Appl Biochem Biotechnol (2009) 158:302–312
DOI 10.1007/s12010-008-8338-5

Environmental Effects on Transglutaminase Production and Cell Sporulation in Submerged Cultivation of *Bacillus circulans*

Claucia Fernanda Volken de Souza · Gilvane Souza de Matos · Simone Hickmann Flôres · Marco Antônio Záchia Ayub

Received: 19 May 2008 / Accepted: 31 July 2008 /
Published online: 21 August 2008

Abstract In this research, the effects of pH, temperature, and oxygen on growth kinetics of a newly isolated strain of *Bacillus circulans* from the Amazon and their correlations with transglutaminase (TGase) production and cell sporulation were investigated. Statistical experimental methods were used to optimize these parameters, while induction of sporulation was achieved by oxygen culture control. Full factorial composite experimental design and response surface methodology were experimentally tested. The model showed that temperature has a positive and significant effect on TGase production ($P<0.05$) while pH and temperature, associated with anoxic conditions, have a marked effect on cell sporulation which is consistently linked with TGase production. The contour plot of results showed that the best culture conditions for TGase production of *B. circulans* were 30°C, initial pH 8.5, and the highest production was obtained in late-stationary culture phase with maximal specific enzyme activity of 655 U g^{-1} of cells (0.37 U/mL). A correlation between enzyme production and cell sporulation, as mediated by oxygen culture conditions, was also demonstrated and, although demonstrated only for *B. subtilis*, it corroborates the molecular mechanisms involved in this process. It can be suggested that *B. circulans* BL32 is a strong biological system for the industrial production of TGases.

Keywords *Bacillus circulans* BL32 · Cell sporulation · Culture conditions optimization · Microbial transglutaminase · Submerged bacterial cultivation

Introduction

Transglutaminase ([TGase]; protein-glutamine γ-glutamyltransferase; EC 2.3.2.13) is an economically important enzyme capable of catalyzing acyl transfer reactions by introducing covalent cross-links between proteins, as well as peptides and various primary amines [1, 2].

C. F. V. de Souza · G. S. de Matos · S. H. Flôres · M. A. Z. Ayub (✉)
Food Science and Technology Institute, Federal University of Rio Grande do Sul, Av. Bento Gonçalves, 9500, P.O. Box 15090, ZIP 91501-970 Porto Alegre, RS, Brazil
e-mail: mazayub@ufrgs.br

Recent developments on the production and uses of this enzyme are on the increase due to its attractive applications in the food industries [2], pharmaceuticals [3], textiles [4], and in the formation of immunoconjugates for ELISA tests and biosensors [5]. For a long time, non-microbial sources of TGase, especially guinea pig liver, were the only commercial preparations available of this enzyme. The scarce source and the complicated separation and purification process for obtaining tissue TGase have resulted in an extremely high price for this enzyme [6] making its use prohibitive in food processing and other low-priced products. In the last few years, however, biotechnological research aiming at the production of microbial TGases [7], along with downstream processing developments [8] and improved fermentation processes [6, 9, 10] are on the increase to boost productivity and to reduce separation costs. Notwithstanding, only *Streptoverticillium* species have so far been used for microbial TGase production.

The efforts to improve TGase production has also to take into account the physiological aspects of spore formation. Although not yet elucidated for *B. circulans*, recent reports on the relationship between cell sporulation and TGase production have been well established for *Bacillus subtilis*. Nutrient limitations will trigger *B. subtilis* sporulation, a process in which TGase has a role in the cross-linking of proteins such as GerQ to generate high-molecular-mass proteins involved in the assembly of the spore coating [11, 12].

Response surface methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the effects of several factors in order to obtain high productivity in several industrial processes. This method has been successfully applied in many areas of biotechnology and bioprocesses, including some studies on optimization of medium to TGase production by *Streptoverticillium* species [13, 14] and *Bacillus circulans* [15]. In biotechnology-based industrial processes, the culture conditions are of critical importance because they affect product concentration, yield, and volumetric productivity. In developing an optimal process for commercial production of microbial metabolites, the major aspects usually considered for improvement are effects of environmental conditions, such as pH and temperature, and the selection of suitable nutrient media. For instance, TGase production by *Streptoverticillium mobaraense* is greatly influenced by physical factors, such as pH [10] and temperature [6], nevertheless the factors were analyzed independently, without an appropriate methodology allowing the overall study of the effects, as well as of their interactions.

In previous works [15, 16], the nutritional factors affecting the TGase production by *Bacillus circulans* BL32 in submerged cultivation and TGase chemical characterization were investigated. In this work, the effects of temperature, pH, and oxygenation of medium, at different incubation times, on cell sporulation and TGase induction was investigated.

Materials and Methods

Microorganism

A strain of *B. circulans*, coded BL32, was used in this study. It has been recently isolated from the Amazon environment and its isolation and characterization were described elsewhere [15, 16]. This strain is deposited in culture collections of The Federal University of Rio Grande do Sul State (Brazil). Before culturing, cells were recovered from frozen stocks in 50% glycerol (*v*/*v*) and were kept at 4°C on Mueller–Hinton agar (Merck) plates.

Inocula Preparation

Erlenmeyer flasks (250 mL) containing 50 mL medium (M1), optimized in our previous work [15], composed of (g L^{-1}): glycerol 9.0, sucrose 2.0, peptone 7.0, tryptone 1.0, Na_2HPO_4 1.0, $MgSO_4 \cdot 7H_2O$ 1.0 and $FeSO_4 \cdot 7H_2O$ 0.1, were inoculated with a single colony from a stock culture and incubated at 30°C in a rotary shaker at 100 rpm and grown to optical density (OD) of 1.0 at 600 nm (approximately 20 h). This culture procedure was then used as the standard inoculum preparation for all experiments.

Cultivation Procedures in Shaker

The effects of pH and temperature on TGase production were investigated on shaker cultures. The initial pH of medium M1 was adjusted according to the experimental design described in this work and were sterilized by autoclaving at 121°C for 15 min. Twenty milliliters of the standard inoculum were transferred to 2 L Erlenmeyer flasks containing 400 mL of medium. Cultivations were conducted in a rotary shaker at 100 rpm under different temperatures for each set of experiment. Samples were periodically removed. TGase activity, biomass, and frequency of sporulation were determined as described below.

Bioreactor Cultivations

The induction of cell sporulation was studied in bioreactor cultures. These cultures were run on a 2-L bioreactor (BBraun model B, Germany), fully equipped with probes for pH, temperature, and dissolved oxygen tension, aeration, and agitation systems. The vessel was filled with 1.9 L of medium M1 and inoculated with 100 mL of a 1.0 OD cell suspension of pre-inoculum as described above. Conditions of culture were 30°C, pH 8.5, controlled with addition of 1 M NaOH solution, and varying conditions of oxygen tension: (a) 400 rpm, 0 vvm air (anoxic conditions throughout cultivation); (b) 400 rpm, 2 vvm air (aerobic conditions throughout cultivation); (c) 400 rpm, initial air supply of 2 vvm up to the end of exponential growth phase, followed by no air supply during the stationary phase.

Experimental Design

The response surface methodology by using a set of experimental designs was performed to determine the optimal levels of the variables, temperature and initial pH, on TGase production. The upper and lower limits were set to be in the range described in the literature and, also, based on our previous experience. A 2^2 full factorial central composite design for two independent variables, each one at five levels with four star points and three replicates at the central point, was employed to fit a second-order polynomial model in which 11 experiments were required for this procedure. In the statistical model, Y denotes either TGase specific activity (U g^{-1} of dry cells) or dry cell weight (g L^{-1}). Table 1 shows the actual levels corresponding to the coded settings, the treatment combinations and responses. The enzyme activity and biomass values were determined after five to nine incubation days but the regression equation was made with the values obtained after 8 days (maximal value of the TGase specific activity).

This design is represented by a second-order polynomial regression model:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (1)$$

Table 1 Process variables used in the CCD, showing the treatment combinations and the mean experimental responses.

Run No	Coded setting levels		Actual levels		TGase specific activity (U g^{-1} of dry cells)					Dry cell weight (g L^{-1})				
	X_1	X_2	X_1	X_2	Day 5	Day 6	Day 7	Day 8	Day 9	Day 5	Day 6	Day 7	Day 8	Day 9
1	−1	−1	5.1	23	174	200	220	237	297	1.20	1.03	0.94	0.97	0.73
2	−1	1	5.1	37	440	531	481	518	552	0.46	0.44	0.43	0.39	0.31
3	1	−1	7.9	23	184	218	343	397	430	1.11	0.93	0.63	0.59	0.50
4	1	1	7.9	37	480	571	618	566	528	0.51	0.46	0.45	0.50	0.46
5	−1.41	0	4.5	30	504	480	606	576	598	0.59	0.58	0.48	0.50	0.48
6	1.41	0	8.5	30	475	540	568	655	636	0.65	0.58	0.56	0.56	0.50
7	0	−1.41	6.5	20	106	120	137	171	218	1.20	1.19	1.10	1.08	0.83
8	0	1.41	6.5	40	411	475	490	369	356	0.50	0.46	0.42	0.45	0.48
9	0	0	6.5	30	333	404	415	434	460	0.88	0.75	0.72	0.70	0.62
10	0	0	6.5	30	400	543	530	528	544	0.76	0.55	0.56	0.57	0.54
11	0	0	6.5	30	341	472	469	497	493	0.85	0.60	0.61	0.59	0.56

The results are the mean of three replications.

$x_1=(X_1-6.5)/1.4$; $x_2=(X_2-30)/7$; x_1 and x_2 are coded values; X_1 and X_2 are the actual values; X_1=initial pH; X_2=temperature (°C)

Where: Y = response variable, β_0 = constant, β_i = coefficient for the linear effect, β_{ii} = coefficient for the quadratic effect, β_{ij} = coefficient for the interaction effect, and x_i and x_j = the coded level of variable X_i and X_j. The above quadratic equation was used to plot surfaces for the variables.

The test factors were coded according to the following equation:

$$x_i = \left(\frac{X_i - X_0}{\Delta X_i}\right) \tag{2}$$

Where: x_i is the *coded* value and X_i is the *actual* value of the ith independent variable, X_0 is the actual value at the center point, and ΔX_i is the step change value.

All statistical experimental designs and results analyses were carried out using Statistica 5.0 software (Statsoft, USA). The statistical analysis of the model was performed in the form of analysis of variance (ANOVA). The significance of the regression coefficients and the associated probabilities $p(t)$ were determined by Student's t test; the second-order model equation significance was determined by Fisher's F test. The variance explained by the model is given by the multiple determination coefficient, R^2. For each variable, the quadratic models were represented as contour plots (2D).

Assay Methods

The colorimetric hydroxamate procedure described by Grossowicz et al. [17] was used for transglutaminase activity determination. Aliquots of 2 mL of the culture were centrifuged at 12,000×g for 5 min at 4°C. The cell-free supernatant was used for the estimation of enzyme activity over the substrate carbobenzoxy-L-glutaminyl-glycine [17]. A calibration curve was prepared with L-glutamic acid γ-monohydroxamate. One enzymatic unit (U) causes the formation of 1 μmol hydroxamic acid per minute. Enzyme was expressed as specific activity (U g^{-1} of dry cells).

The biomass concentration was determined by measuring turbidity at 600 nm and correlating with a dry cell weight calibration curve. To prepare the curve, 10 mL of culture was centrifuged at 3,500×*g* for 20 min at 4°C. Cell pellets were washed twice with cold distilled water and cells were dried in pre-weighed plastic tubes at 80°C to a constant weight in vacuum ovens.

The frequency of sporulation (% spores cells^{-1}) was measured using the technique described by Mansour and Millière [18]. Aliquots of the culture were spread on agar nutrient (Merck) before and after heat treatment at 80°C for 10 min in order to evaluate total cell (before heating) and spore counts (after heating). Plating was made after a series of decimal dilutions in tryptone salt broth (tryptone: 1 g L^{-1}; sodium chloride: 8.5 g L^{-1}). Plates were incubated at 30°C for 24 h and then counted.

All the experiments in this research were carried out in triplicates.

Results and Discussion

The experimental design matrix and results obtained for enzyme activity and dry cell weight are shown in Table 1. The bacterial growth for the interval of days 5 to 9, which corresponds to the higher enzymatic activities, was smaller for temperatures around 37–40°C (experiments 2, 4, and 8) than that observed in lower temperatures (20 and 23°C; experiments 1, 3, and 7). However, the ratio enzyme/dry cell weight (U g^{-1}; Table 1) and volumetric activity (U mL^{-1}) were, in general, higher in temperatures around 37–40°C. The enzyme activity and biomass values were determined after 5 to 9 days of incubation. The highest value for TGase specific activity was obtained after 8 days; consequently, the regression equations were made to this incubation time. Treatment 6 showed the highest level of TGase specific activity (655 U g^{-1} of dry cells; 0.37 U mL^{-1}). This production was obtained at a moderate incubation temperature (30°C) and at high medium initial pH (8.5). Comparatively, for *S. mobaraense*, so far the only bacterium used for the commercial preparation of TGase, the highest activities reported for optimized cultivations were approximately 117 U g^{-1} cells (2.94 U mL^{-1}) [6]. The highest activity experimentally obtained according to the CCD was 35% higher than the activity obtained under conditions previously used in our laboratory (central point of this study); 486 U g^{-1} (0.30 U mL^{-1}) of dry cells (mean of three replications at the central point) at 8 days, initial pH 6.5 and 30°C.

Statistical analysis of results showed that in the studied range, temperature exerts a strong effect on TGase production and biomass formation by *B. circulans* BL32. The significance of each regression coefficient was determined by *t*-values and *P*-values, which are listed in Table 2. The *P*-values suggest that the negative and positive coefficients for the linear effect of temperature are significant for biomass (P=0.0157) and TGase production (P=0.0327), respectively. This confirms that dry cell weight of *B. circulans* BL 32 decreases while enzyme production increases with the elevation of temperature. The high significance for the quadratic effect of temperature in TGase formation (P=0.0312) indicates that even small variations of temperature will significantly affect the enzyme production by *B. circulans*. The experimental results of the CCD design were fitted with second-order polynomial equations by applying multiple regression analysis on the experimental data. Whenever possible, the models were simplified by the elimination of statistically insignificant terms. However, the x_1^2 term ($P\ x_1 \times\ x_1$=0.0933) for TGase specific activity and the $x_1 \times x_2$ term ($P\ x_1 \times x_2$=0.0728) for dry cell weight were maintained in the models because of their magnitudes. We then proposed that the quadratic models should be reduced as described in Eqs. 3 and 4.

Table 2 Coefficient estimates by the regression model for optimization of TGase production and biomass formation.

Independent variables (parameter)	TGase specific activity (U g^{-1})			Dry cell weight (g L^{-1})		
	Coefficient (β)	t value	P value	Coefficient (β)	t value	P value
Intercept	486.33	17.58	0.0032	0.6200	15.34	0.0042
X_1	39.96	2.36	0.1422	−0.0231	−0.93	0.4484
$X_1 \times x_1$	61.27	3.04	0.0933	−0.0537	−1.82	0.2096
X_2	91.25	5.39	0.0327[a]	−0.1951	−7.88	0.0157[a]
$X_2 \times x_2$	−111.48	−5.53	0.0312[a]	0.0637	2.16	0.1629
$X_1 \times x_2$	−28.00	−1.17	0.3628	0.1225	3.50	0.0728

[a] Statistically significant at 95% of confidence level.

The production of TGase may be best predicted by the following model:

$$Y_{\mathrm{TGase}} = \underset{(\pm 27.65)}{486.33} + \underset{(\pm 20.24)}{61.27x_1^2} + \underset{(\pm 16.96)}{91.25x_2} + \underset{(\pm 20.24)}{111.48x_2^2} \tag{3}$$

Where: Y_{TGase} is the predicted response for TGase specific activity (U g^{-1} cell), x_1 in the initial medium pH, and x_2 is the incubation temperature, as coded settings. The lower brackets indicate the associated pure error.

The formation of biomass may be best predicted by the following model:

$$Y_{\mathrm{Drycell}} = \underset{(\pm 0.02)}{0.62} - \underset{(\pm 0.02)}{0.1951x_2} + \underset{(\pm 0.03)}{0.1225x_1x_2} \tag{4}$$

Where: Y_{DryCell} is the predicted response for dry cell weight (g L^{-1}), x_1 is the initial medium pH, and x_2 is the incubation temperature, as coded settings. The lower brackets indicate the associated pure error.

For TGase production and biomass formation, ANOVA shows that the models are very reliable, with R^2 values of 0.9553 and 0.9256, respectively, indicating good agreement between experimental and predicted values. The models explain 95.53 and 92.56% of the variability in the responses. The computed F value of 21.36 for enzyme production and of 12.45 for biomass formation were greater than the tabulated F value, $F_{5,5,0.05}=5.05$, reflecting the statistical significance of the model equations. The lack of fit F values were found to be 0.74 and 1.65 for TGase specific activity and dry cell weight, respectively, they were smaller than the tabulated F value, $F_{3,2,0.05}=19.16$, which implies that the lack of fit is non-significant. This shows that the models, as expressed in Eqs. 3 and 4, provide suitable mathematical models to describe the responses of the experiments concerning TGase production and dry cell weight.

Figure 1a and b shows the shapes of contour plots of incubation temperature against initial pH for TGase production and biomass formation by *B. circulans* BL32, respectively. The plots demonstrate that the production of enzyme could be increased by elevating the medium initial pH, even under moderate temperatures (Fig. 1a). There is a great flexibility for enzyme productivity, either varying pH or temperature, which is interesting for industrial applications. However, maximal enzyme production was achieved at initial pH in between 8.3 and 8.5 and incubation temperatures in between 28 and 36°C. Biomass formation, however, was inversely affected (Fig. 1b). Maximal biomass was achieved at lower values of initial pH, between 4.5 and 5.1, and at lower temperatures, between 20 and 21°C. The optimal response factor levels for TGase production differed from those for the biomass concentration, as is generally the case for secondary metabolites. The effects of pH on TGase production and biomass

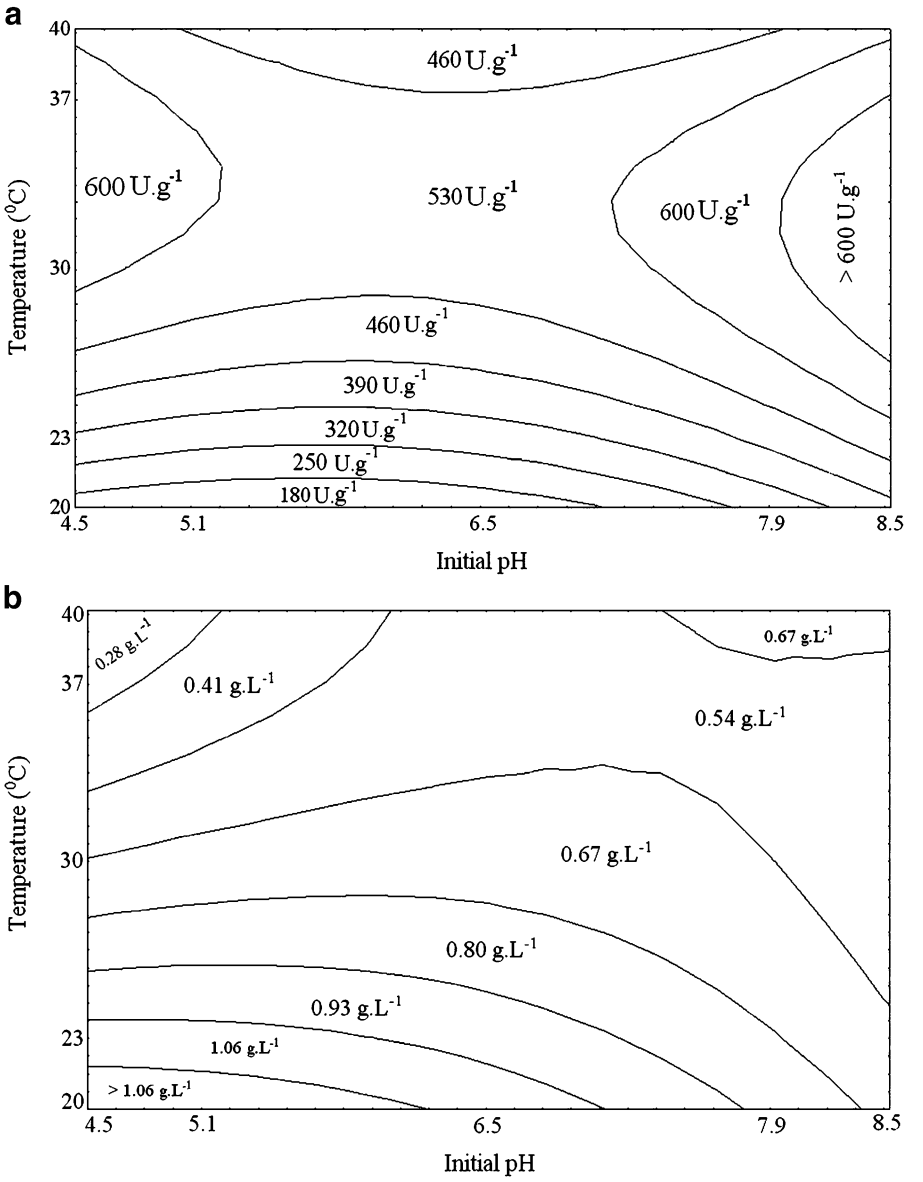

Fig. 1 Contour plot for the effect of temperature vs. initial pH on **a** TGase specific activity and **b** dry cell weight

formation by *B. circulans* BL32 found in our work contrast strongly with results obtained for *S. mobaraense* by Zheng et al. [10]. These authors, evaluating the effects of pH varying in the range of 5.0 to 8.5, observed that, at lower than 6.0 and higher than 7.5, almost no cell growth occurred, and that the best pH for TGase production and biomass formation were in between 6.5 and 7.0. Concerning temperature and its effect on biomass and enzyme production, the results for *B. circulans* obtained in our work contrast with those by Zhu et al. [19], who reported that biomass accumulation is essential for enzyme production by *Streptoverticillium mobaraense*, so far the only microorganism used in commercial-scale

production of TGase. Zheng et al. [6] studying the cell growth and TGase production by *S. mobaraense* in a one-factor-at-a-time optimization experiment at temperatures ranging from 25 to 35°C, found that the ideal temperature for both cell growth and enzyme production by that bacterium was 30°C. These observations might be indicating different mechanisms of these bacteria on the synthesis of TGase.

We speculated whether TGase production by *B. circulans* BL32 would possibly be involved in the sporulation process of this bacterium. In an early study, Kobayashi et al. [20] suggested that this enzyme is expressed during sporulation and plays a role in the assembly of the spore coat proteins of *Bacillus* spp. Recently, Ragkousi and Setlow [11], and Zilhão et al. [12], demonstrated the molecular mechanisms involved in the assembly of spore coat proteins in *B. subtilis* and have shown that TGase plays an essential role in the cross-linking of one of the coat proteins, GerQ. These authors also demonstrated that TGase is produced in the mother cells during the process of sporulation [11, 12]. Our results were showing that temperature and pH were strongly affecting TGase content of *B. circulans* BL32. Probably, the increment of both variables could be acting as inducers of TGase synthesis by mother cells before and during spore formation of *B. circulans*. To test this possibility, we measured the independent effects of pH and temperature on the frequency of sporulation of our strain and results are shown on Fig. 2. As it can be seen, sporulation is linearly stimulated by the increase in temperature, reaching its maximal at 40°C. The effect of pH on cell sporulation was somewhat more complex, with a non-linear response, with the highest value tested (pH 8.5) inducing 100% of spores formation at 192 h of cultivation. This non-linear response can be explained by the fact that extremes of pH will be detrimental to cell metabolism, thus, inducing sporulation. To further confirm the role of spore formation on TGase activity, we cross-checked the effects of varying oxygen concentration of cultures under the optimized values of pH and temperature (Fig. 3). As can be seen, anoxic conditions (Fig. 3a) will have little effect on cell formation and TGase induction, while fully aerated cultures (Fig. 3b) will produce higher amounts of biomass but fails to induce TGase. However, when combining aeration during cell formation (exponential growth) with anoxic conditions during stationary phase, this will result in high spore induction and TGase production.

Further experiments were performed in order to validate the model for the predicted optimal culture conditions for TGase production by our strain (pH 8.5 and temperature 30°C) and results are shown in Fig. 4. After 8 days of cultivation, the TGase specific activity and biomass were 633 U g^{-1} cells (0.37 U mL^{-1}) and 0.58 g L^{-1}, respectively. In this case, the coded settings of the tested variables were x_1=+1.41, x_2=0, with the models predicting TGase activity of 608 U g^{-1} (0.38 U mL^{-1}) and biomass of 0.62 g L^{-1}. Therefore, the models developed were considered to be accurate and reliable for predicting the production of both enzyme and biomass. As expected from our observations on cell sporulation, TGase production is not related to cell growth, being produced from the onset of stationary phase and reaching its maximal after 192 h of cultivation. This time coincides of the maximal frequency of sporulation for all tested conditions (Fig. 2), both for variations in pH and temperature, further demonstrating the correlation between TGase activity and cell sporulation.

Conclusions

The optimal design of process parameters is a relevant aspect to be considered in the development of bioprocesses. The culture conditions for TGase production by *B. circulans*

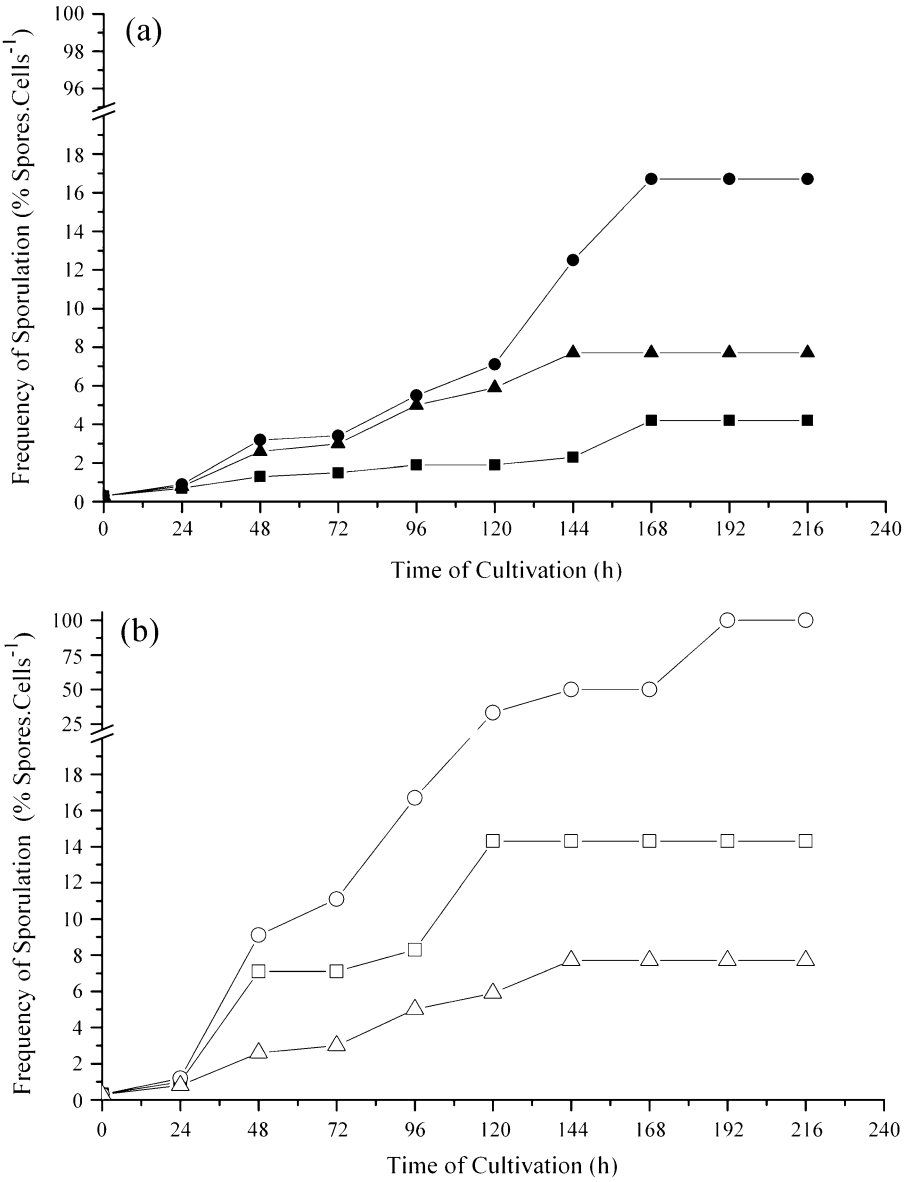

Fig. 2 **a** Effect of temperature in the sporulation of *B. circulans* BL32 at pH 6.5: (*filled square*) 20°C, (*filled upright triangle*) 30°C, (*filled circle*) 40°C. **b** Effect of pH in the sporulation of *B. circulans* BL32 at temperature of 30°C: (*empty square*) 4.5, (*empty upright triangle*) 6.5, (*empty circle*) 8.5

BL32 were optimized using statistical methods. In this study, the experimental results clearly showed that the TGase production by this bacterium is dependent on pH and temperature. Our results showed that the process is flexible concerning good productivities of this enzyme but at 30°C, initial pH 8.5 and 8 days of incubation, the TGase specific activity reached 655 U g^{-1} (0.37 U mL^{-1}) of cells, which is 35% higher than the activity previously obtained in our laboratory and comparable to activities reported for *S. mobaraense*, presently the bacterial model for the production of this enzyme. Our results

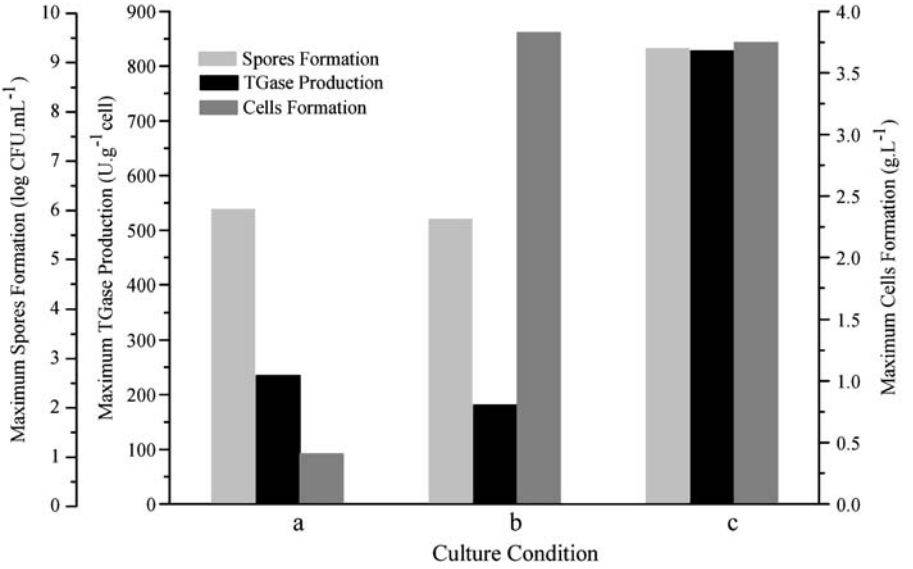

Fig. 3 Effect of oxygen on spore formation, TGase and biomass production in batch cultivations of *B. circulans* BL32 growing at the optimal conditions of pH (8.5) and temperature (30°C); (*A*) 400 rpm, 0 vvm; (*B*) 400 rpm, 2 vvm throughout cultivation; (*C*) 400 rpm, 2 vvm up to the end of exponential phase followed by 400 rpm, 0 vvm to induce sporulation. Results represent the mean of two experiments

also demonstrate a correlation between enzyme production and cell sporulation, as mediated by culture conditions and, although demonstrated only for *B. subtilis*, it corroborates the molecular mechanisms involved in this process. Based on our results, it can be suggested that *B. circulans* BL32 is a strong biological system for the industrial production of TGases.

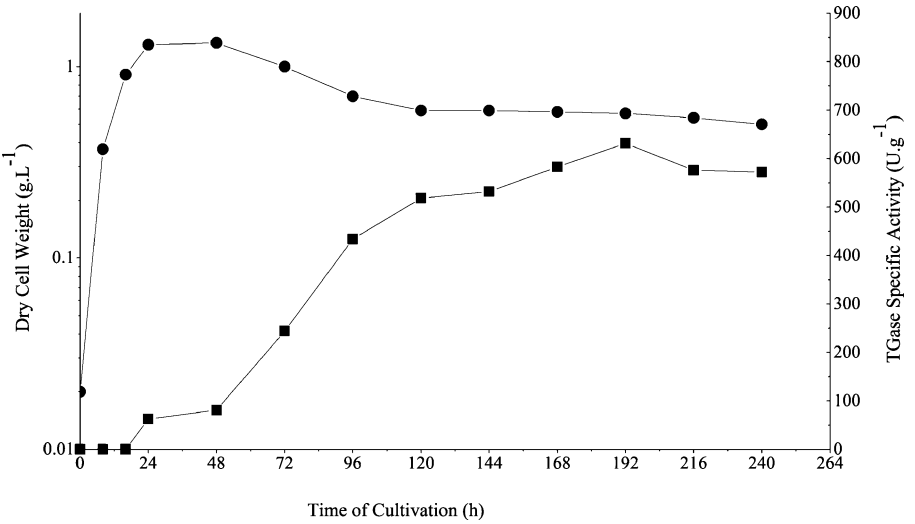

Fig. 4 The growth kinetics of TGase production (model validation) on shaker culture. TGase activity (*filled square*) and cell growth (*filled circle*) profile under the optimal conditions suggested by the model (30°C, pH 8.5). Results are the mean of three experiments

Acknowledgment The authors wish to thank CNPq for their financial support.

References

1. Nonaka, M., Tanaka, H., Okiyama, A., Motoki, M., Ando, H., Umeda, K., et al. (1989). *Agricultural and Biological Chemistry*, *53*, 2619–2623.
2. Zhu, Y., Rinzema, A., Tramper, J., & Bol, J. (1995). *Applied Microbiology and Biotechnology*, *44*, 277–282. doi:10.1007/BF00169916.
3. Bernard, B. K., Tsubuku, S., & Shioya, S. (1998). *International Journal of Toxicology*, *17*, 703–721. doi:10.1080/109158198225928.
4. Cortez, J., Bonner, P. L. R., & Griffin, M. (2004). *Enzyme and Microbial Technology*, *34*, 64–72. doi:10.1016/j.enzmictec.2003.08.004.
5. Josten, A., Meusel, M., Spener, F., & Haalck, L. (1999). *Journal of Molecular Catalysis. B, Enzymatic*, *7*, 57–66. doi:10.1016/S1381-1177(99)00021-1.
6. Zheng, M., Du, G., Guo, W., & Chen, J. (2001). *Process Biochemistry*, *36*, 525–530. doi:10.1016/S0032-9592(00)00229-6.
7. Ando, H., Adachi, M., Umeda, K., Matsuura, A., Nonaka, M., Uchio, R., et al. (1989). *Agricultural and Biological Chemistry*, *53*, 2613–2617.
8. Gerber, U., Jucknischke, U., Putzien, S., & Fuchsbauer, H. L. (1994). *The Biochemical Journal*, *299*, 825–829.
9. Zhu, Y., Rinzema, A., Tramper, J., & Bol, J. (1996). *Biotechnology and Bioengineering*, *50*, 291–298. doi:10.1002/(SICI)1097-0290(19960505)50:3<291::AID-BIT8>3.0.CO;2-B.
10. Zheng, M., Du, G., & Chen, J. (2002). *Enzyme and Microbial Technology*, *31*, 477–481. doi:10.1016/S0141-0229(02)00091-1.
11. Ragkousi, K., & Setlow, P. (2004). *Journal of Bacteriology*, *186*, 5567–5575. doi:10.1128/JB.186.17.5567-5575.2004.
12. Zilhão, R., Isticato, R., Martins, L. O., Steil, L., Völker, U., Ricca, E., et al. (2005). *Journal of Bacteriology*, *187*, 7753–7764. doi:10.1128/JB.187.22.7753-7764.2005.
13. Junqua, M., Duran, R., Gancet, C., & Goulas, P. (1997). *Applied Microbiology and Biotechnology*, *48*, 730–734. doi:10.1007/s002530051124.
14. Téllez-Luis, S., Ramírez, J. A., & Vázquez, M. (2004). *Food Technology and Biotechnology*, *42*, 75–81.
15. Souza, C. F. V., Flôres, S. H., & Ayub, M. A. Z. (2006). *Process Biochemistry*, *41*, 1186–1192. doi:10.1016/j.procbio.2005.12.019.
16. Soares, L. H. B., Assmann, F., & Ayub, M. A. Z. (2003). *Biotechnology and Applied Biochemistry*, *37*, 295–299. doi:10.1042/BA20020110.
17. Grossowicz, N., Wainfan, E., Borek, E., & Waelsch, H. (1950). *The Journal of Biological Chemistry*, *187*, 111–125.
18. Mansour, M., & Millière, J. B. (2001). *Food Microbiology*, *18*, 87–94. doi:10.1006/fmic.2000.0379.
19. Zhu, Y., Rinzema, A., Tramper, J., De Bruin, E., & Bol, J. (1998). *Applied Microbiology and Biotechnology*, *49*, 251–257. doi:10.1007/s002530051165.
20. Kobayashi, K., Suzuki, S., Izawa, Y., Yokozeki, K., Miwa, K., & Yamanaka, S. (1998). *The Journal of General and Applied Microbiology*, *44*, 85–91. doi:10.2323/jgam.44.85.