Enhancement of microbial transglutaminase production from *Streptomyces* sp.

Izzet Turker[1], Gokhan Domurcuk[1], Mehmet Tokatli[1], Hilal Isleroglu[1], Banu Koc[2]

1 – Gaziosmanpasa University, Faculty of Natural Sciences and Engineering, Food Engineering Department, Tokat, Turkey
2 – Gaziantep University, Fine Arts, Gastronomy and Culinary Arts, Gaziantep, Turkey

Abstract

Introduction. Enhancement of microbial transglutaminase (MTGase) production was investigated in terms of creating a model for future studies about the usage of microbial transglutaminase which might become popular in the next years.

Materials and methods. In order to determine the highest enzyme activity, the effects of temperature, pH, medium and type of strains have been investigated. Five different strains were selected to produce MTGase: *Streptomyces mobaraensis* (NRRL B-3729)*, S. ladakanum* (NRRL ISP-5587)*, S. lividans* (NRRL B-12275), *S. sioyaensis* (NRRL B-5408) and *S. platensis* (NRRL B-5486). The fermentation process carried out using two fermentation media based on glucose-starch and soy with different pH and temperatures (6.0, 7.0, 8.0 pH and 20, 30, 40 °C). MTGase activity had been determined for 28 days by hydroxamate-based colorimetric method.

Results and discussions. *S. mobaraensis, S. ladakanum* and *S. lividans* showed greater growth rates than *S. sioyaensis* and *S. platensis*. In that case, *S. mobaraensis, S. ladakanum* and *S. lividans* were selected for the enzyme production. At pH 6.0, the highest enzyme activity (0.036 U/ml) was achieved by *S. mobaraensis* in glucose-starch medium at 30 °C for 14 days and the enzyme activity decreased dramatically after 14th day of fermentation for all strains. At pH 7.0, the highest enzyme activity was seen on 28th day of incubation for *S. mobaraensis* at 30 °C. At pH 8.0, MTGase could not be produced in any of the culture media, or at any temperatures and pH value. For initial pH 6.0 value, the increasing rate of MTGase activity in glucose-starch based medium was higher than in soy based medium at all different temperatures (20, 30 and 40 °C). *S. ladakanum* and *S. lividans* could not produce MTGase for none of the conditions.

Conclusions. *S. mobaraensis* yielded the highest enzyme activity when compared with other strains. Glucose-starch based medium was the most suitable medium for MTGase production. pH and temperature changes affected the enzyme activity and pH 6.0 and 30 °C were the best conditions for the production of MTGase.

Introduction

Transglutaminase (TGase; protein-glutamine-glutamyl-transferase, EC 2.3.2.13) is an enzyme capable of catalyzing acyl transfer reactions by introducing covalent cross-links between proteins as well as peptides and various primary amines [1]. When the amine substrates are absent in the medium, TGase can catalyze the hydrolysis of the γ-carboxyamide group of the glutaminyl residue, resulting in deamination. When the ε-amino group of a peptide-bound lysyl residue is the substrate, peptide chains are covalently connected through by TGase ε-(γ-glutamyl) lysine (G-L) bonds [2]. Transglutaminase has been found in animals, plants, and microorganisms [3]. Recently, TGase has captured peoples' interest due to its potential application for various food products [4, 5].

There are three ways to produce TGase. The first approach is extraction and purification of the enzyme from body fluids and tissues of animals. Factor XIII, extracted from the blood of cattle, was the first commercially produced TGase in Europe [6]. However, the blood enzyme needed thrombin to be active and when used for food products, red pigmentation affected the appearance. The second approach is producing the enzyme by using genetic manipulation. In this purpose, many researchers experimented to produce TGase within the host microorganisms as *E. coli, Bacillus* and *Aspergillus* [7–9]. Although being a low-costed method, because of consumers disclaim, second approach could not be an applicable method to obtain commercial TGase [2]. The third approach is finding an appropriate microorganism and produce TGase by using fermentation technology. Several reviews on the application of microbial transglutaminase (MTGase) in food and other areas are already available in the literature [2, 3, 5, 10].

The production of MTGase by *Streptoverticillium mobaraense* was first reported by Ando et al. [11]. It was reported that MTGase can catalyze acyl transfer reactions by introducing covalent cross-links between proteins like blood and tissue TGase. Furthermore, MTgase do not require Ca^{+2} or thrombin for activation. For commercial production, MTGase can be easily isolated from the culture broth. MTGase from *Streptoverticillium* sp. used for several food applications such as producing polymers of casein and soybean proteins and gelatinizing sodium caseinate and skim milk gels [12, 13].

Streptomyces species are able to produce MTgase in different structures and these species are commonly used for the industrial production of MTGase. Accordingly, researchers are focused on enhancement of the production of MTGase by using *Streptomyces* species in terms of factors affecting enzyme activity such as substrate optimization, metabolic optimization, and extrinsic factors (pH, dissolved oxygen and temperature etc.). Altering the parameters about fermentation may cause to increase the enzyme activity and hereby increasing the enzyme activity may lower the costs of the production [14]. Because of consideration in the usage of MTGase will become popular, in this study enhancement of MTGase production was investigated in terms of creating a model for future studies. For determination of the highest enzyme activity, the effects of temperature, pH, medium and type of strains have been investigated.

Materials and methods

Materials. All the chemicals used were of analytical grade and mainly purchased either from Sigma Chemical Co., Ltd. or Merck Millipore Corporation. Z-Gln-Gly (γ-glutamyl donor substrate), was purchased from Sigma Chemical Co., Ltd. *Streptomyces mobaraensis* NRRL B-3729, *S. ladakanum* NRRL ISP-5587, *S. lividans* NRRL B-12275, *S. sioyaensis* NRRL B-5408 and *S. platensis* NRRL B-5486 were obtained from USDA Agricultural

Research Service. Perkin-Elmer Lambda EZ 201 spectrophotometer was used for the enzyme assays.

Culture conditions and MTGase Production. All strains were pre-cultured on a medium composed of: glucose (4.0 g/L), yeast extract (4.0 g/L) and malt extract (10.0 g/L) at pH 7.2, 30 °C. After 6 days of incubation, *S. mobaraensis, S. ladakanum* and *S. lividans* showed greater growth rates than *S. sioyaensis* and *S. platensis*. In that case, *S. mobaraensis, S. ladakanum* and *S. lividans* were selected for the enzyme production. Two different media were prepared for MTGase production. The first medium based on glucose-starch composed of: glucose (15 g/L), starch (15 g/L), peptone (15 g/L), yeast extract (4 g/L), $MgSO_4$ (2 g/L), $KHPO_4$ (2 g/L) and KH_2PO_4 (2 g/L) [15]. The second medium based on soy composed of: soy (20 g/L), glycerol (10 g/L), peptone (15 g/L), starch (5 g/L), glucose (5 g/L), yeast extract (5 g/L), $CaCO_3$ (5 g/L), $MgSO_4$ (2 g/L), $KHPO_4$ (2 g/L) and KH_2PO_4 (2 g/L) [1]. To determine the effect of different temperatures and pH values on MTGase production, *S. mobaraensis, S. ladakanum* and *S. lividans* were inoculated in both glucose-starch based medium and soy based medium, initial pH values were adjusted to 6.0, 7.0 and 8.0. Samples had been incubated for 28 days at 20, 30 and 40 °C were analyzed to determine MTGase activity.

Determination of MTGase activity. MTGase enzyme activity was determined by Hydroxamate formation with the specific substrate of Z-Gln-Gly. The reaction mixture contained 100 µl enzyme solution and 325 µl substrate solution (200 µl of 200 mM Tris-HCl, 25 µl of 12.5 mM reduced glutathione, 25 µl of 125 mM hydroxylamine and 75 µl of 37.5 mM Z-Gln-Gly). The solution was incubated at 37 °C for 24 hours and then it was ended by adding 425 µl of stop reagent (consisting of 15% TCA and 5% $FeCl_3$). After centrifugation at 11000 rpm for 5 minutes, the absorbance of the supernatant was measured at 525 nm. Assay was also carried out using the enzyme blanks. One unit of MTGase activity was described as the amount of enzyme which caused the formation of 1.0 µmole of hydroxamate per minute by catalyzing the reaction between Z-Gln-Gly and hydroxylamine at pH 6.0 and 37 ºC by using ʟ-glutamic acid γ-monohydroxamate as a standard [16, 17].

Results and discussion

All five lyophilized strains obtained from USDA were pre-cultured on a medium composed of glucose, yeast extract and malt extract. *S. mobaraensis, S. lividans* and *S. ladakanum* were selected for the MTGase production of enzyme. These strains were reproduced once in two days for the first week, and this procedure was repeated every couple of weeks. For preparation of the stock culture, cultured media were centrifuged and bacterial pellet was obtained. Following the centrifugation, sterilized pre-culturing medium containing 30% glycerol (v/v) was added on the bacterial pellet and stock cultures were stored inside cryotubes at -65 °C. For the enzyme production, fresh cultures which obtained from pre-culturing medium were inoculated in (1%) glucose-starch and soy based media. After 28 days of incubation, *S. mobaraensis* yielded the highest enzyme activity. However, *S. ladakanum* and *S. lividans* could not produce MTGase for none of the culture mediums, temperatures and pH value. Some researchers attempted to produce MTGase by genetic modification methods from *S. ladakanum* and *S. lividans* strains which produce MTGase very slightly [18, 19]. On the other hand, according to the Tellez-Luis et al. [20], *S. ladakanum* is able to produce MTGase (0.725 U/ml) in the presence of casein and glycerol in the broth medium. In the same way, Ho et al. [21] produced MTGase (2.40 U/ml) by using agitating fermenter from *S. ladakanum*.
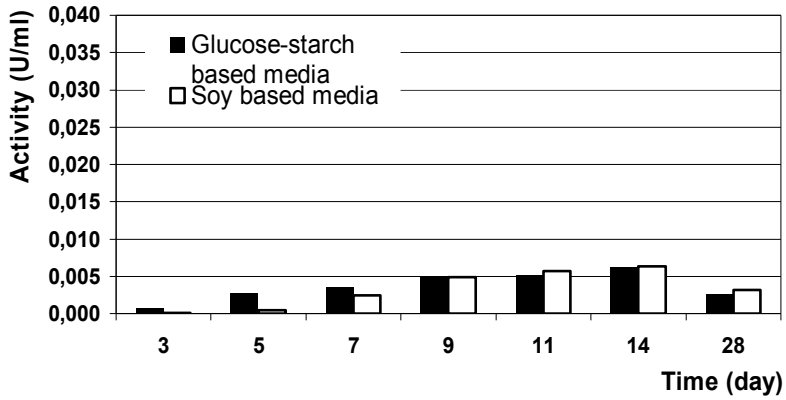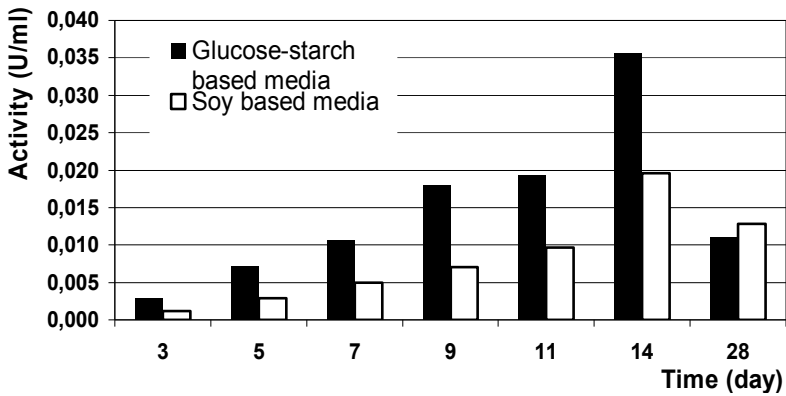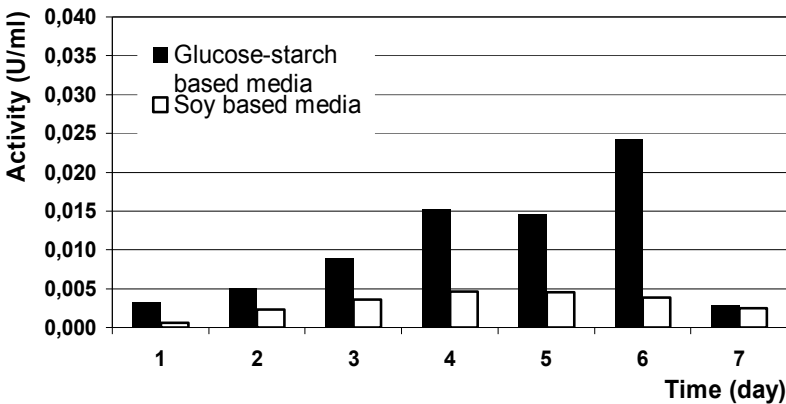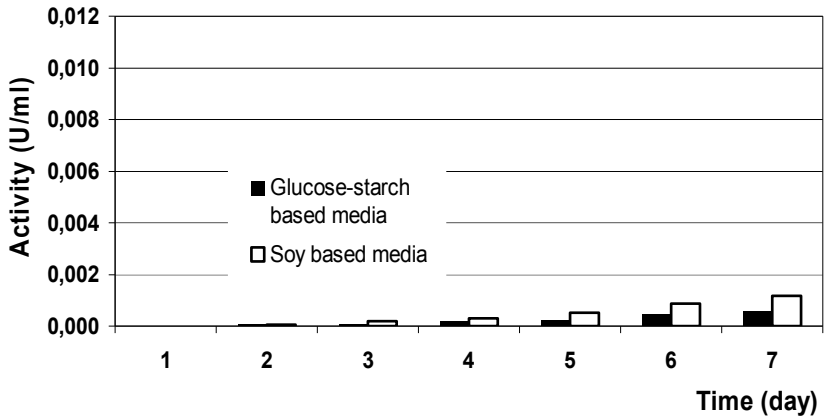
Figure 1. Effect of the different incubation temperatures on the enzyme activity of S. mobaraensis for glucose-starch based medium (dark grey) and soy based medium (light grey) at initial pH of 6.0:
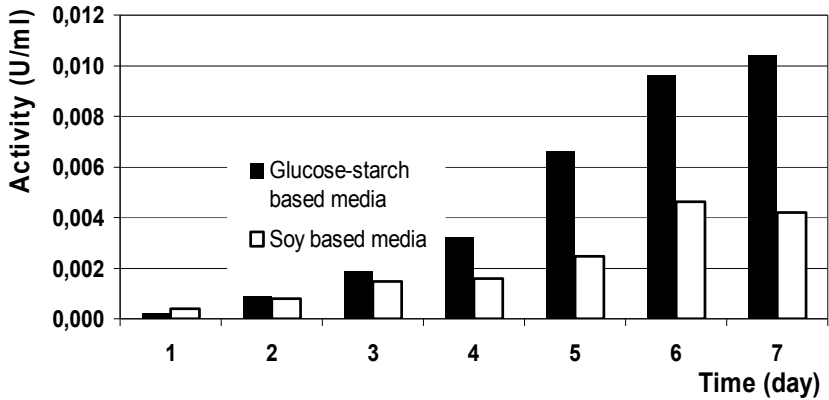*a* – 20 °C, *b* – 30 °C, *c* – 40 °C.

a

b

c

Figure 2. Effect of the different incubation temperatures on the enzyme activity of *S. mobaraensis* for glucose-starch based medium (dark grey) and soy based medium (light grey) at initial pH of 7.0:
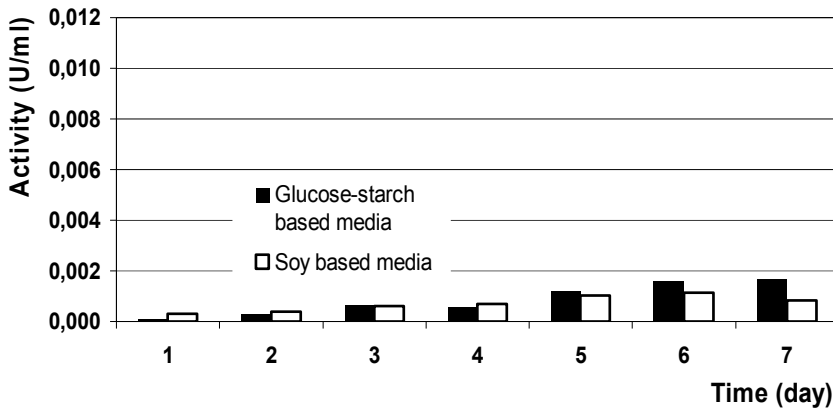*a* – 20 °C, *b* – 30 °C, *c* – 40 °C.

Figure 1 shows that the highest activity for all experimental conditions was found at 30 °C, 14th day of incubation. The results for glucose-starch based medium and soy based medium were 0.036 U/ml and 0.020 U/ml, respectively. In the same way, Zheng et al. [22] carried out the effects of the temperature to the fermentation of *Streptovericillium mobaraense* and reached the highest enzyme activity at 30 °C (2.90 U/ml at pH 6.5). The glucose-starch based medium had the lowest enzyme activity at 20 °C. Likewise, soy based medium showed the lowest enzyme activity at 20 and 40 °C. The highest activity at 20 °C was reached on the 14th day of incubation for both media (~0.006 U/ml). Temperature might considerably affect the specific cell growth rate of microorganisms. Therefore, the amount of MTGase activity can be related with specific cell growth rate of *S. mobaraensis* [22]. Glucose-starch based medium had an acceptable enzyme activity (0.024 U/ml) at 40 °C as well. However, the enzyme activity decreased dramatically after 14th day of incubation for all samples at 20, 30 and 40 °C.

For initial pH 6.0, the increasing rate of MTGase activity in glucose-starch based medium was higher than in soy based medium at all different temperatures (20, 30 and 40 °C). Tellez-Luis et al. [20] reported that using of glycerol and casein in the medium had positive impact on producing MTGase from *S. ladakanum*. On the other hand, in the medium, cross-linking of peptides may occur by MTGase. For our soy based medium, free amino acids may be probably limited the MTGase production.

As shown in Figure 2, at initial pH 7.0, the highest enzyme activities for both glucose-starch based medium and soy based medium at 30 °C were 0.010 U/ml, 0.004 U/ml, respectively similar to initial pH 6.0. We concluded that glucose-starch based medium had high amount of enzyme activity when compared with soy based medium likewise at pH 6.0. For the temperatures 20 and 40 °C, there were slight changes on the MTGase activity. At pH 7.0, the highest enzyme activity was seen on 28th day of incubation unlike pH 6.0. In our study, because of having low enzyme activity at pH 8.0 for both media, the data was not shown in this paper. Meiying et al. [23] stated that *S. mobaraense* cannot grow normally in acidic or slightly alkaline medium. Hydrogen bonds may not be formed; consequently, protein molecule bond formation cannot be established. They suggested that to maximize the specific cell growth rate the pH value should be controlled at 7.0. However, we reached the highest enzyme activity value at initial pH 6.0.

Conclusions

S. mobaraensis showed the highest MTGase activity when compared with *S. lividans* and *S. ladakanum*. MTGase activity of *S. mobaraensis* was crucially affected by medium type, different temperatures and pH values. The effect of temperature (20, 30 and 40 °C), pH (6.0, 7.0 and 8.0) and two different media (glucose-starch based and soy based medium) on the enzyme activity was investigated and it was reached that the highest enzyme activity (0.036 U/ml) at 30 °C, pH 6.0 and in glucose-starch based medium. To maintain maximum MTGase production, in addition to the temperature and pH, other parameters such as dissolved oxygen and agitation may also be changed. Instead of ready to use medium, molasses or waste products can be used as carbon source for the production of MTGase.

References

1. Cui L., Du G., Zhang D., Liu H., Chen J. (2007), Purification and characterization of transglutaminase from a newly isolated Streptomyces hygroscopicus, *Food Chem*, 105, pp. 612–618.
2. Yokoyama, K., Nio N., Kikuchi Y. (2004), Properties and application of microbial transglutaminase, *Appl. Microbiol. Biotechnol*, 64(4), pp. 447– 454.
3. Zhu Y., Tramper J. (2008), Novel applications for microbial transglutaminase beyond food processing, *Trends Biotechnol*, 26, pp. 559−565.
4. Motoki M., Seguro K. (1994), Trends in Japanese soy protein research, *Inform*, 5, pp. 308–313
5. Zhu Y., Rinzema A., Tramper J., Bol J. (1995), Microbial transglutaminase – a review on its production and application in food processing, *Appl. Microbiol. Biotechnol*, 44, pp. 277–82.
6. Wilson S.A. (1992), Modifying meat proteins via enzymatic crosslinking, *Proceedings of the 27th Meat Industry Research Conference*, *Hamilton*, Meal Industry Research Institutes of New Zealand, Mirinz, pp. 247–277
7. Bishop P.D., Teller D.C., Smith R.A., Lasser G.W., Gilbert T., Seale R.L. (1990), Expression, purification, and characterization of human factor XIII in *Saccharomyces cerevisiae.*, *Biochemistry*, 29, pp. 1861–1869.
8. Washizu K., Ando K., Koikeda S., Hirose S., Matsuura A., Akagi H., Motoki M., Takeuchi K. (1994), Molecular cloning of the gene for microbial transglutaminase from Streptoverticillium and its expression in Streptomyces lividans, *Biosci Biotechnol Biochem.*, 58, pp. 82–87.
9. Yokoyama K.I., Nakamura N., Seguro K., Kubota K. (2000), Overproduction of microbial transglutaminase in *Escherichia coli*, in vitro refolding, and characterization of the refolded form, *Biosci. Biotechnol. Biochem.*, 64, pp. 1263–1270.
10. Motoki M., Seguro K. (1998), Transglutaminase and its use for food processing, *Trends Food Sci. Technol.*, 9, pp. 204–210.
11. Ando H., Adachi M., Umeda K., Matsuura A., Nonaka M., Uchio R., Tanaka H., Motoki M. (1989), Purification and characterization of a novel transglutaminase derived from microorganisms, *Agric. Biol. Chem.*, 53, pp. 2613–2617
12. Nonaka, M., Tanaka H., Okiyama A., Motoki M., Ando H., Umeda K., Matsukuwa A. (1989), Polymerization of several proteins by Ca^{2+}-independent transglutaminase derived from microorganisms, *Agric. Biol. Chem.*, 53, pp. 2619–2623.
13. Nonaka M., Sakamoto H., Toiguchi S., Kawajiri H., Soeda T., Motoki M. (1992), Sodium caseinate and skim milk gels formed by incubation with microbial transglutaminase, *J. Food Sci.,* 57, pp. 1214–1218.
14. Zhang D., Zhu Y., Chen J. (2009), Microbial transglutaminase production: understanding the mechanism, *Biotechnol. Genet. Eng. Rev.*, 26, pp. 205–222.
15. Macedo J. A., Sette L.D. , Sato H.H. (2007), Optimization of medium composition for transglutaminase production by a Brazilian soil *Streptomyces sp., Electr. J. Biotechnol.*, 10, pp. 618–626
16. Bourneow C., Benjakul S., Sumpavapol P., Hıkıttıkun A. (2012), Isolation and cultivatıon of transglutaminase-producing bacteria from seafood processing factories, *Innov Rom Food Biotechnol.*, 10, pp. 28–39.
17. Estevinho B.N., Damas A.M., Martins P., Rocha F. (2014), Microencapsulation of β-galactosidase with different biopolymers by a spray-drying process, *Food Res. Int.*, 64, pp. 134–140.

18. Lin Y.S., Chao M.L., Liu C.H., Chu W.S. (2004), Cloning and expression of the transglutaminase gene from Streptoverticillium ladakanum in *Streptomyces lividans*, *Process Biochem.*, 39, pp. 591–598.
19. Kieliszek M., Misiewicz A. (2014), Microbial transglutaminase and its application in the food industry. A review, *Folia Microbiol.*, 59, pp. 241–250.
20. Tellez-Luis S.J., Ramírez J.A., Vázquez M. (2004), Application in restructured fish products of transglutaminase obtained by Streptoverticillum ladakanaum in media made from hydrolysates of sorghum straw, *J. Food Sci.*, 69, pp. 1–5.
21. Ho M.L., Leu S.Z. , Hsieh J.F., Jiang S.T. (2000), Technical approach to simplify the purification method and characterization of microbial transglutaminase produced from *Streptoverticillium ladakanum, J. Food Sci*., 65, pp. 76–80.
22. Zheng M.Y., Du G.C., Chen J. (2002), pH control strategy of batch microbial transglutaminase production with Streptoverticillium mobaraense, *Enzyme Microb. Technol.*, 31, pp. 477–481.
23. Meiying Z., Guocheng D., Jian C. (2002), pH control strategy of batch microbial transglutaminase production with *Streptoverticillium mobaraense*, *Enzyme Microb. Technol.*, 31, pp. 477–481.