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RESEARCH ARTICLE

# Optimization of medium composition for transglutaminase production by a Brazilian soil *Streptomyces* sp.

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**Keywords:** central composite design, fractional factorial design, medium optimization, microbial transglutaminase, *Streptomyces* sp. P20.

**Abbreviations:** ANOVA: analysis of variances
CCD: central composite design
FFD: fractional factorial design
MTGase: microbial transglutaminase
RSM: response surface methodology
TGase: transglutaminase

**Finding a new microbial source of transglutaminase (MTGase) and the study of the medium composition for MTGase production were the goals of this work. A total of 200 actinomycete-like strains were isolated from Brazilian soil samples and two of them named T10b and P20 were selected based on their ability to produce 0.15 U.mL$^{-1}$ and 0.25 U.mL$^{-1}$ of MTGase, respectively. Strain P20 was chosen to continue the study and was identified as *Streptomyces* sp. In order to optimize the MTGase activity, modifications of the usual media composition described for enzyme production were tested. The strategy adopted was: (1) screening experiment for the best carbon and nitrogen sources; (2) fractional factorial design (FFD) to elucidate the key ingredients in the media (the results indicated that the soybean flour, peptone, $KH_2PO_4$ and $MgSO_4.7H_2O$ had a significant effect on MTGase) production and (3) central composite design (CCD) to optimize the concentration of the key components. The experimental results were fitted to a second-order polynomial model at the 95% level of significance ($P < 0.05$). Under the proposed optimized conditions, the model predicted a MTGase activity of 1.37 U.mL$^{-1}$, very closely matching the experimental activity of 1.4 U.mL$^{-1}$.**

Transglutaminase (TGase) (EC 2.3.2.13; protein-glutamine y-glutamiyltranferase) is an enzyme that catalyses an acyl transfer reaction using peptide-bond glutamine residues as acyl donors and several primary amines as acceptors. When the ε-amino groups of the protein-bond lysyl residues are present as acyl receptors, this enzyme is capable of forming intra and intermolecular ε-(γ-Glu)-Lys isopeptide bonds (Soares et al. 2003).

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The covalent cross-links between a number of proteins and peptides introduced by TGase promote the modification of the functional properties of the food proteins (Yokoyama et al. 2004). Therefore, TGase catalysed reactions may be broadly used by food-processing industries, for instance, the creation of new product textures, the modification of viscosity, the alteration of emulsifying and foaming properties, and the improvement of product nutritional value (Zhu et al. 1995; Kwan and Easa, 2003).

Transglutaminases are found in mammalian tissues, plasma, fish, and plants (Pasternack et al. 1998). The mammalian enzymes are $Ca^{+2}$-dependent. However, the relatively small quantities obtained and the complex separation and purification procedures required for these enzymes led to the search for alternative microbiological sources. The first microbial transglutaminase (MTGase) characterized was from an actinomycetes (Ando et al. 1989). Since then, efforts have been made to obtain massive production of this enzyme for commercial applications, especially for the enzymes from *Streptomyces* (Gerber et al. 1994; Zhu et al. 1998a; Zhu et al. 1998b; Zotzel et al. 2003a; Zotzel et al. 2003b).

So far, research has been focused on the isolation and screening of microorganisms for TGase activity, and on purifying and characterizing newly found enzymes. The media compositions used to produce MTGase from *Streptomyces* have been almost the same in every work published since Ando et al. 1989 (Zheng et al. 2001; Zheng et al. 2002; Yan et al. 2005). In contrast, in biotechnology-based industrial processes, the formulation of the culture media is of critical importance because the composition affects product concentration, yield and volumetric productivity. It is also important to reduce the cost of the medium as this may affect the overall process economics (Souza et al. 2006).

The traditional one-at-a-time optimization strategy is simple and useful for screening, and the individual effects of medium components can be seen on a graph without the need to revert to more sophisticated statistical analyses. Unfortunately, this simple method frequently fails to locate the region of optimum response because the joint effects of factors on the response are not taken into account in such procedures. It was reported that the complexities and uncertainties associated with large-scale fermentation usually come from a lack of knowledge of the sophisticated interactions among various factors affecting fermentation (Liu et al. 2005).

Statistically based experimental designs provide an efficient approach to optimization. The fractional factorial design (FFD) is especially suitable to account for the interactions and identify the most significant components in the medium formula. A combination of factors generating a certain optimal response can be identified through factorial design and the use of response surface methodology (RSM) (Junqua et al. 1997).

RSM is a powerful technique for testing multiple process variables because fewer experimental trials are needed compared to the study of one variable at a time. Also, significant interactions between variables can be identified and quantified by this technique.

Taking into account that the soil is a great reservoir of actinomycetes and that there are few reports concerning optimization of culture medium for TGase production, in this paper we report the isolation and screening of Brazilian

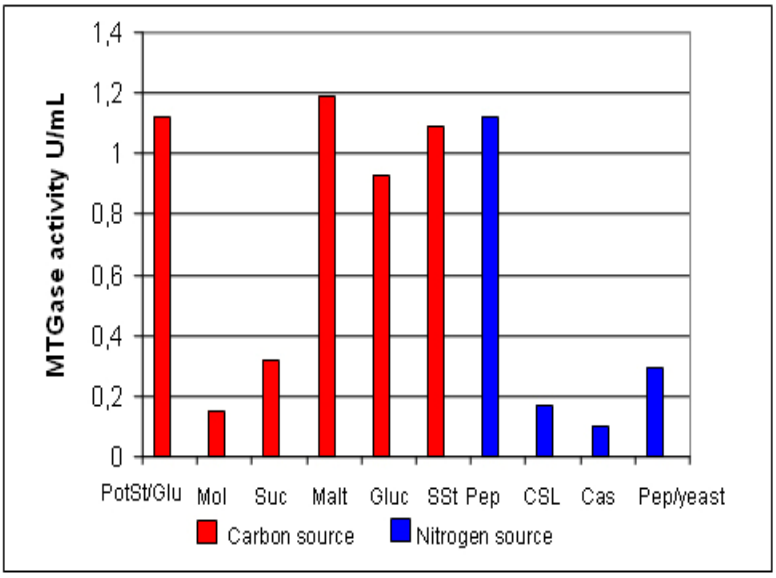


**Figure 1. Effects of carbon and nitrogen sources on MTGase activity.**

**Table 1. Coded levels and real values (in parentheses) for the experimental design and results of the $2^{6-2}$ fractional factorial design.**

| Trial | Powered soy (%) | Potato starch (%) | Peptone (%) | $KH_2PO_4$ (%) | $MgSO_4.7H_2O$ (%) | Glucose (%) | MTGase activity (U/ml) | | |
|---|---|---|---|---|---|---|---|---|---|
| | $x_1$ ($X_1$)[a] | $x_2$ ($X_2$) | $x_3$ ($X_3$) | $x_4$ ($X_4$) | $x_5$ ($X_5$) | $x_6$ ($X_6$) | 72 hrs | 96 hrs | 120 hrs |
| 1 | -1 (0.5) | -1 (0.5) | -1 (1.0) | -1 (0.0) | -1 (0.0) | -1 (0.1) | 0.21 | 0.10 | 0.08 |
| 2 | 1 (2.5) | -1 (0.5) | -1 (1.0) | -1 (0.0) | 1 (0.2) | -1 (0.1) | 0.28 | 0.41 | 0.18 |
| 3 | -1 (0.5) | 1 (3.5) | -1 (1.0) | -1 (0.0) | 1 (0.2) | 1 (0.9) | 0.02 | 0.51 | 1.08 |
| 4 | 1 (2.5) | 1 (3.5) | -1 (1.0) | -1 (0.0) | -1 (0.0) | 1 (0.9) | 0.02 | 0.03 | 0.02 |
| 5 | -1 (0.5) | -1 (0.5) | 1 (2.0) | -1 (0.0) | 1 (0.2) | 1 (0.9) | 0.02 | 0.32 | 0.22 |
| 6 | 1 (2.5) | -1 (0.5) | 1 (2.0) | -1 (0.0) | -1 (0.0) | 1 (0.9) | 0.64 | 0.84 | 0.32 |
| 7 | -1 (0.5) | 1 (3.5) | 1 (2.0) | -1 (0.0) | -1 (0.0) | -1 (0.1) | 0.47 | 0.06 | 0.14 |
| 8 | 1 (2.5) | 1 (3.5) | 1 (2.0) | -1 (0.0) | 1 (0.2) | -1 (0.1) | 0.12 | 0.70 | 0.70 |
| 9 | -1 (0.5) | -1 (0.5) | -1 (1.0) | 1 (0.4) | -1 (0.0) | 1 (0.9) | 0.34 | 0.17 | 0.10 |
| 10 | 1 (2.5) | -1 (0.5) | -1 (1.0) | 1 (0.4) | 1 (0.2) | 1 (0.9) | 0.50 | 0.61 | 1.19 |
| 11 | -1 (0.5) | 1 (3.5) | -1 (1.0) | 1 (0.4) | 1 (0.2) | -1 (0.1) | 0.63 | 1.15 | 1.14 |
| 12 | 1 (2.5) | 1 (3.5) | -1 (1.0) | 1 (0.4) | -1 (0.0) | -1 (0.1) | 0.62 | 1.11 | 1.27 |
| 13 | -1 (0.5) | -1 (0.5) | 1 (2.0) | 1 (0.4) | 1 (0.2) | -1 (0.1) | 0.03 | 0.05 | 0.04 |
| 14 | 1 (2.5) | -1 (0.5) | 1 (2.0) | 1 (0.4) | -1 (0.0) | -1 (0.1) | 0.29 | 0.46 | 0.52 |
| 15 | -1 (0.5) | 1 (3.5) | 1 (2.0) | 1 (0.4) | -1 (0.0) | 1 (0.9) | 0.37 | 0.30 | 0.63 |
| 16 | 1 (2.5) | 1 (3.5) | 1 (2.0) | 1 (0.4) | 1 (0.2) | 1 (0.9) | 0.38 | 0.75 | 0.71 |
| 17 | 0 (1.5) | 0 (2.0) | 0 (1.5) | 0 (0.2) | 0 (0.1) | 0 (0.5) | 0.41 | 0.63 | 1.11 |
| 18 | 0 (1.5) | 0 (2.0) | 0 (1.5) | 0 (0.2) | 0 (0.1) | 0 (0.5) | 0.37 | 0.74 | 1.13 |
| 19 | 0 (1.5) | 0 (2.0) | 0 (1.5) | 0 (0.2) | 0 (0.1) | 0 (0.5) | 0.35 | 0.67 | 1.08 |

[a]xi is the coded value and $X_i$ is the actual value of the *i*th independent variable. The conversion between $x_i$ and $X_i$ is described on equation [1].

soil actinomycetes for TGase activity, and studied the nutritional fermentation conditions in order to maximize the TGase yield.

## MATERIALS AND METHODS

### Selective isolation and preservation of actinomycetes

The actinomycetes strains used in this study were isolated from soil samples collected from the States of São Paulo and Bahia in Brazil.

Nearly five grams of the soil sample were added to 10 mL sterilized distilled water and the suspension was shaken at 200 rpm for 10 min. Aliquots were inoculated onto Petri dishes containing starch-casein media (Küster and Williams, 1964). The plates were incubated at 30ºC for up to 5 days. Actinomycete-like colonies were streaked onto slants of ISP2 media (0.4% yeast extract, 1% malt extract, 0.4% de glucose and agar, pH 7.0 ± 0.2) and checked for purity. Colonies were removed from the agar media and preserved in cryotubes with 10% glycerol solution at -80ºC.

### MTGase-production screening

The ability to produce MTGase was determined by inoculating 1 mL of spore suspension into 250 mL Erlenmeyer flasks containing 50 mL of seed medium (Ando et al. 1989) composed of: 0.2% of peptone; 0.2% $KH_2PO_4$; 0.1% $MgSO_4.7H_2O$; 0.5% of glucose (pH 7.0). The flasks were incubated for 2 days at 30ºC and 200 rpm in a rotational shaker. Aliquots of 15 mL of pre inoculum were transferred to 135 mL of the basal medium (Ando et al. 1989) (2% of peptone; 0.2% of yeast extract; 0.2% $KH_2PO_4$; 0.1% $MgSO_4.7H_2O$; 2% of potato starch; 0.5% of glucose; pH 7.0) in 500 mL Erlenmeyer flasks and cultivated at 30ºC for 5 days at 200 rpm. All runs were made in duplicate. After that, the MTGase activity was measured as described below.

### MTGase activity

Aliquots of 1 mL of culture medium were taken and after centrifugation the enzyme activity was detected in the supernatant by the colorimetric hydroxamate procedure with N-carbo-benzoxy-L-glycine according to Folk and

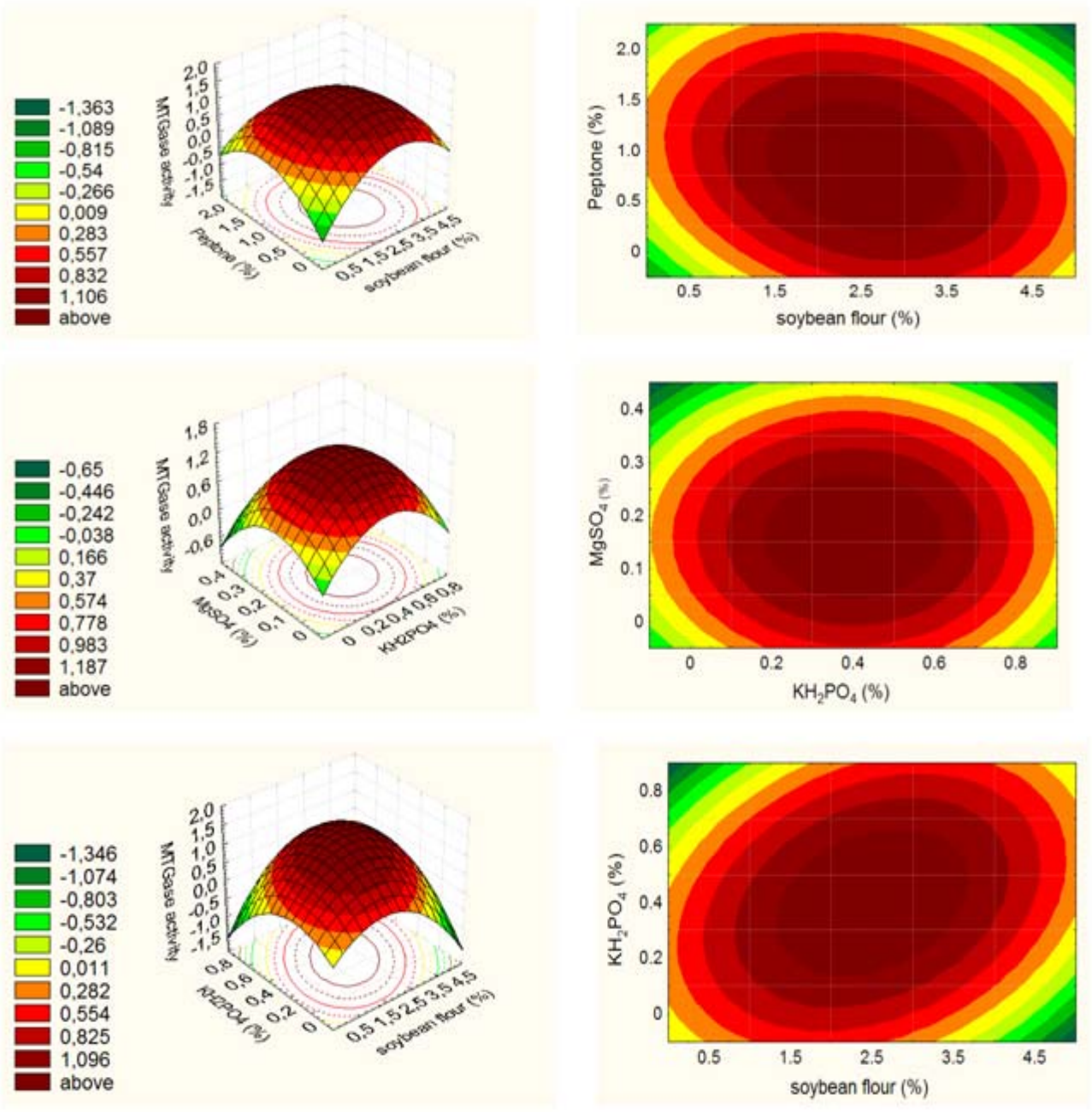


**Figure 2. Contour curve and response surface for the MTGase activity as a function of:**
**(a) peptone *versus* powered soy.**
**(b) $MgSO_4$ *versus* $KH_2PO_4$.**
**(c) $KH_2PO_4$ *versus* soybean flour concentrations, according to the CCD.**

Cole (1966) with some adaptations. Reaction mixture, containing 200 µL of enzyme, 200 µL of 0.2 M pH 6.0 citrate buffer, 25 µL of hydroxylamine, and 75 µL of 0.1 M N-carbo-benzoxy-L-glycine, was incubated at 37ºC for 1 hr and then the reaction was stopped by adding an equal volume (500 µL) of 15% three chloride acetic acid (TCA) containing 5% $FeCl_3$. The absorbance was measured at 525 nm (Beckman coulter DU 640). One unit of MTGase activity was defined as the amount of enzyme which causes the formation of one micromole of hydroxamic acid per min at 37ºC. A calibration curve was prepared using L-glutamic acid y-monohydroxamate.

## Preliminary tests

**Testing high viscosity medium fermentation.** This test consisted of adding the contents of one cryotube (two agar-mycelium cylinders nearly 0.6 cm in diameter) directly into

15 mL of the modified basal medium composed of: peptone (2%); $KH_2PO_4$ (0.2%); $MgSO_4.7H_2O$ (0.1%); potato starch (2%); glucose (0.2%); and soybean flour (2%) (pH 7.0). This was done in 50 mL Erlenmeyer flasks and cultivated for 5 days at 100 rpm in a rotary shaker. An aliquot of 1 mL was taken at 120 hrs, and the enzyme activity measured as described above in section MTGase activity.

**Screening for the optimal sources of carbon and nitrogen.** The effects of different sources of nitrogen and carbon on the MTGase production were investigated with the one-by-one time strategy. The nitrogen sources and corresponding concentrations tested were: peptone 2% with yeast extract 0.2%; peptone 2%; corn steep liquor (CSL) 2%; and casein 2%. The carbon sources were: potato starch 2% with glucose 0.2%; molasses 2%; sucrose 2%; maltodextrin 2%; glycerol 2%; and soluble starch 2%. In the investigation of the nitrogen sources, growth was carried out in the medium containing: 0.2% $KH_2PO_4$; 0.1% $MgSO_4.7H_2O$; 2% soybean flour; 2% potato starch; 0.2% glucose. In the process of screening carbon sources, fermentation was carried out using the medium containing: 0.2% $KH_2PO_4$; 0.1% $MgSO_4.7H_2O$; 2% soybean flour; and 2% peptone.

## The optimization procedure and experimental design

All experimental design results were analyzed using STATISTICA 5.0 for Windows (Statsoft, Inc.).

**Table 2. Coded levels and real values (in parentheses) for the experimental design and results of CCD.**

| Trial | Powered soy (%) $x_1(X_1)$[a] | $KH_2PO_4$ (%) $x_2(X_2)$ | $MgSO_4$ (%) $x_3(X_3)$ | Peptone (%) $x_4(X_4)$ | MTGase (U/ml) 120 hrs | |
|---|---|---|---|---|---|---|
| | | | | | Experimental | Predicted |
| 1 | -1 (1.5) | -1 (0.2) | -1 (0.1) | -1 (0.5) | 0.87 | 0.78 |
| 2 | 1 (3.5) | -1 (0.2) | -1 (0.1) | -1 (0.5) | 0.74 | 0.71 |
| 3 | -1 (1.5) | 1(0.6) | -1 (0.1) | -1 (0.5) | 0.51 | 0.57 |
| 4 | 1 (3.5) | 1(0.6) | -1 (0.1) | -1 (0.5) | 0.99 | 0.93 |
| 5 | -1 (1.5) | -1 (0.2) | 1 (0.3) | -1 (0.5) | 0.67 | 0.86 |
| 6 | 1 (3.5) | -1 (0.2) | 1 (0.3) | -1 (0.5) | 0.72 | 0.79 |
| 7 | -1 (1.5) | 1(0.6) | 1 (0.3) | -1 (0.5) | 0.81 | 0.64 |
| 8 | 1 (3.5) | 1(0.6) | 1 (0.3) | -1 (0.5) | 1.01 | 1.00 |
| 9 | -1 (1.5) | -1 (0.2) | -1 (0.1) | 1 (1.5) | 1.33 | 1.05 |
| 10 | 1 (3.5) | -1 (0.2) | -1 (0.1) | 1 (1.5) | 0.70 | 0.69 |
| 11 | -1 (1.5) | 1(0.6) | -1 (0.1) | 1 (1.5) | 0.82 | 0.83 |
| 12 | 1 (3.5) | 1(0.6) | -1 (0.1) | 1 (1.5) | 0.78 | 0.90 |
| 13 | -1 (1.5) | -1 (0.2) | 1 (0.3) | 1 (1.5) | 0.36 | 0.50 |
| 14 | 1 (3.5) | -1 (0.2) | 1 (0.3) | 1 (1.5) | 0.23 | 0.14 |
| 15 | -1 (1.5) | 1(0.6) | 1 (0.3) | 1 (1.5) | 0.21 | 0.29 |
| 16 | 1 (3.5) | 1(0.6) | 1 (0.3) | 1 (1.5) | 0.44 | 0.36 |
| 17 | -2 (0.5) | 0 (0.4) | 0 (0.2) | 0 (1.0) | 0.56 | 0.54 |
| 18 | 2 (4.5) | 0 (0.4) | 0 (0.2) | 0 (1.0) | 0.49 | 0.54 |
| 19 | 0 (2.5) | -2 (0.0) | 0 (0.2) | 0 (1.0) | 0.57 | 0.71 |
| 20 | 0 (2.5) | 2 (0.8) | 0 (0.2) | 0 (1.0) | 0.81 | 0.71 |
| 21 | 0 (2.5) | 0 (0.4) | -2 (0.0) | 0 (1.0) | 0.90 | 1.02 |
| 22 | 0 (2.5) | 0 (0.4) | 2 (0.4) | 0 (1.0) | 0.65 | 0.56 |
| 23 | 0 (2.5) | 0 (0.4) | 0 (0.2) | -2 (0.0) | 0.91 | 0.91 |
| 24 | 0 (2.5) | 0 (0.4) | 0 (0.2) | 2 (2.0) | 0.49 | 0.53 |
| 25 | 0 (2.5) | 0 (0.4) | 0 (0.2) | 0 (1.0) | 1.43 | 1.37 |
| 26 | 0 (2.5) | 0 (0.4) | 0 (0.2) | 0 (1.0) | 1.17 | 1.37 |
| 27 | 0 (2.5) | 0 (0.4) | 0 (0.2) | 0 (1.0) | 1.50 | 1.37 |

[a] $x_i$ is the coded value and $X_i$ is the actual value of the *i*th independent variable. The conversion between $x_i$ and $X_i$ is described on equation [1].

**Elucidation of the significant components using a FFD.** A $2^{(6-2)}$ FFD was employed to determine the key ingredients that affected TGase production significantly. There were six nutrient factors in the medium, which were: peptone, $KH_2PO_4$, $MgSO_4.7H_2O$, potato starch, soybean flour and glucose. Each factor was examined at a high level (coded +1) and a low level (coded -1), which corresponded to the basal level ± 50%, respectively. The center points were the trials under the basal level conditions (coded 0). Table 1 shows the variables and levels in detail. A ¼ fraction of the full factorial design was adopted; consequently, the experiment included 16 ($2^{6-2}$) combinations plus two replicates at the center point, as shown in Table 1. Each trial was performed only once, and the enzyme activity was measured at 72 hrs, 96 hrs and 120 hrs of fermentation.

**Optimization of key ingredients concentrations using a central composite design (CCD).** The medium components that affected TGase production significantly were optimized using a CCD design. The variables were coded according to the equation [1]:

$$x_i = \frac{X_i - X_0}{\Delta X_i} \qquad [1]$$

where $x_i$ is the coded variable of a nutrient factor, $X_i$ is the natural variable of the nutrient factor. $X_o$ is the value of the natural variable at the center point, and $\Delta X_i$ is the step change value. The variables and levels are shown in Table 2.

The statistical software defined a full CCD design for 4 factors consisting of 24 combinations plus the replicates at the center point (16 cubic points, 8 star points and 2 replicates at the center point to estimate the experimental error and to investigate the suitability of the proposed model); details are illustrated in Table 2. Once the experiments had been performed, the experimental results were fitted to a second-order polynomial function. The Student t-test permitted us to check the statistical significance of the regression coefficients, and the analysis of variances (ANOVA) was performed on the experimental data to evaluate the statistical significance of the model. The model for the response was expressed in terms of coded variables, without taking into account the statistically non-significant terms.

## RESULTS

The strategy employed in this study, comprising selective isolation conditions for actinomycetes using different Brazilian soil samples, was successful for the recovery of about 200 actinomycete pure cultures. These isolates were investigated for TGase production and the results showed that stronger enzyme activity was found in the actinomycete strains T10b (0.15 $U.mL^{-1}$) and P20 (0.25$U.mL^{-1}$). The latter strain was chosen to continue the studies andwas taxonomically identified by molecular methods as *Streptomyces* sp. and subsequently deposited in the Brazilian Collection of Environmental and Industrial Microorganisms (CBMAI) under the number CBMAI 837, to be available for further investigations.

In order to optimize the MTGase activity, modifications of the usual media components reported for enzyme production were tested. Data from the high viscosity medium fermentation test showed that *Streptomyces* sp. (strain P20) presented higher activity on this medium (1.1 $U.mL^{-1}$) when compared with the MTGase activity obtained on the basal medium (0.20 $U.mL^{-1}$) after 120 hrs of fermentation.

The results of the effects of the carbon and nitrogen sources on the MTGase activity produced by *Streptomyces* sp. P20 are shown in Figure 1. Of all nitrogen sources investigated, the most promising was peptone, where the MTGase activity was about 1.12 $U.mL^{-1}$. For the carbon source, the best results were obtained with a mixture of potato starch and glucose (1.12 $U.mL^{-1}$) and with maltodextrin (1.18 $U.mL^{-1}$). Since the results for both carbon sources were not significantly different, the cheapest source (potato starch and glucose) was chosen to continue the study.

The next step was to define each of the media components affecting MTGase production significantly and to determine the best concentrations of each. As a preliminary step for optimization, the most important nutrient factors were screened by applying the two-level FFD as described in materials and methods. The experimental design and the

**Table 3. Effects estimates, standard error, Student's t-test and p-test calculated for the $2^{6-2}$ fractional factorial design.**

| | MTGase activity (U/mL) | | | |
|---|---|---|---|---|
| | **Effect** | **Std. Err.** | **t-value** | **p-value** |
| Mean/Inter. | 0.3195* | 0.0061 | 52.5191 | 0.0004 |
| Powered soy (%) | 0.0943* | 0.0133 | 7.1101 | 0.0192 |
| Potato starch (%) | 0.0384 | 0.0133 | 2.8980 | 0.1013 |
| Peptone (%) | -0.0388 | 0.0133 | -2.9244 | 0.0997 |
| $KH_2PO_4$ (%) | 0.1706* | 0.0133 | 12.8675 | 0.0060 |
| $MgSO_4.7H_2O$ (%) | -0.1207* | 0.0133 | -9.1024 | 0.0118 |
| Glucose (%) | -0.0446 | 0.0133 | -3.3609 | 0.0783 |

*significant factors ($p < 0.05$).

results of the FFD observations are presented in Table 1. TGase production varied greatly from 0.02 to 1.27 $U.mL^{-1}$ with different combinations of the components in the media. The main effects are shown in Table 3.

In order to optimize the key ingredients selected in the media, a CCD was made. The experimental design and results are shown in Table 2. The quadratic model calculated for maximum MTGase activity, after eliminating the statistically insignificant terms ($p > 0.05$), was:

$$Y = 1.3675 - 0.20602x_1^2 - 0.1652x_2^2 - 0.11622x_3 - 0.14376x_3^2 - 0.09508x_4 - 0.16281x_4^2 + 0.106293x_1.x_2 - 0.07399x_1.x_4 - 0.1557x_3.x_4$$

The ANOVA reproduced in Table 4 showed that the model was significant. The Fisher *F*-test ($F_{9;17} = 14.2 > F_{t\ 0.95;9;17} = 2.49$) was 5-6 times higher than the *F*t, and the p-value <0.000001 did in fact demonstrate that this regression was statistically significant at the 95% confidence level. Besides, the $R^2$ (multiple correlation coefficient) of the regression equation obtained I was 0.88 (a value >0.75 indicates aptness of the model), which means that the model can explain 88% of the variation in the response.

Three response surfaces were chosen among the possible combinations as representative of each selected rotation speed to visualize the simultaneous effects of peptone, soybean flour, $KH_2PO_4$ and $MgSO_4.7H_2O$ on MTGase production pattern (Figure 2).

## DISCUSSION

A *Streptomyces* sp. strain was isolated from a Brazilian soil sample as a potential producer of TGase; it was found to be an extracellular producer, with a calcium independent enzyme, which makes it much more interesting and practical for industrial use (Yokoyama et al. 2004).

The fermentation of P20 in the higher viscosity medium was adequate, since a consistent formation of pellets was observed during growth on the liquid medium. According to previous experience in our laboratory, this behavior was already observed for a fungus that was used to growing on a solid medium, when exposed to a liquid medium, and indicated that the microorganism was not well adapted to the medium. Another reason that moved us to try a higher viscosity medium for the enzyme production was the inconsistency of the results obtained in the liquid medium. The lack of repeatability of the values of MTGase activity made it necessary to run several tests. The tests indicated that the microorganism could grow well in a rich liquid, but with no MTGase production at all. On the other hand, MTGase activity reached much higher levels (5.5 times higher) in experiments where the microorganism had some kind of physical support to grow on. These results further supported the hypothesis raised by Yan et al. (2005) that MTGase is probably involved in the formation of covalent bonds between different cell wall proteins (to provide the mycelium growing in *Streptomyces*).

In order to select the best and less expensive sources of nitrogen and carbon for the production of TGase, different compounds were investigated with the one-by-one time strategy. Data from these experiments showed that better results were obtained when peptone was used as the nitrogen source (1.12 $U.mL^{-1}$) and when a mixture of potato starch and glucose (1.12 $U.mL^{-1}$); and maltodextrin (1.18 $U.mL^{-1}$) were used as carbon sources. The results for both carbon sources were not significantly different and for that reason the source with the lower price (potato starch and glucose) was chosen to continue the study. For the next step, the FFD study, the salts of the media were the only independent variables that had not previously been tested; they were used according to the literature data.

Observing the results of the FFD experiment, it was clear that variations in the concentration of potato starch, peptone and glucose did not affect MTGase production significantly at the levels tested. Considering this, the glucose concentration was fixed at the minimum value for the next factorial design, and the concentration of potato starch was fixed at the intermediary level. Since peptone is the most expensive compound in the fermentation medium, its concentration was not fixed at the level -1 for the next study, because we hoped to find an even lower concentration where it would still not interfere with the MTGase activity significantly. On the other hand, the enzyme production was greatly affected by soybean flour, $KH_2PO_4$ and $MgSO_4.7H_2O$ ($p < 0.05$). These three nutrient components and peptone were further investigated in a broader range of concentration within a CCD.

The results from the CCD showed that the optimal

**Table 4. Analysis of variance and regression analyses for the response of the $2^4$ central composite design.**

| Source of variation | Sum of squares | Degrees of freedom | Mean squares | $F_{test}$[a] | p-value |
|---|---|---|---|---|---|
| Regression | 2.4883 | 9 | 0.2765 | 14.2 | <0.00001 |
| Residual | 0.3310 | 17 | 0.0195 | | |
| Lack of fit | 0.2733 | 15 | 0.0182 | | |
| Pure error | 0.0577 | 2 | 0.0288 | | |
| Total | 2.8193 | | | | |

Coefficient of determination: $R^2 = 0.88$

[a] $F_{0.95;9;17} = 2.49$

concentrations of the four key ingredients were basically the same values of the central point: 2.5% soybean flour; 0.4% $KH_2PO_4$; 0.2% $MgSO_4.7H_2O$ and 1.0% peptone. However, it was not possible to reduce the peptone concentration without reducing the MTGase activity. The maximum MTGase activity predicted by the model was calculated to be 1.37 $U.mL^{-1}$. In order to confirm the predicted results and validate the regression obtained, experiments using the improved formula for the media were performed, and a value of 1.4 $U.mL^{-1}$ ($n$ = 3) was obtained. It was shown that the model was adequate to predict the optimization of MTGase production of Streptomyces sp. P20.

The fermentation media for MTGase production has been almost the same in every published work since Ando et al. (1989). In this work we have modified and optimized the fermentation media for MTGase production using statistical methods. From the present study, it is evident that the use of a FFD statistical design and CCD can be used to determine the significant variables and the optimum conditions for MTGase production, respectively. The optimization of the medium resulted not only in an 86% higher MTGase activity than in the media previously cited in the literature, but also in a reduction of the constituents costs and an improvement in repeatability. It is important to observe that the value of 1.4 $U.mL^{-1}$ of MTGase activity obtained in this study is significant when compared with values previously reported in the literature that ranged from 0.25 $U.mL^{-1}$ to 2.5 $U.mL^{-1}$ (Zhu et al. 1995). In addition, the relevance of these results is that the factors leading to the increased activity were identified and are important for further studies.

Considering the results obtained in the present study, the conclusion was that the Brazilian soil isolate *Streptomyces* sp. P20 offers great potential for further investigation on MTGase production. Therefore, experiments involving other aspects of production (optimization of temperature and agitation conditions) and characterization of the enzyme are being carried out. In addition, strain P20 will be taxonomically characterized by using the polyphasic approach, in order to have a more accurate identification.

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