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Optimization Studies for the Production of Microbial Transglutaminase from a Newly Isolated Strain of *Streptomyces* sp.

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Abstract Covalent cross-links between a number of proteins and peptides explain why transglutaminase may be widely used by food processing industries. The objective of this work was optimization of the fermentation process to produce transglutaminase from a new microbial source, the *Streptomyces* sp. P20. The strategy adopted to modify the usual literature media was: (1) fractional factorial design (FFD) to elucidate the key medium ingredients, (2) central composite design (CCD) to optimise the concentration of the key components. Optimization of the medium resulted in not only an 86% increase in microbial transglutaminase activity as compared to the media cited in the literature, but also a reduction in the production cost. Optimal fermentation conditions - namely temperature and agitation rate - were also studied, using CCD methodology. Usual conditions of 30°C and 100 rpm were within the optimal area. All other parameters for enzyme production were experimentally proven to be optimum fermentation conditions.

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

Introduction

Transglutaminase (EC 2.3.2.13; protein-glutamine γ-glutaminyltranferase) is an enzyme that catalyse an acyl transfer reaction using protein or peptide-bond glutamine residues as acyl donors and several primary amines as receptors. When the ε-amino groups of the protein or peptide-bond lysyl residues are present as acyl receptors, this enzyme is capable of forming intra and intermolecular ε-(γ-Glu)-Lys isopeptide bonds (1).

The covalent cross-links between a number of proteins and peptides introduced by transglutaminase promote modification of the functional properties of the food proteins (2). Therefore, transglutaminase are widely used by food processing industries for the purpose of, new product development, modification of the product properties such as viscosity, emulsification foaming, and nutritional values (3,4).

Transglutaminases are found in mammalian tissues, plasma, fish, and plants (5). The mammalian enzymes are Ca^{+2}-dependent. However, the relatively small quantities and the complex separation and purification procedures for these enzymes led to the search for alternative microbial sources. The first microbial transglutaminase (MTGase) was obtained from actinomycetes (6). Since then, efforts have been made to obtain this enzyme in large quantity for commercial applications, especially for the enzymes from *Streptomyces* sp. (7-11).

So far, researches have been focused on the isolation and screening of microorganisms with transglutaminase activity, and the purification and characterization of the enzymes. The media compositions used to produce MTGase from *Streptomyces* sp. have been almost the same in all the papers published since Ando *et al.* (6,12-14). The formulation of the culture media is of critical importance for industrial processes because the composition affects product concen- tration, yield, and volumetric productivity. It is also important to reduce the cost of the medium, as this may affect the overall process economics (15).

The traditional one-at-a-time optimization strategy is simple and useful for screening, and the individual effects of medium components can be seen without the use of 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. It has been 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 (16).

Statistically based experimental designs provide an efficient approach for the optimization of media composition. 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 via a factorial design and the use of response surface methodology (RSM) (17).

RSM is a powerful technique for testing multiple process variables because fewer experimental trials are needed compared to the studies using one variable at a time. Also, significant interactions between variables can be identified and quantified by this technique (18-20).

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Taking into account that the soil is a great reservoir of actinomycetes and that there are few reports concerning the optimization of culture media for transglutaminase production, in this paper we report the isolation and screening of Brazilian soil actinomycetes for transglutaminase activity, and the fermentation conditions in order to maximize the transglutaminase yield.

Materials and Methods

Materials Yeast extract, malt extract, peptone, and agar were from Difco Laboratories (Detroit, MI, USA). KH_2PO_4, $MgSO_4 \cdot 7H_2O$, glycerol, and glucose were from Merck (Darmstadt, Germany). *N*-Carbo-benzoxy-L-glycine and L-glutamic acid γ-monohydroxamate were purchased from Sigma-Aldrich (St. Louis, MO, USA), soybean flour was from Yasmin Food Industry (São Paulo, SP, Brazil) and the potato starch was Yoki Food Industry (São Bernardo do Campo, SP, Brazil).

Selective isolation and preservation of actinomycetes The actinomycetes strains used in this study were isolated from soil samples collected in the States of São Paulo and Bahia in Brazil. About 5 g of the soil sample were added to 10 mL sterilized distilled water and the suspension shaken at 200 rpm for 10 min. Aliquots were inoculated onto solidified starch-casein media (21). The plates were incubated at 30°C for up to 5 days. Actinomycete-like colonies were streaked onto slants of ISP2 media (0.4% yeast extract, 1% malt extract, 0.4% of glucose and agar, pH 7.0±0.2) and single colonies were obtained. Colonies were removed from the agar media and preserved in cryotubes with 10% glycerol solution at –80°C.

Microorganisms selection for MTGase production 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 (6) composed of; 0.2% peptone, 0.2% KH_2PO_4, 0.1% $MgSO_4 \cdot 7H_2O$, and 0.5% glucose (pH 7.0). The flasks were incubated for 2 days at 30°C and 200 rpm in a rotational shaker. Aliquots of 15 mL of preinoculum were transferred to 135 mL of the basal medium (2% peptone, 0.2% yeast extract, 0.2% KH_2PO_4, 0.1% $MgSO_4 \cdot 7H_2O$, 2% potato starch, 0.5% glucose, pH 7.0) in 500-mL Erlenmeyer flasks (6) and cultivated at 30°C for 5 days at 200 rpm. All runs were made in duplicates.

Aliquots of 1 mL of culture medium were taken, and after centrifugation the enzyme activity in the supernatant was detected by the colorimetric hydroxamate procedure with *N*-carbo-benzoxy-L-glycine according to Folk and Cole (22), with some modifications. One unit of MTGase activity was defined as the amount of enzyme causing the formation of 1 μmol of hydroxamic acid/min at 37°C. A calibration curve was prepared using L-glutamic acid γ-monohydroxamate.

Preliminary tests ***Production of MTGase using a high viscosity medium*:** The production of MTGase was tested in a high viscosity medium composed of peptone (2%); KH_2PO_4 (0.2%), $MgSO_4 \cdot 7H_2O$ (0.1%), potato starch (2%), glucose (0.2%), and soybean flour (2%) (pH 7.0). The contents of 1 cryotube (2 agar mycelium cylinders of nearly 6 mm in diameter) were transferred to 50-mL Erlenmeyer flasks containing 15 mL of medium and incubated for 5 days at 100 rpm and 30°C in a rotatory shaker. One mL aliquot was taken after 120 hr, and the enzyme activity measured as described above.

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

Optimization procedure for the production parameters and experimental design All experimental design results were analyzed using STATISTICA 5.0 for Windows (Statsoft, Inc., Tulsa, OK, USA).

***Elucidation of the significant components using a FFD*:** A $2^{(6-2)}$ FFD was employed to determine the key ingredients that significantly affected MTGase production. There were 6 nutrient factors in the medium; peptone, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, 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 1/4 fraction of the full factorial design was adopted and consequently the experiment included 16 (2^{6-2}) combinations plus 2 replicates at the center point, as shown in Table 1. The enzyme activity was measured after 72, 96, and 120 hr of fermentation.

***Optimization of key ingredient concentrations using a CCD*:** The medium components that significantly affected MTGase production were optimized using a CCD design. The variables were coded according to the equation 1:

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad (1)$$

where, x_i is the coded variable of a nutrient factor, X_i is the natural variable of the nutrient factor, X_0 is the value of the natural variable at the center point, and ΔX_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), and the details are given in Table 2. Once the experiments had been performed, the experimental results were fitted to a second-order polynomial function. The Student's *t*-test permitted the statistical significance of the

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

Trial	Soybean flour (%)	Potato starch (%)	Peptone (%)	KH_2PO_4 (%)	$MgSO_4 \cdot 7H_2O$ (%)	Glucose (%)	MTGase activity (U/mL)		
	x_1 (X_1)[1)]	x_2 (X_2)	x_3 (X_3)	x_4 (X_4)	x_5 (X_5)	x_6 (X_6)	72 hr	96 hr	120 hr
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

[1)] 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.

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

Trial	Soybean flour (%) x_1(X_1)[1)]	KH_2PO_4 (%) x_2(X_2)	$MgSO_4$ (%) x_3(X_3)	Peptone (%) x_4(X_4)	MTGase (U/mL) 120 hr	
					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

[1)] 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.

Table 3. Coded levels and real values (in parentheses) for the experimental design and results of CCD for agitation and temperature

Trial	Agitation (rpm) $x_1(X_1)$[1)]	Temperature (°C) $x_2(X_2)$	MTGase (U/mL) 120 hr	
			Experimental	Predicted
1	-1 (115)	-1 (26)	1.5	1.26
2	1 (185)	-1 (26)	1.11	0.76
3	-1 (115)	1 (34)	0.35	0.41
4	1 (185)	1 (34)	0.42	0.37
5	-1.41 (100)	0 (30)	1.05	1.06
6	1.41 (200)	0 (30)	0.52	0.69
7	0 (150)	-1.41 (25)	0,75	0.96
8	0 (150)	1.41 (35)	0.32	0.09
9	0 (150)	0 (30)	0.51	0.52
10	0 (150)	0 (30)	0.54	0.52
11	0 (150)	0 (30)	0.46	0.52
12	0 (150)	0 (30)	0.59	0.52

[1)] 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.

regression coefficients to be checked, and the analysis of variance (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 the statistically non-significant terms into account.

***Temperature and agitation optimization procedure and experimental design*:** The fermentation parameters that influenced the MTGase production were optimized using a CCD design. The variables were coded, as described previously, according to equation 1, and the variables and their levels are shown in Table 3.

The Statistica software defined a full CCD design for 2 factors (2^2), consisting of 9 combinations plus the replicates at the center point (5 cubic points, 4 star points, and 3 replicates at the center point, to estimate the experimental error and investigate the suitability of the proposed model), and the details are presented in Table 3. The experimental results were fitted to a second-order polynomial function and the Student's *t*-test permitted checking of the statistical significance of the regression coefficients. The ANOVA was performed on the experimental data to evaluate the statistical significance of the model. The response model was expressed in terms of coded variables, ignoring the statistically nonsignificant terms.

Influence of inoculum size The influence of inoculum size on MTGase activity was tested with a simple one-variable test. Five flasks of the optimum medium were inoculated with, respectively, 1, 2, 3, 4, and 5 cylinders (6 mm Ø) of the fully-grown agar cultivation of *Streptomyces* sp. P20 cultivated as described previously. All 5 tests were incubated in a rotatory shaker for 5 days under the best conditions of temperature and agitation, and the MTGase activity then measured. This procedure was carried out in triplicate.

Kinetic study MTGase production by *Streptomyces* sp. P20 was observed for 10 days. The microorganism was inoculated into ten 50-mL conical flasks containing 15 mL of optimized medium (2.5% peptone, 0.2% KH_2PO_4, 0.1% $MgSO_4 \cdot 7H_2O$, 2% potato starch, 0.1% glucose, and 2% soybean flour).

Results and Discussion

Microorganisms selection for MTGase production Nearly 200 pure actinomycete cultures were investigated for MTGase production and the actinomycete strains T10b and P20 produced 0.15 and 0.25 U/mL of MTGase activity, respectively. The P20 strain was chosen as a potential producer of extracellular MTGase. The enzyme is calcium independent, which makes it much more interesting and suitable for industrial use (2). The P20 strain was taxonomically identified by molecular methods (16S rDNA gene sequencing and phylogenetic analysis) 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.

Preliminary tests ***Production of MTGase using a high viscosity medium*:** In order to optimize the MTGase production, modifications of the usual media components reported for enzyme production were tested. *Streptomyces* sp. (strain P20) showed higher activity in the high viscosity medium (1.1 U/mL) than in the basal medium (0.20 U/mL) after 120 hr of fermentation. MTGase was more reliably produced in the high viscosity medium. These results further supported the hypothesis raised by Yan *et al.* (12) that MTGase was probably involved in the formation of covalent bonds between different cell wall proteins to provide the mycelium of the *Streptomyces*.

***Selection of the carbon and nitrogen sources for MTGase production*:** The effects of the carbon and nitrogen sources on the MTGase activity produced by *Streptomyces* sp. P20 are shown in Fig. 1. The data from these experiments showed that better results were obtained when peptone was used as the nitrogen source (1.12 U/mL), and when a mixture of potato starch and glucose (1.12 U/mL) or

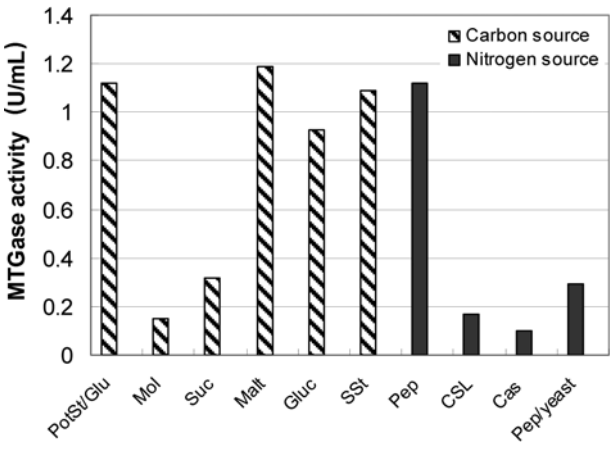

Fig. 1. Effects of carbon and nitrogen sources on MTGase activity.

Table 4. Effects estimates, standard error, Student's *t*-test and *p*-test calculated for the 2^{6-2} FFD

	MTGase activity (U/mL)			
	Effec	Std. Err.	*t*-value	*p*-value
Mean/Inter.	0.3195*[1)]	0.0061	52.5191	0.0004
Soybean flour (%)	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
$MgSO_4 \cdot 7H_2O$ (%)	-0.1207*	0.0133	-9,1024	0.0118
Glucose (%)	-0.0446	0.0133	-3.3609	0.0783

[1)] $*p<0.05$.

Table 5. Analysis of variance and regression analyses[1)] for the response of the 2^4 CCD of the media compounds

Source of variation	Sum of squares	Degrees of freedom	Mean squares	F_{test}[2)]	*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				

[1)] Coefficient of determination: $R^2=0.88$.
[2)] $F_{0,95;9;17}=2.49$.

maltodextrin (1.18 U/mL) was used as the carbon source. Both carbon sources were not significantly different and mixture of potato starch and glucose was chosen for further studies because of lower price.

***Elucidation of the significant components using a FFD*:** The next step was to define the media components that significantly affected the MTGase production and to determine the best concentrations of each. The most important nutrient factors were screened by applying the 2-level FFD described in the material and methods section, and the experimental design and the results of the FFD observations are presented in Table 1. MTGase production varied greatly from 0.02 to 1.27 U/mL with different combinations of the media components, and the main effects are shown in Table 4. Observing the results of the FFD experiment, it was clear that variations in the concentrations of potato starch, peptone, and glucose did not affect MTGase production significantly at the levels tested. Thus the glucose concentration was fixed at the minimum value for the next factorial design, and the concentration of potato starch was fixed at an intermediary level. Since peptone is the most expensive compound in the fermentation medium, its concentration was not fixed, but studied at a lower level of concentration in the next study. On the other hand, the enzyme production was greatly affected by soybean flour, KH_2PO_4, and $MgSO_4 \cdot 7H_2O$ ($p<0.05$). These 3 nutrient components and peptone were further investigated in a broader range of concentrations within a CCD.

***Optimization of key ingredient concentrations using a CCD*:** In order to optimize the key ingredients selected in the media, a CCD was carried out, and 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:

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

The ANOVA reproduced in Table 5 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 <0.00001 did, in fact, demonstrate that this regression was statistically significant at the 95% confidence level. In addition, the multiple correlation coefficient (R^2) of the regression equation obtained 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 amongst the possible combinations as representative of each selected rotation speed to visualize the simultaneous effects of peptone, soybean flour, KH_2PO_4, and $MgSO_4 \cdot 7H_2O$ on the MTGase production pattern (Fig. 2).

The results from the CCD showed that the optimal concentrations of the 4 key ingredients were basically the same as the values at the central point; 2.5% soybean flour, 0.4% KH_2PO_4, 0.2% $MgSO_4 \cdot 7H_2O$, and 1.0% peptone. However, the reduction in peptone concentration decreased the MTGase activity. The maximum MTGase activity predicted by the model was calculated to be 1.37 U/mL. In order to confirm the predicted results and validate the regression obtained, experiments using the improved formula of the medium were performed, and a value of 1.4 U/mL ($n=3$) was obtained, a result that demonstrated the adequacy of the model generated.

In the present work the fermentation medium for MTGase production was modified and optimized using statistical methods. The statistical tools, FFD and CCD, are found to be useful to determine the significant variables and the optimum conditions for MTGase production. The optimization of the medium resulted in not only an increase in MTGase activity from 0.25 to 1.4 U/mL, but also a reduction in the costs of the constituents and an improvement in repeatability. It is important to note that the greatest relevance of these results lies in the factors leading to the increased activity, which may be important in further studies.

***Temperature and agitation optimization procedure and experimental design*:** The study to determine the best conditions of temperature and agitation in the rotatory shaker was made using an experimental design and the results are shown in Table 3. The quadratic model calculated for maximum MTGase activity, after eliminating the statistically insignificant terms ($p>0.05$), was:

$$Y=0.524018-0.13339x_1-0.177777x_1^2-0.30743x_2 +0.114982x_1 \cdot x_2$$

The analysis of variance reproduced in Table 6 shows that the model was significant. The Fisher F-test ($F_{4;7}=6.67>F_{t\,0.95;4;7}=4.12$) was 1.6 times higher than the F_t, and the *p*-value of <0.01 demonstrated that this regression was statistically significant at the 95% confidence level. In addition, the R^2 obtained was 0.79.

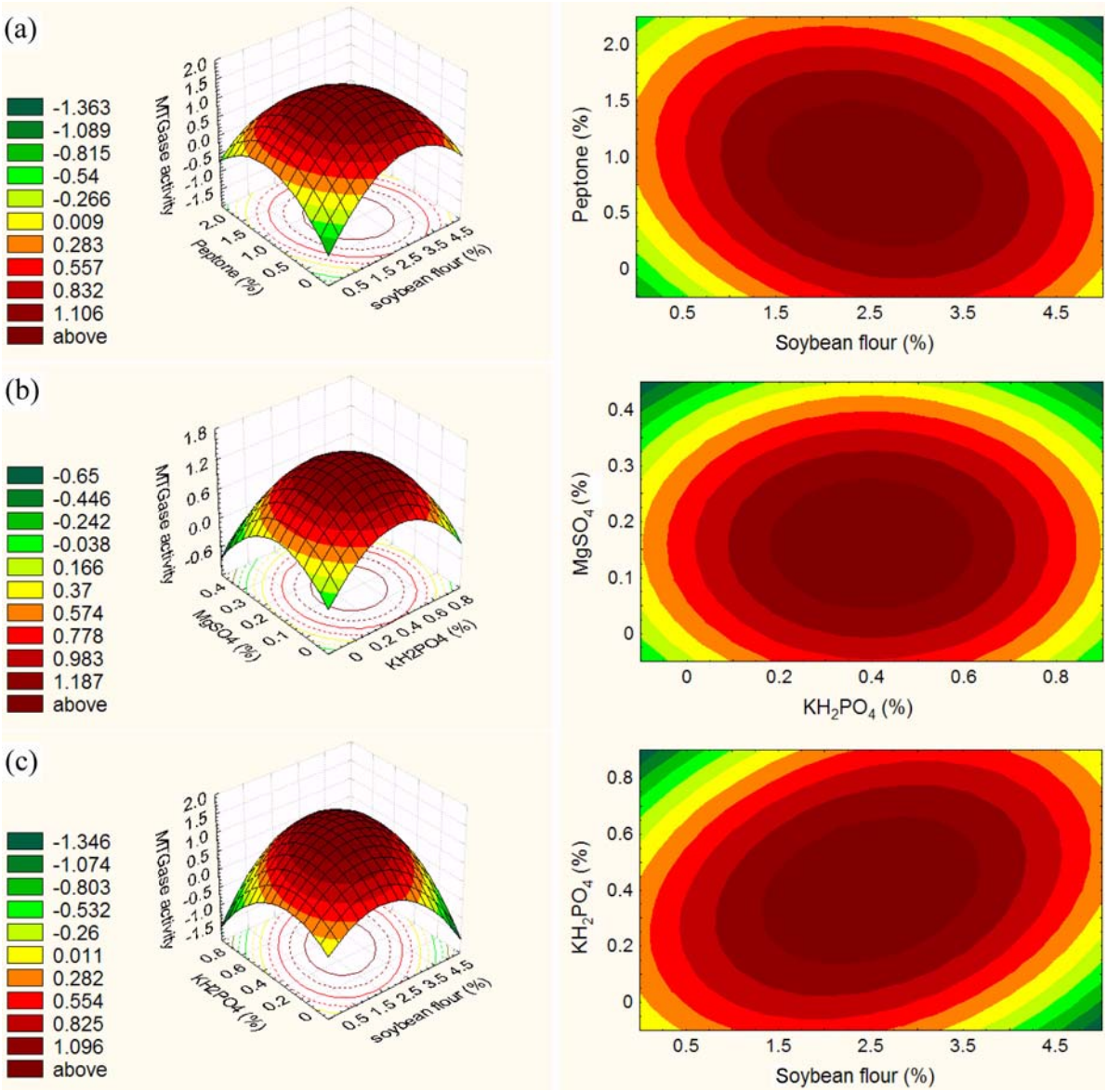

Fig. 2. Contour curve and response surface for the MTGase activity as a function of: (a) peptone vs. soybean flour, (b) $MgSO_4$ vs. KH_2PO_4, (c) KH_2PO_4 vs. soybean flour concentrations, according to the CCD.

The response surface obtained is shown in Fig. 3. It indicates that the highest MTGase activity values were found at the lowest levels of agitation and temperature tested. The best activity obtained in the experimental design was 115 rpm and 26°C.

Table 6. Analysis of variance and regression analyses[1)] for the response of the 2^2 CCD of agitation and temperature

Source of variation	Sum of square	Degree of freedom	Mean square	F_{test}[2)]	*p*-value
Regression	1.1338	4	0.2834	6.6714	<0.01
Residual	0.2974	7	0.0424		
Lack of fit	0.2884	4	0.0721		
Pure error	0.0089	3	0.0029		
Total	1.4312				

[1)]Coefficient of determination: R^2=0.79.
[2)] $F_{0,95;4;7}$=4,12.

The results from the CCD of the effects of temperature and rotation indicated higher MTGase production at 25-30°C or even lower temperatures. To confirm this, the Tukey's test was applied to the means of the values obtained in the single tests under the following conditions: 100 rpm and 25°C, 100 rpm and 30°C, and 100 rpm and 20°C. The means were compared using the Tukey's test at a confidence level of $p<0.05$ and the tests were done in triplicate. The results indicated that at temperatures below 25°C, MTGase production was nearly zero. The other conclusion was that there was no significant difference in the MTGase activity obtained between 30 and 25°C. Thus the enzyme production conditions chosen were the value of 100 rpm indicated by the CCD and a temperature of 30°C.

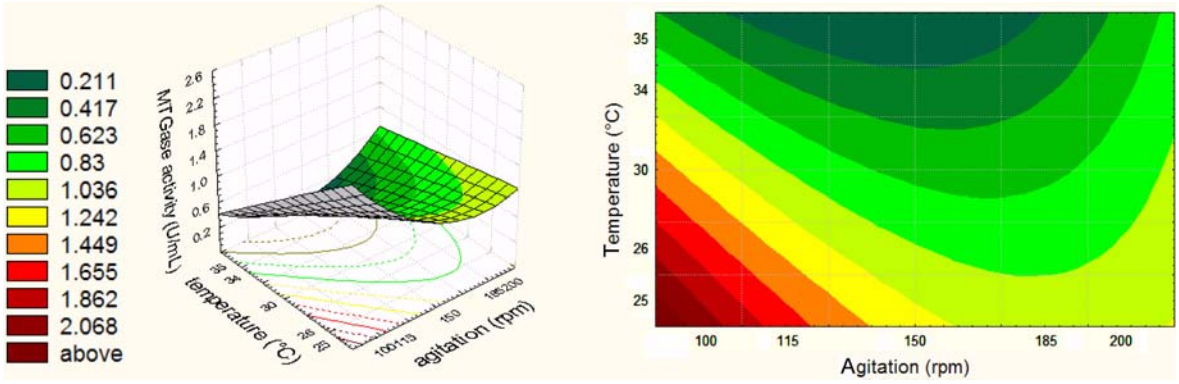

Fig. 3. Contour curve and response surface for the MTGase activity as a function of: temperature vs. agitation, according to the CCD.

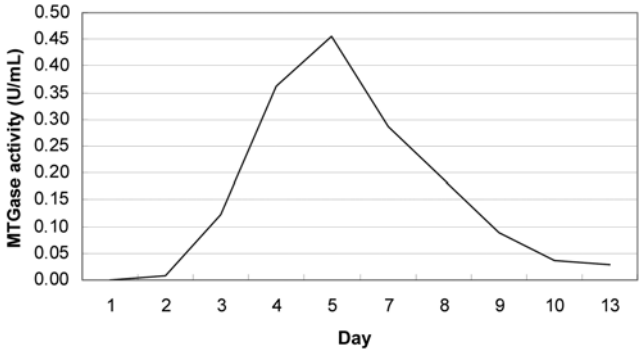

Fig. 4. Production of MTGase during fermentation of *Streptomyces* sp. P20.

Influence of inoculum size and kinetic study The influence of inoculum size on MTGase production was tested and the results analysed using the Tukey's test, admitting a 95% level of significance ($p \leq 0.05$), which indicated there was no statistical significant difference in the MTGase activity obtained using from 1 to 5 agar-mycelium cylinders as the inoculum. The data from the kinetic study of the fermentation process is described in Fig. 4. The inoculum size and kinetic study results indicated that the pre-established parameters of a 2 agar-mycelium cylinder inoculum and 5 days of fermentation were completely adequate.

Considering the results the Brazilian soil isolate *Streptomyces* sp. P20 has great potential for further investigation with respect to MTGase production. Further experiments for the characterization and purification of the enzyme are being carried out.

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