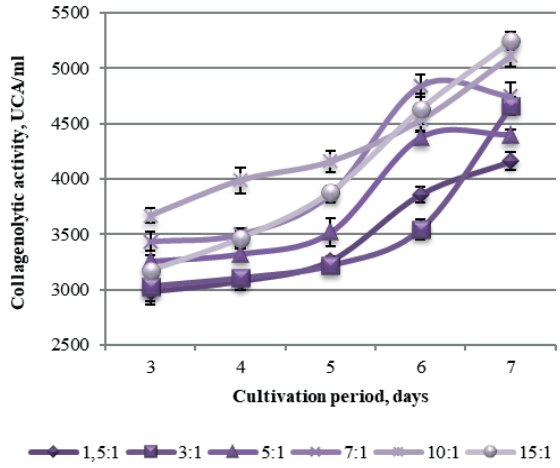

Figure 11. Collagenolytic activity level of basidiomycete culture in case of adjustment of dairy whey mediums pH to 6.5 by 0.1M NaOH

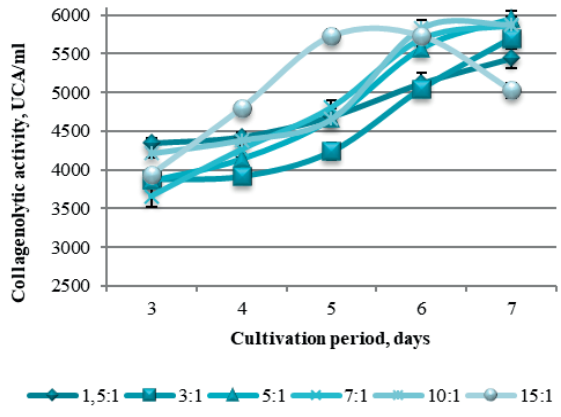

Figure 12. Collagenolytic activity level of producer culture in case of growth in the dairy whey mediums with the addition of 1% of calcium carbonate

According to the data obtained the highest total collagenolytic activity is achieved by growing of producer in medium containing whey and

1% of calcium carbonate on the 7th day of cultivation at the ratios of carbon and nitrogen sources 5:1 and 7:1, and on the 6-7th days of cultivation at a ratio of carbon and nitrogen sources 10:1. The change of dairy whey mediums pH by 0.1M NaOH reduces collagenase activity of producer culture in comparison with the using of dairy whey mediums without any pH correction.

The maximum level of specific collagenolytic activity was observed on the 7th day of fungus cultivation in the medium containing dairy whey at a ratio of carbon and nitrogen sources 15:1, but it is considerably lower than the level achieved in the medium with glucose and urea.

CONCLUSIONS

The use of higher fungi as the producers of collagenase provides an opportunity to realize production of this enzyme under controlled conditions and environments which leads to a relative standardization of product and the possibility of production of sustainable enzyme in commercial scale.

At Microbiological Synthesis Technology department of St. Petersburg State Institute of Technology screening of number of basidiomycetes for the presence of collagenolytic activity was held. It was shown that the deep culture of basidiomycete *Coprinus* spp. has a high level of collagenase activity. Thus, highest fungus *Coprinus* spp. is a promising producer of highly active collagenase.

The lyophilized enzyme preparation was obtained from the culture liquid of basidiomycete *Coprinus* spp. Its specific collagenolytic activity is 1242 UCA/μg of protein. The optimum pH and temperature of its enzymatic activity were determined as a pH of 7.5-7.6, respectively an optimal temperature raging in 35-38ºC.

In order to improve the collagenolytic activity of producer submerged culture the selection of carbon and nitrogen sources and their ratios in the composition of nutrient medium was carried out. According to the information received, the highest total collagenolytic activity is achieved in case of growth of the producer in medium containing urea and dairy whey with addition of 1% of calcium carbonate on the 7th day of cultivation at the ratios of carbon and nitrogen sources 5:1 and 7:1, and on the 6-7th days of cultivation at a ratio of carbon and nitrogen sources 10:1.

The maximum level of specific collagenolytic activity of basidiomycete *Coprinus* spp. culture is observed on the 7th day of cultivation in the medium with glucose and urea at a ratio of carbon and nitrogen sources 15:1.

It should be noted that the replacement of traditionally used expensive carbon (glucose) and nitrogen (peptone) sources in the composition of the nutrient medium by the cheaper ones (dairy whey and urea) will permit us not only to increase the output of the final product - fungal collagenase, but also will significantly reduce the cost of the obtained enzyme preparation. At the same time, it will permit us to use the wastes of cheese and curds productions instead of food product - glucose.

Thus, in this study we have discovered the basidiomycete - producer of collagenolytic enzyme which has not only technological, but also economic advantages in comparison with today's existing preparations of clostridial and crab collagenases.

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REFERENCES

Alipour, H., Raz, A., Zakeri, S., Dinparast Djadid, N. (2016). Therapeutic applications of collagenase (metalloproteases): A review. *Asian Pacific Journal of Tropical Biomedicine*, 6(11), 975-981.

Anzani, C., Prandi, B., Tedeschi, T. et al. (2018). Degradation of Collagen Increases Nitrogen Solubilisation During Enzymatic Hydrolysis of Fleshing Meat. *Waste Biomass*, 9, 1113–1119.

Demina, N. S. (1996). Collagenolytic enzymes produced by microorganisms. *Microbiology*, 65(3), 293-304.

Frantz, S., Paludo L., Stutz, H., Spier, M. (2019). Production of amylases from *Coprinus comatus* under submerged culture using wheat-milling by-products: Optimization, kinetic parameters, partial purification and characterization. *Biocatalysis and Agricultural Biotechnology*, 17, 82-92.

Hu, Y., Tian, G., Zhao, L., Wang, H., Ng, T. B. (2017). A protease-resistant α-galactosidase from Pleurotus djamor with broad pH stability and good hydrolytic activity toward raffinose family oligosaccharides. *Int J Biol Macromol.,* 94 (Pt A), 122-130.

Lowry, O. H., Rosebrough, N. J., Farr A. L., Randall, R. J. (1951). Protein measurement with Folin phenol reagent. *Biol. Chem.*, 193(1), 265-275.

Peralta, R., Silva, B., Kato, C., Seixas, F., Bracht, A. (2017). Enzymes from Basidiomycetes-Peculiar and Efficient Tools for Biotechnology. In *Biotechnology of Microbial Enzymes: Production, Biocatalysis and Industrial Applications* (119–149). Amsterdam, The Netherlands: Elsevier Inc.

Rosen, H. A. (1957). Modified ninhydrin colorimetric analysis for amino acids. *Arch. Biochem. Biophys.*, 67, 10–15.

Rudenskaya, G. N. (2003). Brahiuriny - serine collagenolytic enzymes of crabs. *Bioorganic chemistry,* 29(2), 117-126.

de Souza, P. M., Bittencourt, M. L., Caprara, C. C. et al. (2015). A biotechnology perspective of fungal proteases. *Braz J Microbiol.*, 46(2), 337-346.

Shamtsyan, M., Dmitriyeva, T., Kolesnikov, B., Denisova, N. (2018). Novel milk-clotting enzyme produced by Coprinus lagopides basidial mushroom. *LWT - Food Science and Technology*, 58, 343–347.

Wanderley, M. C., Neto, J. M., Filho, J. L., Lima, C. A., Teixeira, J. A., Porto, A. L. (2017). Collagenolytic enzymes produced by fungi: a systematic review. *Braz J Microbiol*, 48(1), 13-24.