Production of Transglutaminase by *Streptoverticillium ladakanum* NRRL-3191 Using Glycerol as Carbon Source

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Summary

The enzyme transglutaminase (TG) catalyses the formation of covalent bonds between adjacent proteins, thereby improving the gel structure of proteins and has important applications for the food industry. The aims of this work were: (*i*) to elucidate the effect of agitation speed during the biotechnological production of TG by *Streptoverticillium ladakanum* NRRL-3191 using glycerol as carbon source; and (*ii*) to improve TG production by optimising the composition of media based on glycerol, xylose and casein. An agitation speed of 250 rpm and a fermentation time of 72 h resulted in the optimal enzymatic activity (0.628 U/mL) with a productivity of 0.087 U/(mL·h). The composition of media with glycerol, xylose and casein were optimised using an experimental design to improve TG production. The model predicts that the maximum TG activity (0.725 U/mL) can be obtained using glycerol 50.5 g/L and casein 20 g/L without the addition of xylose.

Key words: transglutaminase, glycerol, casein, *Streptoverticillium ladakanum*

Introduction

Transglutaminases (TG, R-glutaminyl peptide: amine γ-glutaminyltransferase, EC 2.3.2.13) catalyses an acyl transfer reaction between γ-carboxyamide groups of glutaminyl residues in proteins. When the primary amine is the ε-amino group of lysine and lysyl residues, ε-(γ-glutamyl)lysine cross-links are formed (*1*).

In prokaryotes, TG activity has been found only in Actinomycetes from the genus *Streptoverticillium* (*2,3*). The two important features of Microbial TG (MTG) are to be extra-cellular and Ca^{2+}-independent. These properties increased the food industry interest for this class of enzymes. MTG has been proposed in industrial processes for the production of modified proteins by incorporation of essential amino acids or glycosyl groups (*4,5*) and for-

mation of thermally stable gels (*6,7*). MTG catalyses the formation of covalent bonds between adjacent proteins, thereby improving the gel structure of proteins. The reaction is the same as of an endogenous TG in food proteins, but the first shows lower deamidation affinity than endogenous TG of fish or pig (*8*).

Nowadays, MTG has been employed to improve the mechanical and textural properties of different proteins contained in foods, including surimi products or restructured fish products (*9,10*). This enzyme is recognised as the one responsible for the setting phenomenon in fish proteins (*11*). However, MTG cannot be used with hydrocolloids such as low methoxyl pectin because a disruptive effect was reported (*12*).

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Fermentation media can represent almost 30 % of the cost for microbial fermentation (*13*). General media employed for the growth of *Streptoverticillium* are not economically attractive due to the high amount of expensive nutrients such as yeast extract and peptone. Although the purification and properties of MTG are known (*14*), the physiological role and the potential inducers are still unknown. For example, with 10 g/L of acetic acid, citric acid, cellobiose, glucose, glycerol, lactose, maltose, mannitol, saccharose or xylose as a carbon source, only media containing glycerol or glucose showed MTG activity. Both casein and glycerol were found to have a significant effect on MTG production. The activity reached 0.331 U/mL with optimum concentrations of casein (38.4 g/L) and glycerol (31.2 g/L) (*15*).

Xylose is a hemicellulosic sugar that can be used as carbon and energy source for growth of microorganisms. The interest in using xylose as a carbon source for microbial proliferation could be enhanced if the fermentation media were prepared from hemicellulosic hydrolysates of a cheap raw material such as sorghum straw or sugar cane bagasse (*16–18*). Moreover, xylose could be a co-carbon source with glycerol for the production of MTG.

The aims of this work were: (*i*) to elucidate the effect of agitation during fermentation of *Streptoverticillium ladakanum* NRRL-3191 using glycerol and (*ii*) to improve MTG production by optimising the composition of media based on glycerol, xylose and casein using an experimental design.

Materials and Methods

Microorganisms and culture conditions

Freeze-dried broths of *Streptoverticillium ladakanum* NRRL-3191 strain were obtained from the Agricultural Research Service Culture Collection (Peoria, Illinois, USA). Microorganisms were maintained on agar plates at 4 °C, and transferred monthly. Proliferation experiments were carried out during 2 days at 26 °C in orbital shakers using 250-mL Erlenmeyer flasks with 50 mL of fermentation medium. The composition of media used for proliferation assays was: yeast extract 2.5 g/L, peptone 10.5 g/L, $MgSO_4$ 0.5 g/L, KH_2PO_4 2 g/L, Na_2HPO_4 5 g/L, sodium casein 38.4 g/L and glycerol 31.2 g/L. In the experimental design, sodium casein, xylose and glycerol concentrations varied as it is explained below. The rest of the nutrients were maintained at the same concentrations.

Analytical methods

Samples were withdrawn from the fermentation media at 48, 72, 96 and 120 h in the first set of experiments and only at 72 h in the experimental design showed below. They were centrifuged (4500 × *g*, 10 min) and supernatants were analysed for glycerol or xylose by HPLC. This was carried out using a Transgenomic ION-300 column (oven temperature = 45 °C) with isocratic elution (flow rate=0.4 mL/min; mobile phase 0.0025 M H_2SO_4) and a refraction index detector. Pellets were washed twice with a solution of sodium chloride 9 g/L in deionised water, centrifuged again and then dried at 102 °C for 48 h, in order to allow the calculation of the biomass concentration on dry weight basis.

The MTG activity was measured by a colorimetric procedure based on the formation of hydroxamate from N-carbobenzoxy-L-glutaminylglycine (*19*). One unit of activity (U) was defined as the amount that causes the formation of 1 μmol hydroxamate in 1 min at 37 °C.

Statistical analysis

All experimental data were carried out in triplicate and mean values and standard deviations were calculated. A second-order multiple regression analysis using least squares regression methodology was performed using Microsoft Excel 2000 (Microsoft Corporation, Redmond, WA, USA, 1999) software. Microsoft PowerPoint 2000 (Microsoft Corporation, Redmond, WA, USA, 1999) was used to plot the experimental data and models.

Results and Discussion

Effect of agitation speed

Several authors have applied low agitation speed (around 140–200 rpm) but they do not justify the use of this low agitation speed (*15,20*). To determine the effect of the agitation speed in flask fermentations, a set of experiments were conducted at 150, 250 or 350 rpm using the composition of culture medium proposed by Junqua *et al.* (*15*). Biomass concentration, glycerol concentration and MTG activity were determined at given times.

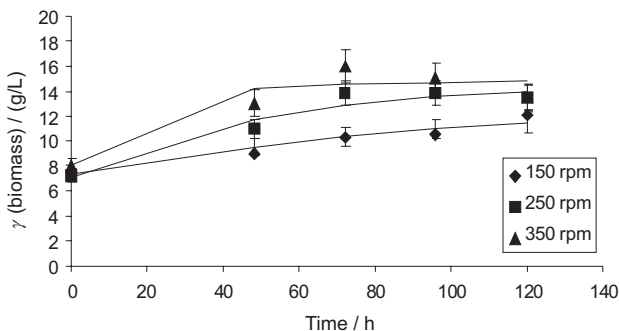The biomass concentration obtained is shown in Fig. 1.

Fig. 1. Dependence of biomass concentrations on the fermentation time for different agitation speed

The highest concentration (16.1±1.1) g/L was obtained at 72 h using 350 rpm. Lower agitation speed gave lower biomass concentration. The biomass concentration was modeled using a mathematical model adopted from a study to describe the fermentative production of lactic acid (*21*):

$$X = \frac{X_0 X_m e^{R_x t}}{X_m - X_0 + (X_0 e^{R_x t})} \qquad /1/$$

where *t* is time, *X* is biomass concentration, X_0 is initial concentration of biomass, X_m is maximum concentration

of biomass, R_x is the rate of biomass formation. The coefficients of the models and the statistical parameters are shown in Table 1. The coefficients r^2 and the value of F-test probability showed a good agreement between experimental and predicted data. The rate of biomass formation increased linearly from 0.017 to 0.063 h^{-1} with

initial glycerol concentration. Using the series of experimental data concerning glycerol concentration/time and the regression parameters of Eq. /1/, the model parameter $Y_{x/s}$ can be calculated for each fermentation medium by non-linear regression using the least-squares method. Table 1 also lists the numerical values of $Y_{x/s}$ and

Table 1. Results of initial concentration of biomass (X_0), maximum concentration of biomass (X_m), rate of biomass formation (R_x) and biomass yield ($Y_{x/s}$) obtained by regression analysis of biomass concentration and glycerol consumption by *Streptoverticillium ladakanum* NRRL-3191 using different agitation speeds. Statistical parameters of the models are also shown

Agitation speed	Biomass production					Glycerol consumption		
	$\dfrac{X_o}{\text{g/L}}$	$\dfrac{X_m}{\text{g/L}}$	$\dfrac{R_x}{\text{h}}$	r^2	F-Test Prob.	$\dfrac{Y_{x/s}}{\text{g/g}}$	r^2	F-Test Prob.
150 rpm	7.28	12.45	0.017	0.95	0.93	0.16	0.90	0.96
250 rpm	7.10	14.28	0.032	0.97	0.95	0.24	0.91	0.97
350 rpm	8.03	14.74	0.063	0.93	0.90	0.24	0.96	0.96

the increase of agitation speed. Using an empirical equation, R_x was related to the agitation speed (AS):

$$R_x = 0.0002\ \text{AS} - 0.0199 \qquad /2/$$

The coefficient r^2 (0.95) confirmed that the empirical equation fits the data well. The coefficients X_0 and X_m were in the range from 7.2 to 8 g/L and 12.5 to 14.7 g/L, respectively. Any clear trend was not observed.
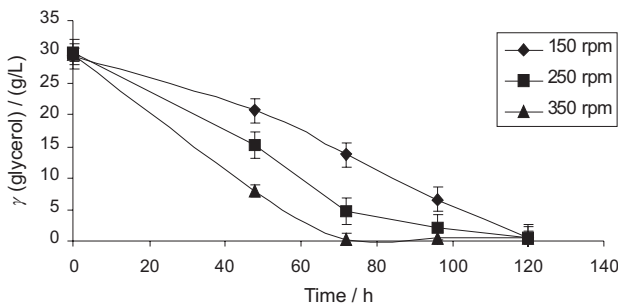

Fig. 2. Dependence of glycerol concentrations on the fermentation time for different agitation speed

Fig. 2 shows the results of glycerol consumption. Using 150 rpm, 120 h are needed for the complete depletion of the carbon source. This time was reduced progressively to 72 h using 350 rpm. The rate of glycerol consumption increased with the increase of agitation speed, which could be explained as the best mass transfer induced by high agitation. At 72 h, 46.5 % of glycerol remained in the culture medium at 150 rpm and 15.5 % in fermentations at 250 rpm. The consumption of glycerol can be expressed by the following equation obtained from the biomass yield ($Y_{x/s}$) definition:

$$S = S_0 - \frac{1}{Y_{X/S}}(X - X_0) \qquad /3/$$

where $Y_{x/s}$ is biomass yield and X and X_0 were defined above, S is the glycerol concentration (g/L) and S_0 is the

statistical parameters obtained for the glucose consumption.

The parameter $Y_{x/s}$ can also be determined using its definition for each fermentation time:

$$Y_{X/S} = \frac{X - X_0}{S_0 - S} \qquad /4/$$

where X, X_o and S were previously defined and S_0 is the initial glycerol concentration (g/L). The $Y_{x/s}$ obtained for regression analysis coincided with the calculated values by the definition for 120 h at 150 rpm (0.16 g/g), 96 h at 250 rpm (0.24 g/g) and 96 h at 350 rpm (0.24 g/g). This suggests that at least 250 rpm should be considered for the optimal consumption of glycerol by *Streptoverticillium ladakanum*.
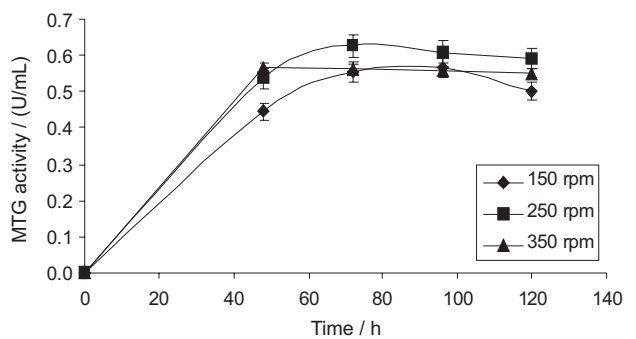

Fig. 3. Dependence of microbial transglutaminase (MTG) activity on the fermentation time with different agitation speed

Fig. 3 shows the results of MTG activity. The highest activity (0.63±0.05) U/mL was detected at 72 h using 250 rpm, and under these conditions, glycerol 5 g/L remained in the medium. This suggests that the presence of glycerol induced the production of MTG although its concentration was low. Using 350 rpm, the MTG activity increased to (0.57±0.24) U/mL at 48 h and remained stable with time. This could be related to the depletion of

glycerol at 72 h and confirm that the production of MTG could be enhanced if a low concentration of glycerol (around 5 g/L) is maintained. This fact could be applied for fed-batch or continuous fermentations. The first fed--batch fermentation was studied but without the addition of casein (*22*).

The product yield $Y_{p/s}$ of an enzyme can be defined as units of activity per g of biomass and the productivity as the units of activity per mL of medium and hour. The highest $Y_{p/s}$ was 52 U/g obtained at 48 h using 150 rpm. At this AS the $Y_{p/s}$ decreased with time until 17 U/g were reached. This trend was observed also at 250 rpm (from 36 to 20 U/g) and 350 rpm (from 26 to 19 U/g).

The volumetric activity of MTG is the main parameter to consider in the selection of optimal operational conditions. Therefore, 250 rpm and 72 h were selected as optimal because they gave an MTG activity of (0.63±0.05) U/mL with a productivity of 0.09 U/(mL·h).

Optimisation of nitrogen and carbon source

Xylose is a carbon source that can be used for biotechnological production of MTG. Junqua *et al.* (*15*) found that its use alone as carbon source does not produce MTG. Due to the interest in the application of xylose, as was commented in the introduction, a strategy for using xylose mixed with glycerol as carbon sources for the production of MTG was studied.

To determine the optimal level of glycerol, xylose and casein to improve MTG activity, an experimental design was conducted in order to obtain mathematical models for response surfaces that can help to find the optimal conditions. The models obtained using the response surface methodology do not have a physical or biophysical meaning, but they are very useful to optimise a process where several interactions occur, such as fermentation process or food process (*11,23,24*).

In our design, MTG production was induced by different levels of glycerol (0, 30, 60 g/L), xylose (0, 30, 60 g/L) and casein (20, 40, 60 g/L). The concentration of glycerol, xylose and casein were considered as opera-

tional variables (denoted G, X and C, respectively) and their effects on the selected dependent variable MTG activity (y) was measured. Fixed conditions were 72 h, 250 rpm and 26 °C.

The range of study for glycerol (0–60 g/L) and casein (20–60 g/L) variables were selected because glycerol 31.2 g/L and casein 38.4 g/L were reported as adequate for MGT production (*15,25*). Table 2 summarises the variables involved in the optimisation of the composition of media for the production of MTG by *Streptoverticillium ladakanum*.

The operational conditions assayed (in terms of dimensional and dimensionless operational variables) as well as the experimental results determined for y are shown in Table 3. The interrelationship between operational and dependent variables was established through an equation including linear interaction and second-order terms:

$$y = b_0 + \Sigma_i b_i x_i + \Sigma_i \Sigma_k b_{ik} x_i x_k \qquad /5/$$

where y and x_i or x_k (i or k=1–3, i≥k) are dependent or independent, normalised variables and b_0, b_i and b_{ik} are regression coefficients calculated from the experimental data by multiple linear regression. Table 4 shows the values of coefficients from the mathematical models and their statistical significance.

The experimental values of MTG activity (y) varied over a wide range (0.09–0.77 U/mL). The analysis of the main experimental trends showed that xylose concentration (dimensional variable X or dimensionless variable x_2) had a negative influence on MTG activity. However, a positive influence of the second order term (b_{22}) of xylose concentration was also observed. This explains the minimum obtained using xylose ≈45 g/L and the increase of MTG activity using xylose >50 g/L.

The coefficient of glycerol concentration (b_1) was not significant, but the second order term (b_{11}) and the interaction glycerol-xylose (b_{12}) were. No influence was found for the term of casein concentration (b_3) or interactions

Table 2. Variables used in the study of optimisation of nitrogen and carbon source in media

(a) Fixed variables			
Agitation	250 rpm		
Temperature	26 °C		
Fermentation time	72 h		
(b) Dimensional independent variables	Nomenclature	Units	Variation levels
Glycerol concentration	G	g/L	(0, 30, 60)
Xylose concentration	X	g/L	(0, 30, 60)
Casein concentration	C	g/L	(20, 40, 60)
(c) Dimensionless, normalised independent variables	Nomenclature	Definition	Variation levels
Dimensionless calcium concentration	x_1	(G-30)/30	(−1, 0, 1)
Dimensionless temperature	x_2	(X-30)/30	(−1, 0, 1)
Dimensionless time	x_3	(C-40)/20	(−1, 0, 1)
(d) Dependent variables	Nomenclature	Units	
MTG	y	U	

Table 3. Operational conditions assayed and experimental results achieved for MTG activity (y_1) as a function of glycerol (G), xylose (X) and casein (C) concentrations

Experiment	Independent variables						Dependent variables
	Dimensional			Dimensionless			
	G	X	C	x_1	x_2	x_3	y
1A	0	0	40	−1	−1	0	0.337
1B	0	0	40	−1	−1	0	0.340
2A	0	30	20	−1	0	−1	0.202
2B	0	30	20	−1	0	−1	0.201
3A	0	30	60	−1	0	1	0.155
3B	0	30	60	−1	0	1	0.155
4A	0	60	40	−1	1	0	0.146
4B	0	60	40	−1	1	0	0.137
5A	30	0	20	0	−1	−1	0.728
5B	30	0	20	0	−1	−1	0.765
6A	30	0	60	0	−1	1	0.628
6B	30	0	60	0	−1	1	0.665
7A	30	30	40	0	0	0	0.192
7B	30	30	40	0	0	0	0.174
8A	30	60	20	0	1	−1	0.165
8B	30	60	20	0	1	−1	0.119
9A	30	60	60	0	1	1	0.119
9B	30	60	60	0	1	1	0.119
10A	60	0	40	1	−1	0	0.650
10B	60	0	40	1	−1	0	0.610
11A	60	30	20	1	0	−1	0.137
11B	60	30	20	1	0	−1	0.128
12A	60	30	60	1	0	1	0.137
12B	60	30	60	1	0	1	0.137
13A	60	60	40	1	1	0	0.012
13B	60	60	40	1	1	0	0.090
14A	30	60	40	0	1	0	0.119
14B	30	60	40	0	1	0	0.101

glycerol-casein (b_{13}) and xylose-casein (b_{23}) on the MTG activity obtained.

The statistical parameters r^2 (0.96), F_{exp} (43.71), and F-test probability (0.91), measuring the correlation and significance of the model, showed a good agreement between experimental and predicted data for the regression.

The resulting variation pattern is shown in Fig. 4. It describes the dependence of the MTG activity on glycerol concentration and xylose concentration at three representative values of casein concentration. Glycerol concentration was the carbon source with more positive influence on the MTG production, suggesting that it also acts as an inducer. The model predicts that the maximum response (0.73 U/mL) can be obtained using glycerol 50.5 g/L and the lowest level of xylose and casein, xylose 0 g/L and casein 20 g/L. Junqua *et al.* (*15*) recommended casein 38.4 g/L to obtain 0.33 U/mL. Our study shows that the casein concentration can be reduced to 20 g/L, decreasing the cost of media.

Table 4. Regression coefficients and statistical significance of the model for nitrogen and carbon source of media

Coefficients	y
b_0	0.1775[a]
b_1	0.0142
b_2	−0.2398[a]
b_3	−0.0205
b_{12}	−0.0956[a]
b_{13}	0.0126
b_{23}	0.0193
b_{11}	−0.0713[a]
b_{22}	0.1828[a]
b_{33}	0.0518[b]
r^2	0.96
F-test prob.	0.91

[a] coefficients significant at 99 % confidence level;
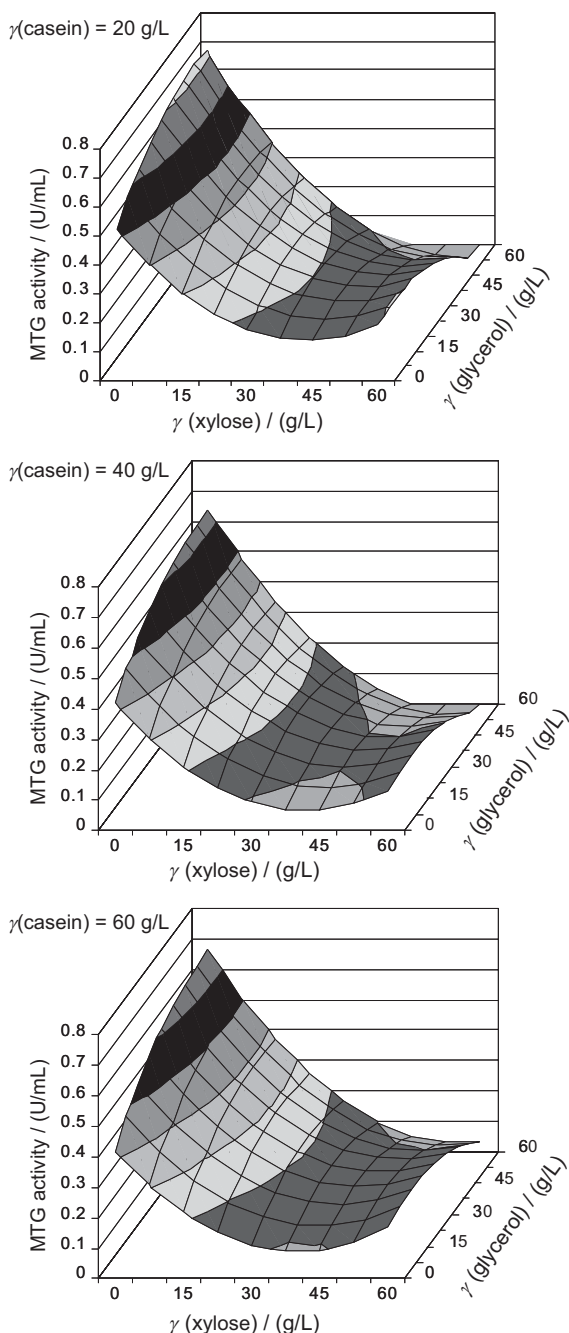[b] coefficients significant at 95 % confidence level

Fig. 4. Calculated dependence of microbial transglutaminase (MTG) activity on glycerol and xylose concentration for different levels of casein concentration

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Proizvodnja transglutaminaze iz *Streptoverticillium ladakanum* NRRL-3191 uz glicerol kao izvor ugljika

Sažetak

Enzim transglutaminaza katalizira stvaranje kovalentnih veza između susjednih proteina, čime se poboljšava njihova koloidna struktura, a ima i važnu primjenu u prehrambenoj industriji. Svrha je ovoga rada bila najprije utvrditi utjecaj brzine miješanja tijekom biotehnološke proizvodnje transglutaminaze iz *Streptoverticillium ladakanum* NRRL-3191 uz glicerol kao izvor ugljika, a zatim poboljšati proizvodnju transglutaminaze optimirajući sastav podloge temeljene na glicerolu, ksilozi i kazeinu. Brzina miješanja od 250 o/min i vrijeme fermentacije od 72 h rezultiralo je optimalnom enzimskom aktivnošću (0,628 U/mL) s produktivnošću od 0,087 U/(mL·h). Sastav podloge s glicerolom, ksilozom i kazeinom optimiran je koristeći eksperimentalnu konstrukciju kako bi se poboljšala proizvodnja transglutaminaze. Model predviđa da se maksimalna aktivnost transglutaminaze (0,725 U/mL) može dobiti koristeći 50,5 g/L glicerola i 20 g/L kazeina bez dodatka ksiloze.