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Production of Microbial Transglutaminase on Media Made from Sugar Cane Molasses and Glycerol

Oscar M. Portilla-Rivera[1], *Simón J. Téllez-Luis*[1], *José A. Ramírez de León*[1] *and Manuel Vázquez*[2]*

[1]Department of Food Science and Technology, UAM Reynosa-Aztlán, Autonomous University of Tamaulipas, Calle 16 y Lago de Chapala s/n, Col. Aztlán, Reynosa, MX-88740 Tamaulipas, Mexico

[2]Department of Analytical Chemistry, Veterinary Faculty, University of Santiago de Compostela-Campus de Lugo, ES-27002 Lugo, Spain

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Summary

Transglutaminase is an enzyme that catalyses an acyl transfer reaction between γ-carboxamide groups of glutaminyl residues and lysine residues in proteins. Due to this property, this enzyme is used for enhancing textural properties of protein-rich food. The transglutaminase used as food additive is obtained by microorganisms, mainly by *Streptoverticillium ladakanum*. On the other hand, sugar cane molasses is a viscous liquid rich in noncrystallized carbohydrates (saccharose, glucose and fructose). In this work, the feasibility of using sugar cane molasses as a carbon source for the production of microbial transglutaminase by *Streptoverticillium ladakanum* NRRL 3191 has been studied. Carbon sources including sugar cane molasses (60 g of total sugars per L), glycerol (60 g/L) and their mixture in a ratio of 1:1 (30 g/L of each) were evaluated. Time course of microbial growth, transglutaminase activity and carbon source consumption were determined every 24 h during 120 h of fermentations at three agitation speeds (200, 300 or 400 rpm). The results showed that with the increase in agitation speed, the biomass concentration increased up to 8.39 g/L in the medium containing sugar cane molasses alone or the mixture of molasses and glycerol. The highest transglutaminase activity was obtained at 400 rpm in the medium containing a mixture of molasses and glycerol, reaching 0.460 U/mL, while in the medium containing sugar cane molasses alone, the activity was 0.240 U/mL, and using glycerol alone it was 0.250 U/mL. These results show that sugar cane molasses is a suitable medium for transglutaminase production when it is combined with glycerol.

Key words: transglutaminase, glycerol, glucose, sugar cane molasses

Introduction

Transglutaminase (*R*-glutaminyl-peptide:amine γ-glutamyltransferase, EC 2.3.2.13) is an enzyme that catalyses an acyl transfer reaction between γ-carboxamide groups of glutamine residues and ε-amino groups of lysine residues (*1*).

The endogenous transglutaminase enzyme is used in fish muscle proteins to form a translucent and highly deformable gel after being solubilized with salt when incubated below 40 °C. This phenomenon is called setting. Some microorganisms also produce the enzyme transglutaminase. Both endogenous and microbial transglutaminase show similar optimal temperature for inducing setting in tropical fish species (40 °C) because this temperature is optimal for myosin denaturation (*2*).

*Corresponding author; E-mail: manuel.vazquez@usc.es

This enzyme is used in food technology to restructure protein-rich food such as meat and fish products. Furthermore, stability, water binding, mechanical and textural properties can be enhanced (*3,4*). The increasing demand for vegetarian food, the utilisation of novel proteins as functional ingredients from soy, peas, sesame and sunflower open new horizons for transglutaminase applications (*5*).

Microbial transglutaminase is considered an efficient but expensive additive for binding muscle proteins to produce restructured products. *Streptoverticillium ladakanum* NRRL 3191 is the main source of microbial transglutaminase. The main characteristic of the microbial transglutaminase is that it is extracellular and Ca^{2+}-independent (*6*). These properties increase the interest in this type of enzymes for the food industry.

Fermentation media can represent almost 30 % of the cost of a microbial production (*7*). General media employed for the growth of *Streptoverticillium* are not economically attractive and some economical media for the production of transglutaminase based on wastes from the agroindustry have been studied. For example, Téllez-Luis *et al*. (*8*) studied the transglutaminase production on hydrolysates of sorghum straw, obtaning up to 0.348 U/mL. A high transglutaminase activity (0.725 U/mL) was obtained using glycerol (1,2,3-propanetriol or glycerine) as carbon source by Téllez-Luis *et al.* (*9*), corroborating previous results about the beneficial effect of glycerol and casein on the production of transglutaminase by *S. ladakanum* (*10*). However, glycerol is a chemical with a high cost (approx. 20 $ per L) and it is of interest to find alternative raw materials of low cost for preparing fermentation media. One of these materials can be sugar cane molasses with a cost lower than 0.5 $ per L.

Sugar cane industry in countries like Mexico is facing a high pressure to improve their profitability. Different studies are being conducted to give added value to the by-products and to increase profitability of this industry (*11,12*). Sugar cane molasses is a by-product of the sugar cane industry. It contains monosaccharides (glucose and fructose) and a disaccharide (saccharose) in high concentrations that in the process of commercial sugar production do not crystallize, which makes their recovery difficult. Nowadays this by-product is mainly used for the animal feed, fermentation to ethanol and production of yeast (*13*). Biotechnological applications for sugar cane molasses have been studied for the production of a lot of additives and enzymes such as β-D-galactosidase by *Kluyveromyces marxianus* (*14*), glucosyltransferase by *Erwinia* sp. (*15*), ethanol by *Zymomonas mobilis* (*16*), lactic acid by *Enterococcus faecalis* (*17*), citric acid by *Aspergillus niger* (*18*) and sorbitol by *Zymomonas mobilis* (*19*). Until now, molasses has not been reported as a carbon source for the production of microbial transglutaminase.

The aim of this work is to elucidate if the biotechnological production of transglutaminase by *S. ladakanum* NRRL 3191 grown in the medium made from sugar cane molasses is attainable. Additionally, the effect of agitation speed and the mixture of sugar cane molasses with glycerol have been studied.

Materials and Methods

Raw material

Sugar cane molasses was obtained from a local factory (Ciudad Mante, Tamaulipas, Mexico). Glycerol was obtained from Sigma (USA).

Freeze-dried broths of wild *S. ladakanum* NRRL 3191 strain were obtained from the Agricultural Research Service Culture Collection (Peoria, IL, USA). Microorganisms were maintained on agar plates at 4 °C, and transferred monthly.

Culture media were prepared using diluted sugar cane molasses (60 g/L of total sugars), glycerol (60 g/L) or their mixture (sugar cane molasses with 30 g/L of total sugars and 30 g/L of glycerol) supplemented with (in g/L): sodium casein 38.4, peptone 10.5, yeast extract 2.5, Na_2HPO_4 5, KH_2PO_4 2, and $MgSO_4$ 0.5.

Experiments were carried out in duplicate during 120 h at 26 °C in orbital shakers (agitation speed: 200, 300 or 400 rpm) using 250-mL Erlenmeyer flasks with 100 mL of culture medium.

Previous studies showed that the heat sterilization of the whole medium produces reactions that affect the fermentation, decreasing the production of transglutaminase (*8,9*). Therefore, media were sterilized in an autoclave, except sugar cane molasses, which was sterilized by filtration through a membrane (0.2 μm) before mixing with the rest of the media.

Analytical methods

Samples were withdrawn from the fermentation media at 24, 48, 72, 96 and 120 h and centrifuged (4500×*g*, 10 min). Supernatants were analyzed for glucose, fructose, saccharose and glycerol by HPLC and for transglutaminase activity by a colorimetric procedure.

HPLC analysis was carried out using a Transgenomic ION-300 column (oven temperature=45 °C) with isocratic elution (flow rate=0.4 mL/min; mobile phase: H_2SO_4 0.0025 M) and a refraction index detector. Pellets were washed twice with a solution of 9 g of sodium chloride per L in deionized water, centrifuged again and then dried at 102 °C for 48 h, in order to allow the calculation of biomass concentration on dry mass basis.

Transglutaminase activity was measured by a colorimetric procedure based on the formation of hydroxamate from *N*-carbobenzoxy-L-glutaminylglycine (*20*). One unit of activity (U) was defined as the amount that causes the formation of 1 μmol of hydroxamate in 1 min at 37 °C.

Statistical analysis

All experimental data were carried out in duplicate, and mean values and standard deviations were calculated. Fitting of models by nonlinear regression analysis using least squares regression was performed applying Microsoft Excel 2003 (Microsoft Corporation, Redmond, WA, USA) software. Microsoft PowerPoint 2003 (Microsoft Corporation, Redmond, WA, USA) was used to plot the experimental data and models.

Results and Discussion

Fermentations on sugar cane molasses

The composition of the original sugar cane molasses was (in g/L): saccharose 609.8, glucose 116.5, and fructose 158.3. Total sugar concentration (sum of saccharose, glucose and fructose) was 884.6 g/L. This concentration is too high to be fermented. In order to assess the possibility of using sugar cane molasses for culturing *S. ladakanum* NRRL-3191, the experiments were carried out expecting a concentration of around 60 g of total sugars per L as adequate on the basis of previous works (*8,9*). Therefore, the molasses was diluted with distilled water to obtain a fermentation medium with a total sugar concentration close to 60 g/L.

Several authors applied low agitation speed (around 140–200 rpm), but they did not justify it (*10,21*). Téllez-Luis *et al*. (*9*) found that 250 rpm allowed higher transglutaminase activity than lower speeds using glycerol as a carbon source.

A set of experiments was conducted at 200, 300 or 400 rpm using the culture medium nutrients described above, which were proposed by Junqua *et al.* (*10*), and using the diluted sugar cane molasses as a carbon source. The aim was to determine the effect of agitation speed in flask fermentations. Biomass, glucose, fructose and saccharose concentrations and transglutaminase activity were determined at given times.

The highest biomass concentration of (8.39±0.61) g/L (Fig. 1) was obtained after 72 h at 400 rpm. The increase of agitation speed enhanced the biomass production. This can be due to the fact that an increase in agitation speed increases the oxygenation of the culture media. Dissolved oxygen is one of the main factors that affect microbial growth and the formation of transglutaminase by *S. ladakanum* (*22*).

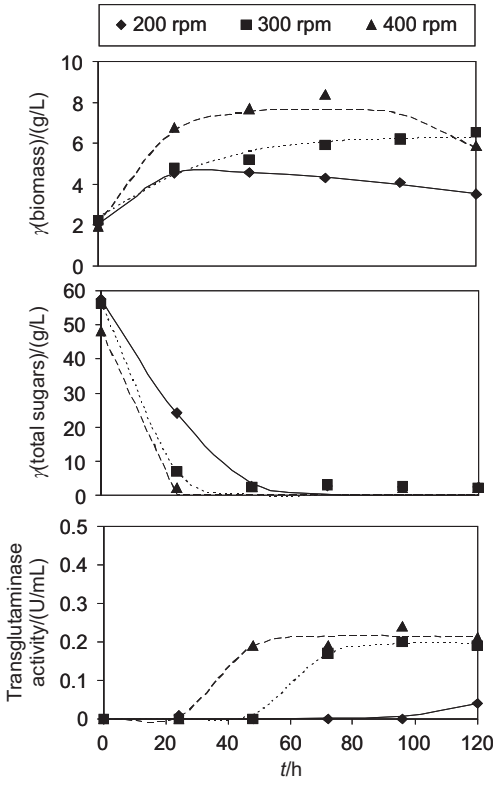

Fig. 1. Dependence of biomass concentration, total sugar concentration and microbial transglutaminase activity on the fermentation time of *Streptoverticillium ladakanum* NRRL 3191 grown on molasses at different agitation speeds

Glucose, fructose and saccharose concentrations were given as total sugar concentration, as shown in Fig. 1. The initial total sugar concentrations were 57.62, 56.15 and 48.13 g/L for fermentations at 200, 300 and 400 rpm, respectively. The initial concentration of glucose was 8.91 g/L at 200 rpm, which increased to 11.68 g/L in 24 h and then decreased to 0.4 g/L in 72 h. This behaviour showed that the decomposition of saccharose generated glucose, which was then consumed. The rate of glucose consumption increased with the agitation speed, thus the increase of glucose in 24 h was not detected in the fermentations at 300 or 400 rpm. All sugars were consumed in 48 h at 200 rpm and in 24 h at 300 and 400 rpm, with residual values remaining (2–3 g/L).

The agitation speed showed a beneficial effect on transglutaminase activity. At 200 rpm, the activity was not detected until 120 h and when it was detected, it was very low (0.040 U/mL). The initial transglutaminase activity was detected at 72 h and 300 rpm (0.170 U/mL), and at 48 h and 400 rpm (0.190 U/mL). The highest transglutaminase activity (0.240 U/mL) was obtained at 96 h and 400 rpm.

The use of kinetic models allows predicting the results that can be obtained under operational conditions that were not experimentally studied. The obtained parameters also help to understand the overall process.

To determine the biomass concentration of *S. ladakanum*, Téllez-Luis *et al*. (*9*) used a mathematical model adopted from a study to describe the fermentation of lactic acid based on the logistic equation:

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

where t is time, X is biomass concentration, X_0 is initial concentration of the biomass, X_m is maximum concentration of the biomass, and R_x is the rate of biomass formation.

The logistic equation is not adequate for fitting fermentations when a decrease in the biomass concentration is observed at the end of the fermentation. Therefore, in this study a new model was developed, including a rate of biomass death (R_d):

$$X=\frac{X_0 X_m e^{(R_x t - R_d t^2)}}{X_m - X_0 + \left(X_0 e^{(R_x t - R_d t^2)}\right)} \qquad /2/$$

The new parameter (R_d) depends on the time squared. Thus, its effect increased with time and caused a decrease in the biomass concentration at the end of the fermentation.

Table 1 shows the results of fitting the experimental data to the model and the statistical parameters r^2 and F-test probability. The model predicts that the biomass concentration increases with the agitation speed, from 4.83 g/L at 200 rpm to 7.80 g/L at 400 rpm, confirming that the dissolved oxygen is one of the main factors of the biomass production. The values of the rate of bio-

Table 1. Results of initial biomass concentration (X_0), maximum biomass concentration (X_m), rate of biomass formation (R_x) and rate of biomass death (R_d) obtained by regression analysis of biomass concentration of *Streptoverticillium ladakanum* NRRL 3191 grown on molasses using different agitation speeds. Statistical parameters of the models are also shown

Agitation speed/rpm	X_0 g/L	X_m g/L	R_x h^{-1}	R_d h^{-1}	r^2	*F*-test
200	2.07	4.83	0.134	0.00002	0.9967	0.9960
300	2.37	6.46	0.061	0.00022	0.9677	0.9507
400	2.06	7.80	0.139	0.00102	0.9131	0.9060

mass formation were 3–4 orders of magnitude higher than the values of the rate of biomass death. The rate of biomass death showed an exponential evolution with the agitation speed of 0.00002 h^{-1} at 200 rpm, 0.00022 h^{-1} at 300 rpm and 0.00102 h^{-1} at 400 rpm.

The coefficient r^2 showed a good agreement between experimental and predicted data for all regressions in the range of 0.91–0.99. The values of *F*-test probability showed that the model for 400 rpm is the least accurate.

With the obtained models, it is possible to predict any biomass concentration in the range of time of the study (0–120 h). The maximum biomass concentration (7.71 g/L) was predicted at 68.3 h and 400 rpm.

To determine the total sugar concentration, a model based on the prediction of a global substrate uptake rate was fitted:

$$S=\frac{S_0 S_m e^{-SUR\cdot t}}{S_m-S_0+\left(S_0 e^{-SUR\cdot t}\right)} \qquad /3/$$

where t is time, S is total sugar concentration (sum of saccharose, glucose and fructose), S_0 is initial concentration of sugars, S_m is maximum concentration of sugars and SUR is the substrate uptake rate.

Table 2 shows the values obtained by fitting the experimental data. The substrate uptake rate increased with the agitation speed from 0.096 h^{-1} at 200 rpm to 0.791 h^{-1} at 400 rpm. This high rate at 400 rpm is consistent with the low concentration of sugars obtained at 24 h (2.34 g/L). Both coefficients r^2 and *F*-test probability showed a good fitting for all agitation speeds.

Table 2. Results of initial total sugar concentration (S_0), maximum total sugar concentration (S_m) and substrate uptake rate (SUR) obtained by regression analysis of total sugar consumption by *Streptoverticillium ladakanum* NRRL 3191 grown on molasses using different agitation speeds. Statistical parameters of the models are also shown

Agitation speed/rpm	S_0 g/L	S_m g/L	SUR h^{-1}	r^2	*F*-test
200	57.63	68.39	0.096	0.9971	0.9413
300	56.14	56.49	0.295	0.9978	0.9254
400	48.13	46.74	0.791	0.9997	0.9102

An empirical model analogue to the biomass model was developed to express the transglutaminase activity:

$$P=\frac{P_0 P_m e^{\left(R_e t-R_{dn}t^2\right)}}{P_m-P_0+\left(P_0 e^{\left(R_e t-R_{dn}t^2\right)}\right)} \qquad /4/$$

where t is time, P is transglutaminase activity, P_0 is initial transglutaminase activity, P_m is maximum transglutaminase activity, R_e is the rate of enzyme formation, and R_{dn} is the rate of enzyme denaturation.

Table 3 shows the values obtained by fitting this model for each agitation speed. Both r^2 coefficient and *F*-test probability showed a good fitting for all agitation speeds except 200 rpm, where *F*-test probability showed a value of 0.8883, indicating that the model is not accurate. This can be due to the fact that the activity was detected only at 120 h.

Table 3. Results of initial transglutaminase activity (P_0), maximum transglutaminase activity (P_m), rate of enzyme formation (R_e) and rate of enzyme denaturation (R_{dn}) obtained by regression analysis of transglutaminase activity generated by *Streptoverticillium ladakanum* NRRL 3191 grown on molasses using different agitation speeds. Statistical parameters of the models are also shown

Agitation speed/rpm	P_0 U/mL	P_m U/mL	R_e h^{-1}	R_{dn} h^{-1}	r^2	*F*-test
200	0.000	0.100	0.088	0.0000	0.9686	0.8883
300	0.000	0.196	0.193	0.0000	0.9973	0.9680
400	0.000	0.216	0.400	0.0022	0.9790	0.9640

The values obtained for the regression coefficients showed that the highest transglutaminase activity predicted was 0.216 U/mL at 400 rpm. However, the values of the enzyme denaturation rate (0 h^{-1} at 200 and 300 rpm, but 0.0022 h^{-1}at 400 rpm) suggest that the excessive increase in the agitation speed can cause stress that leads to denaturation. The enzyme activity also grew when the sugars were depleted. The same was observed by Junqua *et al.* (*10*). This could be because transglutaminase can be produced in the medium like zymogene during the time that the microorganism consumes the sugars. The zymogene is hydrolyzed by proteases to give the transglutaminase. This can also happen when the sugars have been depleted (*23*,*24*).

The results show that sugar cane molasses is a suitable carbon source for the production of transglutaminase, but the maximum transglutaminase activity obtained was low compared to the activity obtained by *S. mobaraense* CBS 20778 mutant using a medium based on starch, which reached up to 3.32 U/mL in 6 days (*22*).

Fermentations on glycerol

New fermentations were conducted using glycerol as a carbon source to compare the results to those obtained with sugar cane molasses. Fig. 2 shows the kinetics of the fermentations performed on glycerol. The highest biomass concentration ((10.03±0.20) g/L) was obtained at 120 h and 400 rpm. The increase of the agitation speed clearly increased the biomass production.

The values of the regression parameters for biomass equation are shown in Table 4. The model gave a maxi-

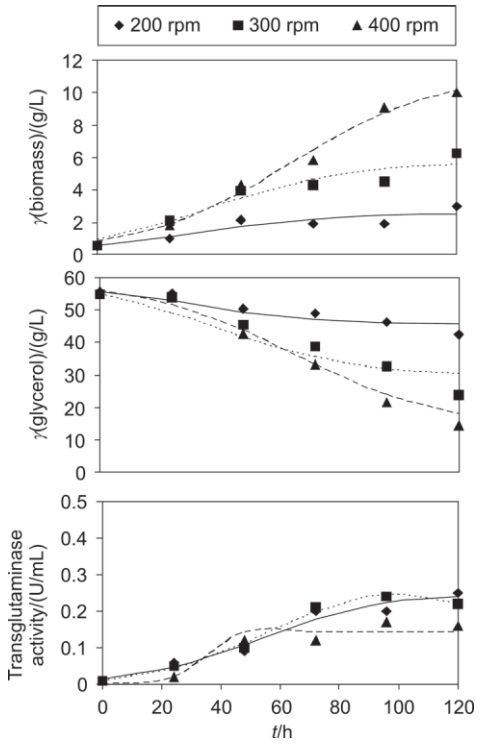

Fig. 2. Dependence of biomass concentration, glycerol concentration and microbial transglutaminase activity on the fermentation time of *Streptoverticillium ladakanum* NRRL 3191 grown on glycerol at different agitation speeds

Table 4. Results of initial biomass concentration (X_0), maximum biomass concentration (X_m), rate of biomass formation (R_x) and rate of biomass death (R_d) obtained by regression analysis of biomass concentration of *Streptoverticillium ladakanum* NRRL 3191 grown on glycerol using different agitation speeds. Statistical parameters of the models are also shown

Agitation speed/rpm	X_0 g/L	X_m g/L	R_x h^{-1}	R_d h^{-1}	r^2	*F*-test
200	0.53	2.55	0.045	0.00000	0.8170	0.7890
300	0.86	6.31	0.050	0.00015	0.9296	0.8864
400	0.73	12.47	0.045	0.00008	0.9901	0.9812

mum biomass concentration of 12.47 g/L, but at 120 h the predicted maximum was 10.16 g/L. The values of biomass formation rate were in the range of 0.045–0.050 h^{-1}. Thus no effect of agitation speed on the rate of biomass formation was observed. These values are lower than those obtained for sugar cane molasses (0.061–0.139 h^{-1}). The values of the rate of biomass death are also lower, because the final biomass concentrations obtained are higher when using glycerol than when using sugar cane molasses.

Glycerol was not consumed totally. Residual concentrations of 42.2, 23.8 and 14.4 g/L were obtained in 120 h at 200, 300 or 400 rpm, respectively. Eq. 3 was used to fit the glycerol concentration, but in this case, *S* is glycerol concentration, S_0 is initial glycerol concentration and S_m is maximum glycerol concentration. Table 5 shows the results of the regression. All r^2 coefficients and *F*-test probability showed a good fitting. The substrate uptake rate increased with the agitation speed, like in the case of sugar cane molasses, but the values were much lower (from 0.010 to 0.030 h^{-1}). This confirms the obtained high residual concentrations.

Table 5. Results of initial glycerol concentration (S_0), maximum glycerol concentration (S_m) and substrate uptake rate (SUR) obtained by regression analysis of glycerol consumption by *Streptoverticillium ladakanum* NRRL 3191 grown on glycerol using different agitation speeds. Statistical parameters of the models are also shown

Agitation speed/rpm	S_0 g/L	S_m g/L	SUR h^{-1}	r^2	*F*-test
200	55.65	65.39	0.010	0.9776	0.9916
300	54.76	63.34	0.021	0.9882	0.9494
400	55.76	61.03	0.030	0.9941	0.9724

The pattern for transglutaminase activity at 200 and 300 rpm was similar to the maximum values (around 0.240 U/mL), as it can be seen in Fig. 2. Using 400 rpm, the transglutaminase activity was lower. The results of fitting the Eq. 4 for the fermentations on glycerol shown in Table 6 can help to understand this behaviour. The rate of enzyme formation increases with the agitation speed. This rate is lower than that obtained using sugar cane molasses at 200 and 300 rpm, and similar to that at 400 rpm. The main difference is in the rate of enzyme denaturation, which is much higher in the fermentation on glycerol (0.00102 h^{-1}) than in the fermentation on sugar cane molasses (0.0022 h^{-1}). Thus, the transglutaminase activity obtained was only 0.170 U/mL in glycerol in 96 h at 400 rpm, while in molasses it was 0.240 U/mL. The low value obtained in glycerol can be explained by the higher viscosity of the medium containing glycerol and by the increase of the stress due to the effect of the transglutaminase on sodium caseinate, generating bonds into adjacent molecules of caseinate (*25*). The maximum transglutaminase activity obtained was 0.250 U/mL in 120 h at 200 rpm. The maximum productivity was 0.0025 U/(mL·h) obtained in 96 h at 300 rpm. These results compare very well with those obtained in a synthetic medium containing glycerol by *Bacillus circulans*, where transglutaminase activity of 0.194 U/mL was obtained in 8 days (*26*).

Table 6. Results of initial transglutaminase activity (P_0), maximum transglutaminase activity (P_m), rate of enzyme formation (R_e) and rate of enzyme denaturation (R_{dn}) obtained by regression analysis of transglutaminase activity generated by *Streptoverticillium ladakanum* NRRL 3191 grown on glycerol using different agitation speeds. Statistical parameters of the models are also shown

Agitation speed/rpm	P_0 U/mL	P_m U/mL	R_e h^{-1}	R_{dn} h^{-1}	r^2	*F*-test
200	0.016	0.249	0.050	0.00000	0.9645	0.9644
300	0.011	1.812	0.065	0.00033	0.9923	0.9929
400	0.000	0.143	0.453	0.00102	0.9116	0.9980

Fermentations on mixtures of sugar cane molasses and glycerol

A medium was prepared mixing diluted sugar cane molasses (around 30 g/L of total sugars) and glycerol (30 g/L) to obtain a final concentration of around 60 g/L to compare it with the previous fermentations. Fig. 3 shows the kinetics of the fermentations performed in the mixture of sugar cane molasses and glycerol. The highest biomass concentration ((10.98±0.32) g/L) was obtained in 96 h using the highest agitation speed (400 rpm). Lower agitation speeds were not adequate for the biomass production, the biomass concentration remained in values close to 3–4 g/L.

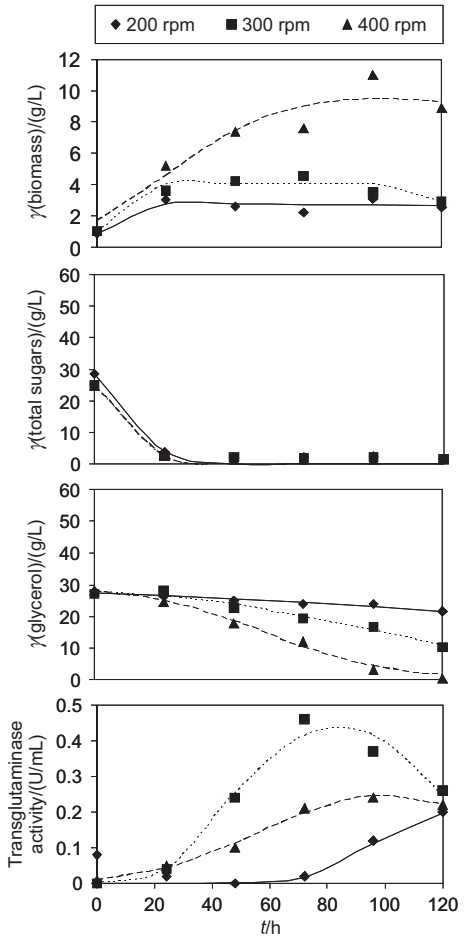

Fig. 3. Dependence of biomass concentration, total sugar concentration, glycerol concentration and microbial transglutaminase activity on the fermentation time of *Streptoverticillium ladakanum* NRRL 3191 grown on the mixture of molasses and glycerol at different agitation speeds

The models obtained for biomass were significant with values of r^2 and *F*-test probability over 0.90 for 300 and 400 rpm. The values of the regression parameters are shown in Table 7. The maximum predicted biomass concentration was 11.90 g/L for the fermentation at 400 rpm. Using this agitation speed, the values of the rate of biomass formation and biomass death were in the middle of those of the fermentation on glycerol (lower) and on sugar cane molasses (higher).

Table 7. Results of initial biomass concentration (X_0), maximum biomass concentration (X_m), rate of biomass formation (R_x) and rate of biomass death (R_d) obtained by regression analysis of biomass concentration of *Streptoverticillium ladakanum* NRRL 3191 grown on the mixture of molasses and glycerol using different agitation speeds. Statistical parameters of the models are also shown

Agitation speed/rpm	X_0 g/L	X_m g/L	R_x h^{-1}	R_d h^{-1}	r^2	*F*-test
200	0.88	2.78	0.633	0.00000	0.8495	0.9134
300	1.05	3.98	0.714	0.00581	0.9032	0.9151
400	1.65	11.90	0.064	0.00032	0.9144	0.8762

The consumption of carbon sources was different for sugars and glycerol. All the sugars were consumed in 24 h, less than 4 g/L remaining in the media at the end of the fermentations. Glycerol began to be consumed after 24 h, when sugars were depleted. Tables 8 and 9 show the values of coefficients of the models for sugars and glycerol, respectively.

Table 8. Results of initial total sugar concentration (S_0), maximum sugar concentration (S_m) and substrate uptake rate (SUR) obtained by regression analysis of total sugar consumption by *Streptoverticillium ladakanum* NRRL 3191 grown on the mixture of molasses and glycerol using different agitation speeds. Statistical parameters of the models are also shown

Agitation speed/rpm	S_0 g/L	S_m g/L	SUR h^{-1}	r^2	*F*-test
200	28.61	29.61	0.217	0.9947	0.8846
300	24.92	26.04	0.222	0.9931	0.8771
400	24.97	25.31	0.271	0.9967	0.9121

Table 9. Results of initial glycerol concentration (S_0), maximum glycerol concentration (S_m) and substrate uptake rate (SUR) obtained by regression analysis of glycerol consumption by *Streptoverticillium ladakanum* NRRL 3191 grown on the mixture of molasses and glycerol using different agitation speeds. Statistical parameters of the models are also shown

Agitation speed/rpm	S_0 g/L	S_m g/L	SUR h^{-1}	r^2	*F*-test
200	27.34	31.58	0.009	0.9266	0.9407
300	27.92	30.07	0.026	0.9685	0.9695
400	27.79	29.33	0.049	0.9920	0.9492

The values of SUR for total sugars were in the range of 0.217–0.271 h^{-1}, *i.e.* similar to those of the fermentation of sugar cane molasses at 300 rpm. The values of SUR for glycerol were a little higher than the values of the fermentation of glycerol alone.

The transglutaminase activity obtained at 200 rpm (0.200 U/mL at 120 h) and 400 rpm (0.240 U/mL at 96 h) was similar to that obtained using sugar cane molasses or glycerol separately. However, the production at 300 rpm was 0.460 U/mL in 72 h, decreasing to 0.260

U/mL in 120 h. Table 10 shows the results of fitting the models for enzyme activity. At 300 rpm, the rate of enzyme formation was 0.168 h^{-1}, a value close to that of the fermentation of sugar cane molasses alone (0.193 h^{-1}). The rate of enzyme denaturation was 0.00100 h^{-1} at 300 rpm, similar to the value of the fermentation of glycerol alone (0.00102 h^{-1}). The maximum activity obtained in the fermentation at 300 rpm could be explained by a high denaturation rate at 400 rpm and by diffusional problems at 200 rpm, which disappear at 300 rpm.

Table 10. Results of initial transglutaminase activity (P_0), maximum transglutaminase activity (P_m), rate of enzyme formation (R_e) and rate of enzyme denaturation (R_{dn}) obtained by regression analysis of transglutaminase activity generated by *Streptoverticillium ladakanum* NRRL 3191 grown on the mixture of molasses and glycerol using different agitation speed. Statistical parameters of the models are also shown

Agitation speed/rpm	P_0 U/mL	P_m U/mL	R_e h^{-1}	R_{dn} h^{-1}	r^2	*F*-test
200	0.000	0.213	0.104	0.00000	0.9999	0.9934
300	0.001	0.598	0.168	0.00100	0.9739	0.9721
400	0.010	1.040	0.069	0.00035	0.9922	0.9978

The model predicts a maximum transglutaminase activity of 0.433 U/mL at 83.75 h and 300 rpm. The productivity at 72 h and 300 rpm was 0.0064 h^{-1} and the product yield was 100 U/g.

The comparison of the transglutaminase production by *S. ladakanum* in the mixture of sugar cane molasses and glycerol with other substrates is favourable. For example, the production of transglutaminase in hydrolysates of sorghum straw (*8*) gave an activity of 0.348 U/mL at 72 h. Moreover, the use of sugar cane molasses does not require additional treatments like in the case of using any kind of hydrolysates.

Conclusion

The mixture of sugar cane molasses and glycerol is an adequate culture medium for the production of transglutaminase by *Streptoverticillium ladakanum*. The results obtained suggest a synergistic effect of both carbon sources, duplicating the production of that obtained when they were used separately.

The glucose, fructose and saccharose of the sugar cane molasses were consumed in 24–48 h, but glycerol was not consumed totally in 120 h. The highest transglutaminase activity (0.460 U/mL) was obtained in the mixture of sugar cane molasses and glycerol after 72 h at 300 rpm.

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