J Ind Microbiol Biotechnol (2008) 35:1677–1685
DOI 10.1007/s10295-008-0432-8

ORIGINAL PAPER

Solid state bioreactor production of transglutaminase by Amazonian *Bacillus circulans* BL32 strain

Claucia Fernanda Volken de Souza · Júlio Xandro Heck · Marco Antônio Záchia Ayub

Received: 7 March 2008 / Accepted: 30 July 2008 / Published online: 12 August 2008

Abstract In this work, we investigated the production of transglutaminase (TGase) by an Amazonian isolated strain of *Bacillus circulans* by solid-state cultivation (SSC). Several agro-industrial residues, such as untreated corn grits, milled brewers rice, industrial fibrous soy residue, soy hull, and malt bagasse, were used as substrates for microbial growth and enzyme production. Growth on industrial fibrous soy residue, which is rich in protein and hemicellulose, produced the highest TGase activity (0.74 U g^{-1} of dried substrate after 48 h of incubation). A 2^3 central composite design was applied to determine the optimal conditions of aeration, cultivation temperature and inoculum cell concentration to TGase production. The best culture conditions were determined as being 0.6 L air min^{-1}, 33 °C and 10 $\log_{10}$ CFU g^{-1} of dried substrate, respectively. Under the proposed optimized conditions, the model predicted an enzyme production of 1.16 U g^{-1} of dried substrate, closely matching the experimental activity of 1.25 U g^{-1}. Results presented in this work point to the use of this newly isolated *B. circulans* strain as a potential alternative of microbial source for TGase production by SSC, using inexpensive culture media.

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

J. X. Heck
Technical School, Federal University of Rio Grande do Sul State, Av. Bento Gonçalves, 9500, P. O. Box 15090,
Porto Alegre, RS ZC 91501-970, Brazil

Keywords Transglutaminase · *Bacillus circulans* BL32 · Solid-state cultivation · Industrial fibrous soy residue · Optimization

Introduction

Transglutaminases (TGase; protein-glutamine γ-glutamyltransferase; EC 2.3.2.13) are a family of enzymes that catalyzes acyl transfer reactions using peptide-bond glutamine residues as acyl donors and several primary amines as acceptors. These reactions introduce covalent cross-links between proteins, as well as peptides and various primary amines [18]. TGase has been found in animal and plant tissues and microorganisms [31]. The microbial enzyme has widespread and growing applications in the food processing industry. Recent research has shown that TGase can be employed in many others attractive applications such as, pharmaceuticals [3], textiles [6], tissue engineering [19], and in the formation of immunoconjugates to be applied in ELISA and biosensors [11]. To date, only one microbial TGase, that from the actinomycete *Streptoverticilium mobarense*, has met commercial applications [31]. However, the potential commercial uses of this enzyme have not been achieved because of lack of mass production and also because some of its properties, such as specificity of pH and temperature, are not consistent with a variety of substrates and processes [3].

Commercial TGase production depends largely on culture conditions [13, 14], medium composition [29, 32] and downstream processing [7, 28]. Therefore, the development of low-cost processes for the production of transglutaminases would allow for more extensive industrial applications. Presently, submerged cultivation (SmC) is the only technology used for the commercial production of TGase.

However, solid-state cultivation (SSC) systems offer many advantages over commonly used SmC, and it has gained renewed interest in recent years, often being employed for the production of many metabolites due to several economical and engineering advantages [20] including higher productivity, simplified operation, low investments, low energy requirements, less water output, better product recovery, and lack of foam build-up [10, 22, 30]. The latter is a problem when TGase is obtained in SmC [27]. Furthermore, agro-industrial residues are generally considered to be the best substrates for the enzyme production by SSC. Thus, these processes are of special economic interest for countries with abundant biomass and agro-industrial residues, as they can be used as cheap raw materials [5].

In SSC-based industrial bioprocesses, the determination of optimal culture conditions is of critical importance because it affects product concentration, yield and volumetric productivity. The studies of culture conditions by SSC that use conventional single factor optimization does not allow for the interaction of these variables with each other. This method is also time consuming and requires large numbers of experiments to be carried out in order to determine the optimal level of each factor [4]. The response surface methodology (RSM) is a time saving method, which reveals the interactions that occur among the different culture conditions, building models and evaluating the effects of several factors in order to obtain high productivity [16]. So far, there have been no reports in the literature on the optimization of culture conditions using statistical design concerning the production of TGase by SSC.

In a previous study, we compared the TGase production of *Bacillus circulans* BL32 on SmC and SSC, in which productivity by SSC was higher than in SmC [27]. However, in that early work, conditions for both forms of cultures were not optimized. It is important to optimize the process parameters in order to obtain high TGase production by this microorganism by SSC. Therefore, the purpose of this study was to evaluate the effects of different solid substrates and culture conditions such as aeration, cultivation temperature, and inoculum cell concentration on this enzyme production in order to determine its viability as a production system.

Material and methods

Microorganism and inoculum preparation

A strain of *Bacillus circulans*, coded BL32, which was isolated from the aquatic environment of the Amazon rain forest, was used in this study. Its isolation and characterization were described elsewhere [28, 29]. Previous to culture, cells were recovered from frozen stocks in glycerol and were kept at 4 °C on Mueller-Hinton agar plates (Merck, Germany). The inocula for all experiments by SSC were prepared in 1,000-mL Erlenmeyer flasks containing 160 mL of Luria–Bertani (LB) medium. Flasks were inoculated from a single colony from agar plates and incubated for 24 h, 37 °C and 150 rpm.

Bioreactors cultivations

Bioreactor experiments were carried out in 500-mL cylindrical bioreactors (60-mm diameter; 170-mm height) designed and constructed in our laboratory [9]. Bioreactors were loaded with the solid substrates soaked with basic salt solution with the following composition (g L^{-1}): 0.2 $MgSO_4$; 1.0 KH_2PO_4; 1.0 K_2HPO_4; 1.0 NH_4NO_3; 0.02 $CaCl_2$; 0.05 $FeCl_2$. Bioreactors were inoculated with 20 mL of inoculum.

Screening of solid substrates

Preliminary studies were carried out in order to evaluate the effect of the different solid substrates, such as corn grits (CG), milled brewers rice (MBR), industrial fibrous soy residue (IFSR, which is a by-product of isolated soybean protein production, rich in sugars and proteins), soy hull (SH) and malt bagasse (MB) on the TGase production by *B. circulans* BL32. These substrates were obtained locally. Soy hull was milled and sieved to obtain particles smaller than 1 mm in size and the malt bagasse was oven dried at 60 °C for 12 h, in order to prevent decomposition during storage. The substrates were used without any other pretreatments. Twenty grams of the dried substrates were soaked with basic salt solution until they were saturated and then sterilized at 121 °C for 15 min. The inoculum cell concentration was adjusted to 10^6 CFU g^{-1} of dried substrate (DS). The initial pH of cultures was that of the respective substrate without adjustment, while the moisture of cultures was that attained by the maximum water absorption capacity of each substrate. Cultures were run at 30 °C for up to 168 h. During cultivation, water-saturated sterile air was supplied at a constant flow of 0.25 L air min^{-1}. The substrate in which the highest enzyme production was achieved in this step was selected and used in the subsequent experiments.

Optimization of process parameters

The influence of the process parameters on TGase production by *B. circulans* BL32 by SSC was tested performing a statistical experimental design. A 2^3 full factorial central composite design for three independent variables, each one at five levels with six star points and

four replicates at the central point, was employed to fit a second-order polynomial model in which 18 experiments were required for this procedure. The test variables chosen in this study were: aeration, cultivation temperature and inoculum cell concentration. Five levels of each variable were chosen, the upper and lower limits of them were set to be in the range described in the literature and also based on our previous experience. In the statistical model, Y denotes units of TGase activity (U g^{-1} of dried substrate).

Table 1 shows the actual levels corresponding to the coded settings, the treatment combinations and responses. The enzyme activity values were determined from inoculation time (0 h) to 96 h of incubation, but the regression equation was constructed with the values obtained after 48 h, when maximal values of the TGase activity were achieved.

The experimental design is represented by a second-order polynomial regression model, Eq. (1), to generate contour plots:

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

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:

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

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 [4].

All statistical experimental designs and results analyses were carried out using Statistica 7.0 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 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).

Enzyme extraction

Enzyme recovery from cultivated medium was performed by the addition of 140 mL of distilled water to the total content of the bioreactors and extracted under agitation at 150 rpm during 30 min. The enzymatic extract was then centrifuged at 17,000g for 15 min. The extract was filtered

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

Treatment	Coded setting levels			Actual levels			TGase activity (U g^{-1} of dried substrate)			
	x_1	x_2	x_3	X_1	X_2	X_3	24 h	48 h	72 h	96 h
1	−1	−1	−1	0.2	30	6	0.72 ± 0.05	0.77 ± 0.06	0.55 ± 0.04	0.55 ± 0.04
2	−1	−1	+1	0.2	30	9	0.76 ± 0.05	1.06 ± 0.09	0.91 ± 0.09	0.83 ± 0.07
3	−1	+1	−1	0.2	44	6	0.22 ± 0.01	0.29 ± 0.02	0.29 ± 0.01	0.27 ± 0.01
4	−1	+1	+1	0.2	44	9	0.35 ± 0.02	0.42 ± 0.03	0.31 ± 0.02	0.30 ± 0.02
5	+1	−1	−1	0.8	30	6	0.82 ± 0.07	0.89 ± 0.08	0.49 ± 0.04	0.44 ± 0.01
6	+1	−1	+1	0.8	30	9	0.81 ± 0.05	0.95 ± 0.06	0.83 ± 0.03	0.52 ± 0.02
7	+1	+1	−1	0.8	44	6	0.42 ± 0.01	0.51 ± 0.03	0.46 ± 0.02	0.43 ± 0.02
8	+1	+1	+1	0.8	44	9	0.47 ± 0.03	0.77 ± 0.07	0.64 ± 0.06	0.59 ± 0.04
9	−1.68	0	0	0	37	7.5	0.09 ± 0.01	0.12 ± 0.01	0.29 ± 0.01	0.27 ± 0.01
10	+1.68	0	0	1	37	7.5	0.77 ± 0.05	0.82 ± 0.04	0.61 ± 0.05	0.57 ± 0.04
11	0	−1.68	0	0.5	25	7.5	0.21 ± 0.02	0.57 ± 0.03	0.53 ± 0.04	0.51 ± 0.04
12	0	+1.68	0	0.5	49	7.5	0.03 ± 0.00	0.18 ± 0.01	0.18 ± 0.01	0.15 ± 0.01
13	0	0	−1.68	0.5	37	5	0.78 ± 0.05	0.84 ± 0.07	0.50 ± 0.04	0.39 ± 0.04
14	0	0	+1.68	0.5	37	10	0.77 ± 0.08	0.96 ± 0.08	0.64 ± 0.06	0.59 ± 0.06
15	0	0	0	0.5	37	7.5	0.86 ± 0.07	0.91 ± 0.07	0.75 ± 0.05	0.65 ± 0.03
16	0	0	0	0.5	37	7.5	0.81 ± 0.06	0.88 ± 0.06	0.73 ± 0.03	0.51 ± 0.01
17	0	0	0	0.5	37	7.5	0.78 ± 0.07	0.82 ± 0.07	0.72 ± 0.05	0.54 ± 0.04
18	0	0	0	0.5	37	7.5	0.79 ± 0.05	0.81 ± 0.06	0.77 ± 0.04	0.60 ± 0.03

The results are the mean of three replications. x_1, x_2 and x_3 are coded values. X_1, X_2 and X_3 are the actual values. X_1 = aeration (L min^{-1}); X_2 = temperature (°C); X_3 = inoculum cell concentration (log $_{10}$ CFU g^{-1} of DS). $x_1 = \frac{X_1 - 0.5}{0.3}; x_2 = \frac{X_2 - 37}{7}; x_3 = \frac{X_3 - 7.5}{1.5}$

Table 2 Chemical composition of solid substrates tested for TGase production by *B. circulans* BL32

Components (%)	Substrates				
	CG	MBR	IFSR	SH	MB
Moisture	11.5 ± 0.6	10.3 ± 0.3	9.1 ± 0.2	9.9 ± 0.3	6.6 ± 0.1
Ash	0.3 ± 0.0	0.2 ± 0.0	3.3 ± 0.2	4.0 ± 0.3	3.9 ± 0.2
Proteins	7.0 ± 0.5	6.6 ± 0.3	23.3 ± 0.9	10.8 ± 0.4	19.1 ± 0.6
Lipids	0.6 ± 0.0	0.6 ± 0.0	0.2 ± 0.0	1.1 ± 0.1	6.2 ± 0.3
Total carbohydrates	80.6 ± 1.2	82.3 ± 0.7	64.1 ± 1.4	74.2 ± 1.2	64.2 ± 1.3
Fibers	0.2 ± 0.0	0.2 ± 0.0	14.1 ± 0.5	34.8 ± 0.9	14.3 ± 0.6
Reducing sugars	n.d.	n.d.	n.d.	n.d.	2.2 ± 0.1
Non-reducing sugars	n.d.	n.d.	2.7 ± 0.2	2.3 ± 0.1	2.5 ± 0.1

The results are the mean of three replications

n.d. not detected, *CG* corn grits, *MBR* milled brewers rice, *IFSR* industrial fibrous soy residue, *SH* soy hull, *MB* malt bagasse

through a Whatman No. 1 filter paper to obtain a clear extract and assayed for TGase activity.

Assay techniques

The chemical composition of the substrates was determined as described by AOAC [2]. The pH value of the sample of cultures was assayed following dilution of 5 g in 50 ml of distilled water, and by using a pHmeter DM 20 (Digimed, Brazil). Reducing sugars were measured according to Miller [15]. Proteolytic activity was measured by the azocasein method, according to Sarath et al. [26], in which one enzymatic unit (U) is defined as the amount of enzyme needed to produce the change in one unit of absorbance under the conditions of the method (40 °C, 40 min). TGase activity was determined by the hydroxamate procedure [8], in which a calibration curve is prepared with L-glutamic acid γ-monohydroxamate and one enzymatic unit (U) causes the formation of 1 μmol hydroxamic acid per minute at 37 °C. Enzyme was expressed as activity per gram of dried substrate (U g^{-1} of dried substrate).

Results and discussion

Selection of substrate

Substrates provide the required energy and nutrients for the microorganism to grow and produce the desired metabolites [21]. The selection of a suitable solid substrate for the production of enzyme is a primary-key factor, which involves the choice of agro-industrial residues for microbial growth and product formation. In the present study, we tested five different substrates, which are abundant worldwide: corn grits (CG), milled brewers rice (MBR), industrial fibrous soy residue (IFSR), soy hull (SH), and malt bagasse (MB). Their complete chemical composition, as dry matter, is shown in Table 2 and results for enzyme production by *B. circulans* BL32 on them are shown in Fig. 1. The highest TGase production (0.74 U g^{-1} of dried substrate) was achieved with IFSR after an incubation time of 48 h. Maximal TGase activities for MB and SH were 0.33 and 0.21 U g^{-1} of dried substrate after 48 and 120 h, respectively, while for CG and MBR there were no detectable activities. The results suggest that the carbohydrate:protein ratio is an important factor for TGase production. The carbohydrate:protein ratio of enzyme-producing substrates are: IFSR (2.7), MB (3.4) and SH (6.9), whereas for the non-producing ones the ratios are CG (11.5) and MBR (12.5). In a previous work, we observed that C:N initial ratio of the culture medium influence in the TGase production by *B. circulans* BL32 [29]. The activity increases exponentially from 1 to 5 C:N ratio, suggesting

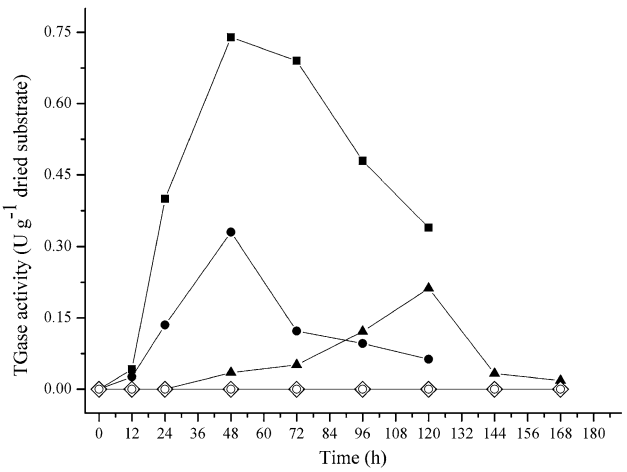

Fig. 1 SSC TGase production by *B. circulans* BL32 on different substrates. IFSR, industrial fibrous soy residue (*filled square*); *MB* malt bagasse (*filled circle*), *SH* soy hull (*filled triangle*), *CG* corn grits (*open circle*), *MBR* milled brewers rice (*open diamond*)

that nitrogen limitation is important for enzyme production, probably by stimulating the sporulation process of cells. Based on these results, IFSR was used as the substrate for the production of transglutaminase in subsequent experiments.

Optimization of solid-state cultivation conditions

Cultivation factors that strongly influence microbial growth and enzyme activities by SSC are reported to be the temperature, aeration, and inoculum cell concentration [1, 9, 17, 21, 23]. Therefore, in this work, Central Composite Design (CCD) was used to investigate the best conditions of these three variables for TGase production by *B. circulans* BL32 and determined as growth kinetics.

The experimental design matrix and results obtained for enzyme activities are shown in Table 1. TGase activities values were determined in a time span of 96 h of incubation, being the highest values of TGase activity observed in 48 h. Therefore, the regression equations were constructed considering this incubation time.

TGase activity varied from 0.12 to 1.06 U g^{-1} according to different levels of process parameters. The lowest values of TGase activity were obtained without aeration and at the highest incubation temperature (49 °C). The upper temperature level tested probably impaired cell metabolism, while cultivation without aeration might be indicating either the need of air for cell metabolism and enzyme production, or the increased difficulty of heat dissipation; therefore, negatively affecting the results. Heat dissipation in SSC bioreactors have been reported as major scale-up problems and must be adequately addressed [20, 22]. Treatment 2 (Table 1) showed the highest TGase activity (1.06 U g^{-1} of dried substrate), which was obtained at a moderate incubation temperature (30 °C). In SmC, the best temperatures for maximum transglutaminase activities by *B. circulans* BL32 were within the range of 28 and 36 °C (our results, not published yet). Meiying et al. [13] studying the TGase production by *S. mobaraense* in SmC, using a one-factor-at-a-time experimental procedure, testing temperatures in between 25 to 35 °C, have found highest enzyme production at 30 °C. For this same substrate, IFSR, Heck et al. [9] have shown that temperature significantly affects xylanase activities of *B. circulans*, the enzyme that is involved in the hydrolysis of xylans to xylo-oligosaccharides.

The significance of each regression coefficient was determined by t values and P values, listed in Table 3. Although the P values for the negative coefficient for the linear effect of temperature ($Px_2 = 0.0009$), and the positive coefficient for the linear effect of aeration ($Px_1 = 0.0022$), were highly significant, the model also shows the significant interaction between them ($Px_1x_2 = 0.0258$). Therefore, treating them separately may not reflect their real influence on the TGase production (e.g., optimum aeration changes along with incubation temperature). This interaction is essential and it would be of difficult solution using the *one-variable-at-a-time* approach and possibly explains the differences of results found in this work with those obtained by Soares et al. [27] in relation to the effect of aeration on TGase production by *B. circulans* BL32 by SSC.

The inoculum cell concentration was also an important factor for the production of TGase. The positive coefficient for the linear effect of inoculum cell concentration (x_3), with a value <0.0130, is highly significant. This result indicates that TGase production by *B. circulans* increases with elevated initial inoculum cell concentration. The physiological role of microbial transglutaminase has

Table 3 Effect and coefficient estimates by the regression model for optimization of TGase production by SSC by *B. circulans* BL32

Independent variables (parameter)	Effect	Coefficient (β)	Standard error (β)	t value	P value
Intercept (β_0)	0.8481	0.8481	0.0239	35.42	<0.0001
x_1^*	0.2573	0.1287	0.0260	9.92	0.0022
$x_1x_1^*$	−0.2106	−0.1053	0.0270	−7.81	0.0044
x_2^*	−0.3421	−0.1710	0.0260	−13.18	0.0009
$x_2x_2^*$	−0.2777	−0.1389	0.0270	−10.30	0.0020
x_3^{**}	0.1379	0.0690	0.0260	5.31	0.0130
$x_3x_3^{**}$	0.0935	0.0467	0.0270	3.47	0.0404
$x_1x_2^{**}$	0.1400	0.0700	0.0339	4.13	0.0258
x_1x_3	−0.0250	−0.0125	0.0339	−0.74	0.5144
x_2x_3	0.0100	0.0050	0.0339	0.29	0.7873

x_1, x_2 and x_3 are the coded values of variables aeration (L min^{-1}), temperature (°C) and inoculum cell concentration (log $_{10}$ CFU g^{-1} of DS), respectively

* Statistically significant at 99% of confidence level

** Statistically significant at 95% of confidence level

recently been linked to the sporulation process in which it 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 [24, 34]. According to Kobayashi et al. [12], this enzyme is expressed during sporulation and plays a role in the assembly of the spore coat proteins of the genus *Bacillus* sp. Zhu et al. [33] reported that biomass accumulation is essential for enzyme production, due to the fact that TGase is produced before the sporulation phase. One possible explanation for the positive influence of inoculum size on TGase activity might be that more biomass is formed, triggering a faster sporulation process. TGase production contrasts with findings for other bio-products for which higher concentrations of inocula are reported to be inhibitory when done by SSC. According to Adinarayana et al. [1], high inoculum cell concentrations are inhibitory for the production of cephalosporin C by *Acremonium chrysogenum* by SSC. The same was observed by Murthy et al. [17] for cyclosporine-A produced by *Tolypocladium inflatum*, and by Ramachandrana et al. [25] for phytase produced by *Rhizopus* spp. On the other hand, a 10% (v/w) inoculum of 3.6×10^6 cells mL^{-1} was found to be optimum for xylanase production by *Bacillus pumilus* when using SSC with wheat bran as substrate [23].

The second-order effect of aeration, temperature, and inoculum cell concentration were highly significant ($Px_1x_1 = 0.0044$, $Px_2x_2 = 0.0020$ and $Px_3x_3 = 0.0404$, respectively). The high significance of second-order variables in the model indicates that they can act as limiting factors. Therefore, even small variations in their values, will alter TGase production to a considerable extent.

Whenever possible, the model was simplified by the elimination of statistically insignificant terms. Then, the quadratic model should be reduced to:

$$Y = 0.8481 + 0.1287x_1 - 0.1053x_1x_1 - 0.1710x_2 - 0.1389x_2x_2 + 0.0690x_3 + 0.0467x_3x_3 + 0.0700x_1x_2 \quad (3)$$

Where, Y is the predicted response to the TGase activity (U g^{-1} of dried substrate), x_1 the aeration (L min^{-1}), x_2 the temperature (°C) and x_3 is the inoculum cell concentration (log $_{10}$ CFU g^{-1} of dried substrate) as coded settings.

Analysis of variance (ANOVA) was employed to determine the significance of the second-order polynomial model. The model was highly significant ($P < 0.01$), with an $R^2 = 0.86$, explaining 86% of the total variation. This shows that Eq. 3 provides a suitable model to describe the response of the experiment relative to TGase production by SSC.

The contour shapes (Fig. 2) were plotted on the basis of the model equation in order to investigate the interaction among the variables and to determine the optimum concentration of each factor for maximum TGase production

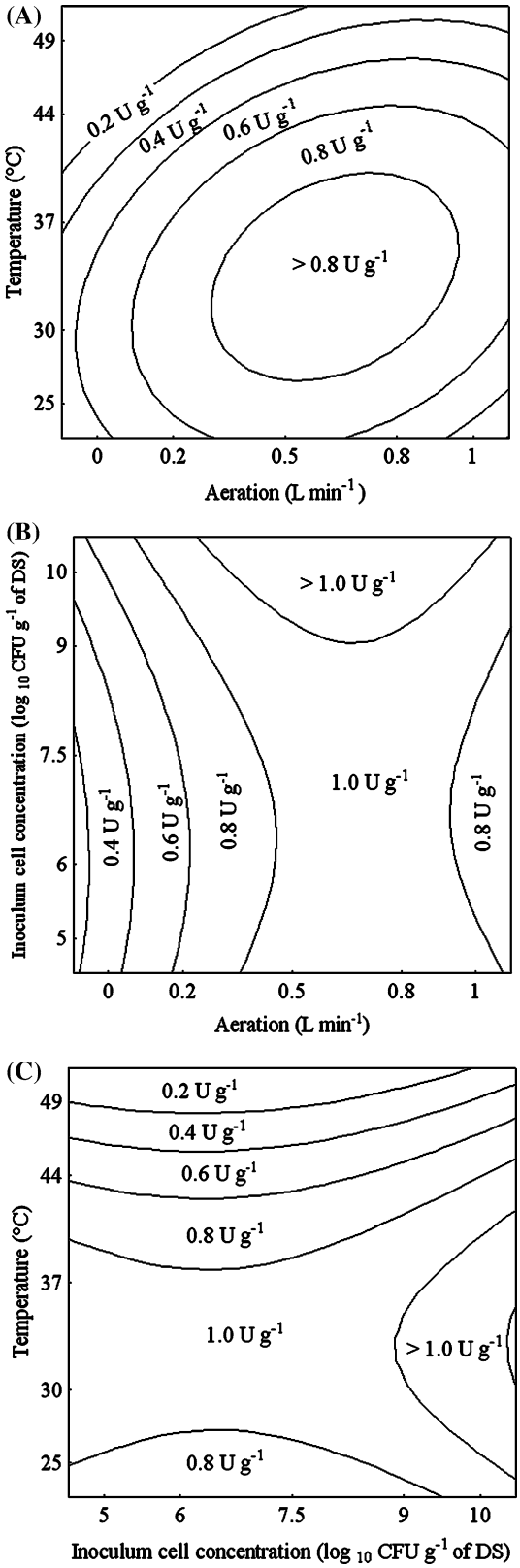

Fig. 2 Contour plot for the effect of **a** aeration × temperature, **b** aeration × inoculum cell concentration and **c** inoculum cell concentration × temperature on transglutaminase production by *B. circulans* BL32 under SSC. The variable that is not plotted is fixed at zero level in all of the three graphs

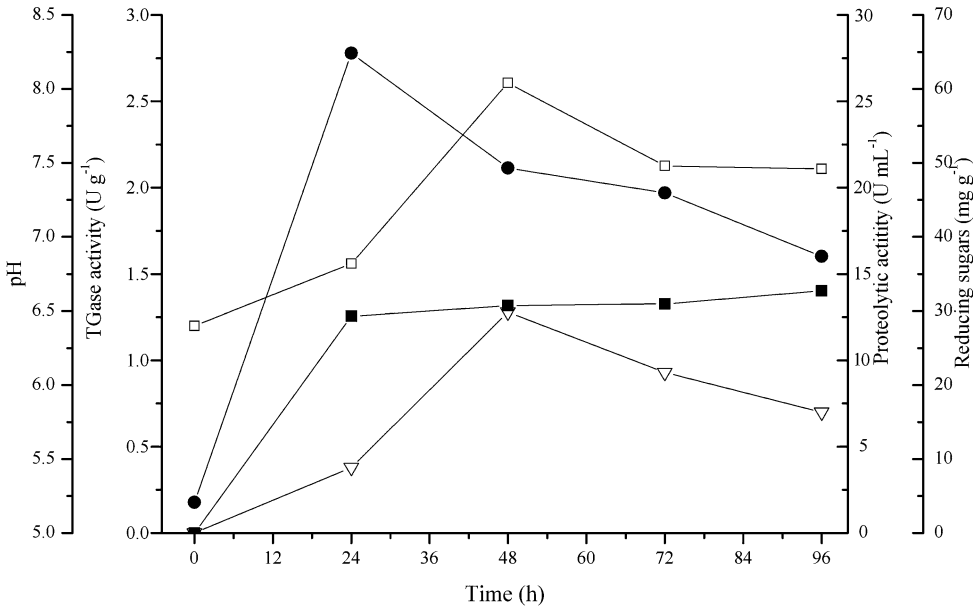

Fig. 3 Kinetics of TGase production by *B. circulans* BL32 under the optimal conditions suggested by the model. Aeration = 0.6 L min^{-1}; temperature = 33 °C; and inoculum cell concentration = 10 log $_{10}$ CFU g^{-1} of dried substrate. pH (*open square*); TGase activity (*open inverted triangle*); proteolytic activity (*filled square*); reducing sugars (*filled circle*)

by *B. circulans* BL32. In the Fig. 2a is depicted the shapes contour of aeration versus temperature of incubation, showing that there is a significant mutual interaction between these two variables. The TGase production tends to increase with both moderate aeration and cultivation temperature. When incubation temperatures were set at the extremes of the studied interval, low TGase activities were obtained. Low temperature may lead to slow bacterium metabolism, whilst high temperatures might induce enzyme inactivation. Figure 2b shows contour plot of aeration against inoculum cell concentration. The production of TGase is increased for high inoculum cell concentration under moderate aeration. Figure 2c shows contour of temperature against inoculum cell concentration with results suggesting an optimum TGase production at temperatures between 27 and 35 °C and again at high inoculum cell concentration. The non-elliptical nature of contour plots (Fig. 2b, c) depicts that there is no mutual interaction between inoculum cell concentration and each of the other two variables. Thus, the contour plots revealed that the optimal range of process values for TGase production by *B. circulans* on IFSR is: aeration, 0.55 – 0.65 L air min^{-1}; temperature, 30–37 °C; and inoculum cell concentration, 9.5–10 log $_{10}$ CFU g^{-1} DS. The polynomial model gives the optimal levels for the three process variables to be: aeration of 0.6 L air min^{-1}, temperature of 33 °C, and inoculum cell concentration of 10 log $_{10}$ CFU g^{-1} DS, with a predicted highest TGase production of 1.16 U g^{-1} of activity. The experimental validation of the model, at the optimized process parameters, produced TGase activity of 1.25 U g^{-1} of dried substrate (mean of three experiments). The activity of transglutaminase achieved under optimized culture conditions was 1.7-fold higher than the non-optimized conditions (0.74 U g^{-1} of dried substrate; Fig. 1).

Production of TGase under the optimized conditions

Figure 3 shows the kinetics of *B. circulans* BL32 on IFSR. It shows that the enzyme production increased progressively with incubation time to a maximum of 1.28 U g^{-1} dried substrate at 48 h of cultivation. TGase activity declined during further incubation, which could have been due to either increased proteolytic activity during cultivation or the complete cell sporulation towards the end of the run. Variations in reducing sugars concentration and pH followed expected patterns of hydrolysis and liberation of basic aminoacids by proteolytic enzymes. However, the most important observation is the fact that, in submerged cultivations of *B. circulans* BL32 on starch [27] and on glycerol [29], it takes 6–8 days for a maximum enzyme activity. In contrast, for the solid-state substrate bioprocess, this time is reduced to only 2 days, which is advantageous in terms of cost for enzyme production on a large scale. This effect might be explained by faster sporulation by SSC versus SmC. Moreover, in the SmC, the maximum TGase activity was 27.6 U g^{-1} reducing sugars (for starch [27]), whereas in SSC we obtained 47.4 U g^{-1} reducing sugars, which represents an increase of 42% in productivity.

Conclusions

The search for alternative sources and the improvement of enzyme production and productivity are major goals of

bioprocess engineering. These are essential features for cost reductions and cleaner production that ultimately leads to wider industrial uses of a specific enzyme. Microbial TGase is a case in particular, since it is entirely marketed by one single company from one single microorganism. *Bacillus circulans* BL32, recently isolated from the Amazonian environment, is a promising source for this highly valued enzyme. Furthermore, if SSC with agro-industrial residues used as substrates can be employed for enzyme production, then inexpensive and efficient bioprocess alternatives are possible. In the present work, several cultivation parameters were studied through the use of CCD, in order to improve TGase production by *Bacillus circulans* BL32 by SSC. The highest productivity of TGase (1.28 U g^{-1} of dried substrate) was achieved in only 48 h of cultivation, under optimized process parameters. This activity is 70% higher than with non-optimized SSC conditions and 42% higher than activities obtained in long time SmC. Presented results show the potential of SSC using an abundant and inexpensive agro-residue (IFSR) as substrate, and also points to the use of *B. circulans* BL32 as an alternative biological source for the efficient production of TGase on a large scale, commercially viable process.

Acknowledgments The authors wish to thank the Brazilian Bureau for Scientific and Technological Development (Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq) for the financial support of this research.

References

1. Adinarayana K, Prabhakar T, Srinivasulu V, Anitha Rao M, Jhansi Lakshmi P, Ellaiah P (2003) Optimization of process parameters for cephalosporin C production under solid state fermentation from *Acremonium chrysogenum*. Process Biochem 39:171–177. doi:10.1016/S0032-9592(03)00049-9
2. Association of Official Analytical Chemists (1996) Official methods of analysis. AOAC, Gaithersburg, USA
3. Bernard BK, Tsubuku S, Shioya S (1998) Acute toxicity and genotoxicity studies of a microbial transglutaminase. Int J Toxicol 17:703–721. doi:10.1080/109158198225928
4. Box GP, Hunter JS (1978) Statistics for experiment: an introduction to design, data analysis and model building. John Wiley and Sons, New York
5. Castilho LR, Medronhob RA, Alves TLM (2000) Production and extraction of pectinases obtained by solid state fermentation of agroindustrial residues with *Aspergillus niger*. Bioresour Technol 71:45–50. doi:10.1016/S0960-8524(99)00058-9
6. Cortez J, Bonner PLR, Griffin M (2004) Application of transglutaminases in the modification of wool textiles. Enzyme Microb Technol 34:64–72. doi:10.1016/j.enzmictec.2003.08.004
7. Gerber U, Jucknischke U, Putzien S, Fuchsbauer HL (1994) A rapid and simple method for the purification of transglutaminase from *Streptoverticillium mobaraense*. Biochem J 299:825–829
8. Grossowicz N, Wainfan E, Borek E, Waelsch H (1950) The enzymatic formation of hydroxamic acids from glutamine and asparagines. J Biol Chem 187:111–125
9. Heck J, Soares LHB, Ayub MAZ (2005) Optimization of xylanase and mannanase production by *Bacillus circulans* strain BL53 on solid-state cultivation. Enzyme Microb Technol 37:417–423. doi:10.1016/j.enzmictec.2005.02.015
10. Holker U, Hofer M, Lenz J (2004) Biotechnological advantages of laboratory scale solid state fermentation with fungi. Appl Microbiol Biotechnol 64:175–186. doi:10.1007/s00253-003-1504-3
11. Josten A, Meusel M, Spener F, Haalck L (1999) Enzyme immobilization via microbial transglutaminase: a method for the generation of stable sensing surfaces. J Mol Catal B Enzym 7:57–66. doi:10.1016/S1381-1177(99)00021-1
12. Kobayashi K, Suzuki S, Izawa Y, Yokozeki IK, Miwa IK, Yamanaka S (1998) Transglutaminase in sporulating cells of *Bacillus subtilis*. J Gen Appl Microbiol 44:85–91. doi:10.2323/jgam.44.85
13. Meiying Z, Guocheng D, Wangfang G, Jian C (2001) A temperature-shift strategy in batch microbial transglutaminase fermentation. Process Biochem 36:525–530. doi:10.1016/S0032-9592(00)00229-6
14. Meiying Z, Guocheng D, Jian C (2002) pH control strategy of batch microbial transglutaminase production with *Streptoverticillium mobaraense*. Enzyme Microb Technol 31:477–481. doi:10.1016/S0141-0229(02)00127-8
15. Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal Chem 31:426–428. doi:10.1021/ac60147a030
16. Montgomery DC (1991) Design and analysis of experiment. John Wiley and Sons, New York
17. Murthy MVR, Mohan EVS, Sadhukhan AK (1999) Cyclosporin-A production by *Tolypocladium inflatum* using solid state fermentation. Process Biochem 34:269–280. doi:10.1016/S0032-9592(98)00095-8
18. Nonaka M, Tanaka H, Okiyama A, Motoki M, Ando H, Umeda K et al (1989) Polymerization of several proteins by Ca^{2+}-independent transglutaminase derived from microorganism. Agric Biol Chem 53:2619–2623
19. Orban JM, Wilson LB, Kofroth JA, El-Kurdi MS, Maul TM, Vorp DA (2004) Crosslinking of collagen gels by transglutaminase. J Biomed Mater Res Part A 68A:756–762
20. Pandey A, Selvakumar P, Soccol CR, Nigam P (1999) Solid state fermentation for the production of industrial enzymes. Curr Sci 77:149–162
21. Pandey A, Soccol CR, Mitchell D (2000) New developments in solid-state fermentation: I-bioprocesses and products. Process Biochem 35:1153–1169. doi:10.1016/S0032-9592(00)00152-7
22. Pandey A (2003) Solid state fermentation. Biochem Eng J 13:81–84. doi:10.1016/S1369-703X(02)00121-3
23. Poorna CA, Prema P (2007) Production of cellulase-free endoxylanase from novel alkalophilic thermotolerent *Bacillus pumilus* by solid-state fermentation and its application in wastepaper recycling. Bioresour Technol 98:485–490. doi:10.1016/j.biortech.2006.02.033
24. Ragkousi K, Setlow P (2004) Transglutaminase-mediated crosslinking of GerQ in the coats of *Bacillus subtilis* spores. J Bacteriol 186:5567–5575. doi:10.1128/JB.186.17.5567-5575.2004
25. Ramachandrana S, Roopesha K, Nampoothiria KM, Szakacsb G, Pandey A (2005) Mixed substrate fermentation for the production of phytase by *Rhizopus* spp. using oilcakes as substrates. Process Biochem 40:1749–1754. doi:10.1016/j.procbio.2004.06.040
26. Sarath G, Motte RS, Wagner FW (1989) Proteases assay methods. In: Beyon RJ, Bond JS (eds) Proteolytic enzymes: a practical approach. IRL, Oxford
27. Soares LHB, Assmann F, Ayub MAZ (2003) Production of transglutaminase from *Bacillus circulans* on solid-state and

submerged cultivations. Biotechnol Lett 25:2029–2033. doi:10.1023/B:BILE.0000004397.43058.3e
28. Soares LHB, Assmann F, Ayub MAZ (2003) Purification and properties of a transglutaminase produced by a *Bacillus circulans* strain isolated from the Amazon environment. Biotechnol Appl Biochem 37:295–299. doi:10.1042/BA20020110
29. Souza CFV, Flôres SH, Ayub MAZ (2006) Optimization of medium composition for the production of transglutaminase by *Bacillus circulans* BL32 using statistical experimental methods. Process Biochem 41:1186–1192. doi:10.1016/j.procbio.2005.12.019
30. Viniegra-Gonzalez G, Favela-Torres E, Aguilar CN, Romero-Gomez SD, Diaz-Godinez G, Augur C (2003) Advantages of fungal enzyme production in solid state over liquid fermentation systems. Biochem Eng J 13:157–167. doi:10.1016/S1369-703X(02)00128-6
31. Zhu Y, Rinzema A, Tramper J, Bol J (1995) Microbial transglutaminase—a review on its production and application in food processing. Appl Microbiol Biotechnol 44:277–282. doi:10.1007/BF00169916
32. Zhu Y, Rinzema A, Tramper J, Bol J (1996) Medium design based on stoichiometric analysis of microbial transglutaminase production by *Streptoverticillium mobaraense*. Biotechnol Bioeng 50:291–298. doi:10.1002/(SICI)1097-0290(19960505)50:3<;291::AID-BIT8>;3.0.CO;2-B
33. Zhu Y, Rinzema A, Tramper J, De Bruin E, Bol J (1998) Fed-batch fermentation dealing with nitrogen limitation in microbial transglutaminase production by *Streptoverticillium mobaraense*. Appl Microbiol Biotechnol 49:251–257. doi:10.1007/s002530051165
34. Zilhão R, Isticato R, Martins LO, Steil L, Völker U, Ricca E et al (2005) Assembly and function of a spore coat-associated transglutaminase of *Bacillus subtilis*. J Bacteriol 187:7753–7764. doi:10.1128/JB.187.22.7753-7764.2005