Annals of Agri-Bio Research **24** (2) : 273-276, 2019

Medium Optimization for Enhanced Collagenase Production by *Aspergillus* Fungi

R. BLIEVA*, ZH. SULEIMENOVA, G. ULTANBEKOVA[1], N. AKMUKHANOVA[1], N. ALTYBAEVA[1] AND A. NURMAHANOVA[1]

Research and Production Enterprise Antigen Co. Ltd., Almaty, Kazakhstan
**(e-mail : raubil@mail.ru; Mobile : +77077259059)*

(Received : May 22, 2019; Accepted : July 18, 2019)

ABSTRACT

Collagenases are among the most important hydrolytic enzymes which have great potential in various industrial processes such as leather, detergent, textile, etc. Although many microorganisms produce this enzyme, in the recent period the most commonly used for their industrial application are *Aspergillus* fungi. Among media components, the carbon and nitrogen sources are the valuable nutritional factors for cost-efficient enzyme production. The aim of the present study was the selection of suitable carbon and nitrogen sources of *Aspergillus awamori 16* and *Aspergillus awamori 22* mixed cultures for maximal production of extracellular protease. Sucrose (4.2 U/ml) and peptone (4.8 U/ml) were found as the best carbon and nitrogen sources, respectively.

Key words : Collagenase, *Aspergillus* fungi, carbon sources, nitrogen sources

INTRODUCTION

Collagenase is one of the most important enzymes used for the processing of meat raw materials (Guo-Yan *et al.,* 2012; Purslow, 2018; Marciniak *et al.,* 2018). Studies in the area of production and use of collagenases are now focused at searching for microorganisms that are capable of intensive synthesis of these enzymes (Rani and Pooja, 2018; Bhagwat and Dandge, 2018). Collagenases from microbial sources have dominated applications in industrial sectors (Yi and Li, 2013; Sharma *et al.*, 2017; Pequeno *et al.,* 2019). Despite the fact that among microorganisms that produce collagenase there are bacteria, fungi, and actinomycetes, in the recent period micromycetes got wide application. Among the many advantages offered by the production of enzymes by fungi are low material costs coupled with high productivity, faster production and the ease of enzymes modification. Besides, fungal enzymes are commonly used in industries due to the feasibility of obtaining enzymes at high concentration in the fermentation medium (Monteiro *et al.,* 2015).

Many widespread microorganisms secrete a significant amount of biocatalysts into the environment, which greatly facilitates the task of their isolation and purification. The ability to control the enzymes production through the regulation of their biosynthesis and the selection of nutrient media allows not only to increase the yield of enzymes, but also to obtain enzyme preparations with certain properties. In enzyme production processes, even small improvements have been significant for commercial success (Reddy *et al.,* 2008).

On industrial scale, the production of collagenases has been affected by a variety of physico-chemical factors, such as the composition of the growth medium, the type of strain, cell growth, methods of cultivation, inoculum concentration, time of incubation, pH, temperature, salinity, carbon, nitrogen and mineral sources (Qureshi *et al.,* 2011; Israel-Roming *et al.,* 2015; Pant *et al.,* 2015). However, composition of the cultivation medium (carbon and nitrogen sources) plays significant role on enzymes production.

On the other hand, in natural environments microorganisms live in mixed populations, while in laboratory conditions monocultures are mainly used (Benoit-Gelber *et al.,* 2017). Mixed cultures of *Aspergillus* fungi have shown interesting features for enzyme production. In this research optimization of media components (e. g. carbon and nitrogen sources) for growth

[1]Al-Farabi Kazakh National University, Almaty, Kazakhstan.

and production of extracellular protease by *Aspergillus awamori 16* and *Aspergillus awamori 22* mixed cultures was investigated.

MATERIALS AND METHODS

Association of mixed fungi *A. awamori 16* and *A. awamori 22* (own collection) was used in this study. The microorganisms were maintained on potato dextrose agar at 4°C. For inoculum preparation, 25 ml of sterile distilled water was added to the 5-day-old culture grown on potato dextrose agar plate and scraped aseptically with inoculating loop. This suspension with spore concentration of 1.3 x 10^7 cells/ml was used as inoculum for the fungal cultivation.

Collagenase production was quantified using a standard Czapek-Dox medium. This basic medium already contained balanced levels of nutrients for the enzyme production. The standard Czapek-Dox production medium containing (g/l) : $NaNO_3$ – 5.0, KH_2PO_4 – 1.0; $MgSO_4$ – 0.5; KCL – 0.5 and $FeSO_4$ – 0.01 was supplemented with different carbon sources like sucrose, glucose, fructose, galactose, maltose, lactose and starch. Each source was used at concentration of 10 g/l. Liquid broth was inoculated with fungal suspension at concentration of 1.3 x 10^7 spores/ml in 250 ml Erlenmeyer flasks with a working volume of 50 ml and incubated in a rotary shaker (210 rpm) for 72 h at 30°C.

The production medium containing (g/l) : KH_2PO_4 – 1.0; $MgSO_4$ – 0.5; KCL – 0.5; $FeSO_4$ – 0.01 and sucrose – 10.0 was supplemented with different organic and inorganic nitrogen sources like $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, NH_4NO_3, KNO_3, yeast extract, peptone, casein hydrolysate and gelatin in concentration of 5.0 g/l. The 250 ml Erlenmeyer flasks (50 ml per flask) were inoculated with fungal suspension at concentration of 1.3 x 10^7 spores/ml and incubated at 30°C on a rotary shaker at 210 rpm for 72 h.

Collagenase activity was determined by spectrophotometric method. To 20 mg collagen from bovine tendon (Sigma) suspended in 3.8 ml Tris buffer (0.02 M Tris, 0.005 M $CaCl_2$, pH 7.4) was added 200 µl collagenase solution (1 mg/ml in Tris buffer) to make a total volume of 4.0 ml. The mixture was incubated at 40°C for 3 h or 70°C for 30 min. The reaction mixtures were centrifuged in a microfuge for 10 min at 14,000 rpm. 1.5 ml of supernatant was mixed with 4.5 ml of 5 N HCl and kept in a drying oven at 110°C for 16 h (overnight) for complete hydrolysis of soluble peptides. The hydrolysate was then analyzed for hydroxyproline content as follows : the hydrolysate was diluted 25 times with distilled water. To 1.00 ml of diluted hydrolysate 1.00 ml of chloramine-T solution was added and the mixture was allowed to stand at room temperature for 20 min. 1.00 ml of colour reagent was added after this period and the reaction mixture was transferred to a 60°C. water bath and incubated for 15 min. Tubes were removed and allowed to cool down to room temperature. Absorbance at 600 nm was measured. The experiments were carried out in triplicates and standard deviation was determined. To determine the significance, the data were analyzed using Microsoft excel software 2010.

RESULTS AND DISCUSSION

Various carbon sources were supplemented in the production medium to study their effect on extracellular collagenase production. The collagenase activity ranged from 1.2 to 6.8 U/ml. Among the carbon sources, sucrose supported moderate growth and collagenase production in *A. awamori 16* and *A. awamori 22* mixed cultures with enzymatic activity of 4.2 U/ml (Table 1).

Table 1.Effect of carbon sources on collagenase production in *A. awamori 16* and *A. awamori 22* mixed cultures

Carbon source	pH	Collagenase activity (U/ml)
Sucrose	6.2	6.8±0.9
Glucose	5.9	3.2±0.6
Fructose	6.0	1.9±0.3
Galactose	6.5	1.2±0.4
Maltose	6.1	4.0±0.7
Lactose	6.0	1.6±0.6
Starch	6.5	1.5±0.6

As can be seen from data presented in Table 1 all other carbon sources used had less effect on collagenase production. All other monosaccharides and disaccharides used had a little effect on collagenase production. It is known that disaccharides contain a higher content of carbon atoms (4.21 mol/l) than monosaccharides when used in the same concentrations. However, none of the disaccharides used, except of sucrose, did not affect collagenase activity. The reason for this may be that the α-D-glucopyranolyl-β-D-

fructofuranoside bond in sucrose makes carbon atoms more accessible to the fungus than other sugars.

Thus, all data obtained indicate that sucrose is the most efficient source for collagenase production by fungal association of *A. awamori 16* and *A. awamori 22* mixed culture. Screening of nitrogen sources allowing the maximum collagenase production was performed in nutrient medium with 10 g/l sucrose as a carbon source.

Nitrogen is very important in metabolism of microorganisms especially in the synthesis of enzymes and other proteins. So, nitrogen has been always one of the important components of fermentation substrate/media. Sodium nitrate was replaced with various inorganic (ammonium sulfate, ammonium hydrogen phosphate, ammonium nitrate, potassium nitrate) and organic (yeast extract, peptone, casein hydrolysate, gelatin) nitrogen sources at equivalent nitrogen concentration.

Peptone was found to be the best nitrogen source giving maximum enzyme activity (7.1 U/ml), while other nitrogen sources had less effect on collagenase production (Table 2).

Table 2. Effect of nitrogen sources on collagenase production in *A. awamori* 16 and *A. awamori* 22 mixed cultures

Nitrogen source	pH	Collagenase activity (U/ml)
$(NH_4)_2SO_4$	5.8	6.6±0.6
$(NH_4)_2HPO_4$	5.5	5.8±1.6
NH_4NO_3	4.9	3.5±1.4
KNO_3	5.8	1.9±0.8
Yeast extract	5.0	3.8±1.1
Peptone	5.6	7.1±0.6
Casein hydrolysate	5.1	5.8±0.6
Gelatin	4.8	3.3±0.4

For the selection of optimal concentrations of carbon and nitrogen sources for collagenase production 64 nutrient media with different concentration of sucrose and peptone were used (Table 3).

The highest enzyme activity (8.1 U/ml) was observed in variant with sucrose and peptone at concentration of 2.0 and 1.0%, respectively. In other variants, collagenase activity ranged from 2.1 to 7.9 U/ml.

CONCLUSION

Among various carbon and nitrogen sources examined, 2% sucrose and 1% peptone were found to show maximum collagenase activity.

Table 3. Effect of various concentrations of sucrose and peptone on collagenase biosynthesis in *A. awamori 22* and *A. awamori 16*

Sucrose (%)	Peptone (%)	Collagenase activity (U/ml)
0.25	0.25	2.2±0.8
	0.5	2.1±1.5
	0.75	3.3±1.2
	1.0	2.9±1.3
	1.25	3.3±1.6
	1.5	2.5±1.1
	1.75	3.1±1.2
	2.0	3.2±0.9
0.5	0.25	3.3±1.8
	0.5	2.9±0.7
	0.75	3.2±0.5
	1.0	3.7±0.6
	1.25	3.8±1.5
	1.5	2.9±1.2
	1.75	3.6±1.1
	2.0	4.0±0.9
0.75	0.25	4.2±0.9
	0.5	4.9±0.7
	0.75	4.5±1.5
	1.0	5.5±1.3
	1.25	5.6±1.2
	1.5	5.3±0.8
	1.75	6.0±0.4
	2.0	4.8±1.1
1.0	0.25	5.9±1.4
	0.5	4.9±0.8
	0.75	5.5±1.7
	1.0	5.5±1.8
	1.25	4.2±0.6
	1.5	5.3±1.1
	1.75	5.0±0.4
	2.0	5.4±1.1
1.25	0.25	4.5±1.0
	0.5	5.5±1.8
	0.75	4.9±0.6
	1.0	5.0±0.7
	1.25	5.1±0.5
	1.5	4.8±0.5
	1.75	5.3±0.9
	2.0	4.9±1.1
1.5	0.25	5.5±1.3
	0.5	4.7±1.1
	0.75	5.2±1.0
	1.0	5.0±0.8
	1.25	4.6±1.0
	1.5	5.9±0.9
	1.75	6.7±0.5
	2.0	7.1±0.6
1.75	0.25	6.8±0.7
	0.5	6.2±0.4
	0.75	6.4±0.5
	1.0	7.9±1.0
	1.25	7.2±1.8
	1.5	7.4±1.2
	1.75	6.9±1.1
	2.0	8.1±1.2
2.0	0.25	6.8±0.9
	0.5	7.2±0.4
	0.75	6.9±0.4
	1.0	8.1±0.6
	1.25	7.7±1.2
	1.5	7.5±1.1
	1.75	7.3±1.5
	2.0	7.9±0.9

The optimal fermentation medium for the production of protease by *A. awamori 16* and *A. awamori 22* mixed cultures in submerged cultivation was as follows (g/l) : sucrose – 10.0; KH_2PO_4 – 1.0; $MgSO_4$ – 0.5; KCL – 0.5; $FeSO_4$ – 0.01 and peptone – 5.0. The results presented in this work, therefore, suggest the possibility of secretion of collagenase by *A. awamori 16* and *A. awamori 22* mixed cultures using locally available substrates as carbon and nitrogen sources and its subsequent application in industries.

ACKNOWLEDGEMENT

This study was conducted with financial support from Ministry of Education and Sciences of the Republic of Kazakhstan (Grant # AP05132402).

REFERENCES

Benoit-Gelber, I., Gruntjes, T., Vinck, A., van Veluw, J. G., Wösten, H. A. B., Boeren, S., Vervoort, J. J. M. and de Vries, R. P. (2017). Mixed colonies of *Aspergillus niger* and *Aspergillus oryzae* cooperatively degrading wheat bran. *Fungal Genet. Biol.* **102** : 31-37.

Bhagwat, P. K. and Dandge, P. B. (2018). Collagen and collagenolytic proteases : A review. *Biocatal. Agric. Biotechnol.* **15** : 43-55.

Guo-Yan Zhao, Ming-Yang Zhou and Hui-Lin Zhao (2012). Tenderization effect of cold-adapted collagenolytic protease MCP-01 on beef meat at low temperature and its mechanism. *Food Chem.* **134** : 1738-1744.

Israel-Roming Florentina, Luta Gabriela, Balan Daniela, Gherghina Evelina and Calina Petruta Cornea (2015). Time and temperature stability of collagenase produced by *Bacillus licheniformis. Agric. Sci. Procedia.* **6** : 579-584.

Marciniak, A., Suwal, S., Naderi, N., Pouliot, Y. and Doyen, A. (2018). Enhancing enzymatic hydrolysis of food proteins and production of bioactive peptides using high hydrostatic pressure technology. *Trends Food Sci. Technol.* **80** : 187-198.

Monteiro Paula de Souza, Mona Lisa de Assis Bittencourt and Carolina Canielles Caprara (2015). A biotechnology perspective of fungal protease. *Braz. J. Microbiol.* **46** : 337-346.

Pant, Gaurav, Prakash, Anil and Bera, Sayantan (2015). Production, optimization and partial purification of protease from *Bacillus subtilis. J. Taibah. Univ. Sci.* **9** : 50-55.

Pequeno, A. C. L., Arruda, A. A., Silva, D. F., Duarte, Neto, J. M. W., Silveira Filho, V. M., Converti, A., Marques, D. A. V., Porto, A. L. F. and Lima, C. A. (2019). Production and characterization of collagenase from a new Amazonian *Bacillus cereus* strain. *Prep. Biochem. Biotechnol.* **4** : 1-9.

Purslow, P. P. (2018). Contribution of collagen and connective tissue to cooked meat toughness : some paradigms reviewed. *Meat Sci.* **144** : 127-134.

Qureshi, A. S., Bhutto, M. A., Khushk, I. and Dahot, M. U. (2011). Optimization of cultural conditions for protease production by *Bacillus subtilis* EFRL 01. *Afr. J. Biotechnol.* **10** : 5173-5181.

Rani, S. and Pooja, K. (2018). Elucidation of structural and functional characteristics of collagenase from *Pseudomonas aeruginosa. Process Biochem.* **64** : 116-123.

Reddy, L. V., Wee, Y. J., Yun, J. S. and Ryu, H. W. (2008). Optimization of alkaline protease production by batch culture of Bacillus sp. RKY3 through Plackett-Burman and response surface methodological approaches. *Bioresour. Technol.* **99** : 2242-2249.

Sharma Kanupriya Miglani, Kumar, Rajesh, Panwar, Surbhi and Kumar, Ashwani (2017). Microbial alkaline proteases : Optimization of production parameters and their properties. *J. Genet. Eng. Biotechnol.* **15** : 115-126.

Yi Qing Li and Li Marek Peter (2013). Iverson commercial proteases : Present and future. *FEBS Lett.* **587** : 1155-1163.