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STATISTICAL OPTIMIZATION OF MINERAL SALT AND UREA CONCENTRATION FOR CELLULASE AND XYLANASE PRODUCTION BY *Penicillium echinulatum* IN SUBMERGED FERMENTATION

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Abstract - *Penicillium echinulatum* S1M29 is a mutant with cellulase and xylanase production comparable to the most studied microorganisms in the literature. However, its potential to produce these enzymes has not been fully investigated. This study aimed at optimizing salt and urea concentrations in the mineral solution, employing the response surface methodology. A 2^{5-1} Fractional Factorial Design and a 2^3 Central Composite Design were applied to elucidate the effect of salts and urea in enzyme production. Lower concentrations of KH_2PO_4 (2.0 $g.L^{-1}$), $(NH_4)_2SO_4$ (1.4 $g.L^{-1}$), $MgSO_4.7H_2O$ (0.375 $g.L^{-1}$) and $CaCl_2$ (0.375 $g.L^{-1}$) were most suitable for the production of all enzymes evaluated. Nevertheless, higher concentrations of urea (0.525 $g.L^{-1}$) gave the best results for cellulase and xylanase production. The maximum FPase (1,5 $U.m.L^{-1}$), endoglucanase (7,2 $U.m.L^{-1}$), xylanase (30,5 $U.m.L^{-1}$) and β-glucosidase (4,0 $U.m.L^{-1}$) activities obtained with the planned medium were, respectively, 87, 16, 17 and 21% higher when compared to standard medium. The experimental design contributed to adjust the concentrations of minerals and urea of the culture media for cellulase and xylanase production by *P. echinulatum*, avoiding waste of components in the medium.
Keywords: Cellulolytic enzyme; Experimental design; Medium composition; Shake flask.

INTRODUCTION

Lignocellulosic biomass is an abundant and renewable source of carbohydrates for microbial conversion to chemicals and fuels (Geddes *et al*., 2011). Cellulose, the principal constituent of plant biomass, is a linear polymer of glucose units, which can be hydrolyzed by the action of endogluconases, cellobiohydrolases and β-glucosidases (Ahamed and Vermette, 2008). Cellulose consists of linear chains of hundreds or thousands of glucose molecules, whereas hemicellulose is a branched polymer consisting of a mixture of energy-rich glucose and sugar monomers (Zhang *et al*., 2012). Composed mainly of D-xylose, xylan is the most common hemicellulose and it is the second most abundant biopolymer found in nature (Terrasan *et al*., 2010). Xylanase is the major component of a group of enzymes, and acts by depolymerizing the xylan molecules in to monomers (Goulart *et al*., 2005).

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Ethanol produced from renewable biomass is attracting attention as an alternative energy source. However, during the production of ethanol from lignocellulosic biomass, the main problems are related to hydrolysis (Kang *et al*., 2004). The large amount of enzyme required for enzymatic conversion of hemicelluloses and cellulose to fermentable sugars severely impacts the cost effectiveness of this technology (Xiros and Christakopoulos, 2009).

Cellulase and xylanase production can be conducted by submerged fermentation technology (SmF) or solid-state fermentation (SSF). According to the literature, SmF is the most used technology for microbial production of cellulases (Sukumaran *et al*., 2005). A submerged fungal culture is recognized as a complex multiphase, multicomponent process where cell growth and product formation are influenced by a large number of operating parameters, such as culture broth composition, temperature, pH, shear stress, initial inoculum, dissolved oxygen, rheology and fungal morphology (Patel *et al*., 2009). It is a well-established fact that optimization of culture medium and culture conditions influence enzyme production (Juhász *et al*., 2005; Shanmugam *et al*., 2008).

Filamentous fungi are the major source of cellulases and hemicellulases (Gusakov *et al*., 2007). Several fungal species belonging to the genera *Penicillium* should be considered for the production of second-generation biofuels (Gusakov, 2011). *Penicillium echinulatum* has been identified as a potential candidate for cellulase and xylanase production because its secreting capacity is almost equivalent to the best *T. reesei* strains (Camassola and Dillon, 2010; Dillon *et al*., 2006).

Statistical methods have also been designed for bioprocess optimization (Cheng *et al*., 2012; Coelho *et al*., 2011; Singh and Kaur, 2012). Combinatorial interactions of process variables with the production of the desired compound are numerous and the optimum processes may be developed using an effective experimental design procedure (Muthuvelayudham and Viruthagiri, 2010). Response Surface Methodology is one of the most practical optimization methods. It enables one to identify the effects of individual variables and to efficiently seek the optimum conditions for a multivariable system. With this methodology, the effect of interaction of various parameters can be understood, generally resulting in high production yields and a lower number of experiments (Han *et al*., 2009; Hao *et al*., 2006).

In the current study, the Central Composite Design (CCD) and Fractional Factorial Design (FFD) were used to evaluate the effects of the mineral solution components described by Mandels and Reese (1957) on cellulase and xylanase production by the mutant *Penicillium echinulatum* S1M29. Response surface methodology is also applied to predict the optimum yield of cellulases and xylanases.

MATERIALS AND METHODS

Microorganism

P. echinulatum S1M29, obtained from the mutant strain 9A02S1 (*Deutsche Sammlung von Mikroorganismen und Zellkulturen* – DSM 18942) after several steps of mutagenesis, was used in this study (Dillon *et al*., 2011). The strain was grown and maintained on cellulose agar (C-agar) consisting of distilled water containing 1% (v/v) swollen cellulose, 10% (v/v) of the mineral solution described by Mandels and Reese (1957), 0.1% (w/v) proteose peptone (Oxoid L85), and 2% (w/v) agar. The strain was grown on C-agar slants for up to 7 days at 28 °C until conidia were formed.

Medium and Cultivation Conditions

The production medium consisted of 1% (w/w) cellulose *Celuflok E*®, 0.5% (w/w) sucrose, 0.2% (w/w) soybean meal, 0.5% (w/w) wheat bran, 0.05% (w/w) *Prodex*®, 0.1% (v/v) *Tween 80*®, 10% (v/v) mineral standard solution based on Mandels and Reese (1957) composed of (g/L): KH_2PO_4, 20; $(NH_4)_2SO_4$, 14; $MgSO_4.7H_2O$, 3; $CO(NH_2)_2$ (urea), 3; $CaCl_2$, 3; $FeSO_4.7H_2O$, 0.05; $MnSO_4.H_2O$, 0.0156; $ZnSO_4.7H_2O$, 0.014 and $CoCl_2.6H_2O$, 0.02. Different concentrations of urea and salts $(NH_4)_2SO_4$, KH_2PO_4, $CaCl_2$, $MgSO_4.7H_2O$ of the mineral solution were tested (Table 1).

The experiments were conducted in 500-mL Erlenmeyer flasks with 100 mL of production medium. After autoclaving at 121 °C for 15 min, the flasks were inoculated with a conidial suspension ($1x10^7$ conidia.mL^{-1}, counting in a Neubauer chamber) and kept at 28 °C, under agitation of 180 rpm for 120 h. All assays were tested in triplicate and the mean values were calculated.

Enzymatic Assays

The filter paper activity (FPase) was measured by using Whatman Nº. 1 filter paper as substrate, according to Camassola and Dillon (2012). Endoglucanase activity was determined according to Ghose (1987), using 2% (w/v) carboxymethyl cellulose in

0.05 M sodium citrate buffer (pH 4.8). The β-glucosidase activity was measured using ρ-nitrophenyl-β-D-glucopiranoside (Daroit *et al*., 2008). Xylanase activity was determined according to Bailey *et al*. (1992), using 1% oat spelled xylan (w/v). The concentrations of reducing sugars were estimated with dinitrosalicylic acid, according to Miller (1959).

Medium Composition Optimization

A 2^{5-1} Fractional Factorial Design was carried out to evaluate the effects of the mineral solution components used in the enzyme production medium, which resulted in 16 different assays with three replications at the center point, totaling 19 assays (Table 1). A 2^3 Central Composite Design was performed after analyzing the effects of the five variables (Table 3). The experiment included six axial points and six central point replicates, totaling 20 assays. The effects of the variables, the significance of the multiple regression coefficients and graphical analysis of the data were determined using the Statistica 5.0 software and the confidence interval was 95%. The experiments were conducted up to 120 h and the time of fermentation employed for experimental designs was 96 h, because the main activities were found in this time.

The regression parameters were fitted to a polynomial equation with the coded variables (Eq. (1)):

$$Y_i = B_0 + B_1x_1 + B_2x_2 + B_3x_3 + B_{11}x_1^2 + B_{22}x_2^2 + B_{33}x_3^2 + B_{12}x_1x_2 + B_{23}x_2x_3 + B_{13}x_1x_3 \quad (1)$$

where Yi is the predicted response, B_0 is the intercept term, B_1, B_2, and B_3 are linear effects B_{11}, B_{22}, B_{33} are squared effects, B_{12}, B_{23}, B_{13} are interaction terms and x_1, x_2, x_3 are independent variables, respectively.

To verify the accuracy of the optimal conditions, the assay with the best results was repeated in triplicate using Erlenmeyer flasks. The activities were compared with the standard mineral solution (MS standard) described by Mandels and Reese (1957), and also compared with two solutions in which salts and urea were concentrated 1.5-fold (MS 1.5) and 2-fold (MS 2.0) relative to the standard solution. The other culture medium components and process conditions were the same as in the experimental design assays. Graphs representing the enzymatic activities were developed in the PrismGraphPad® software version 3.0. The same software was used to perform an analysis of variance with Tukey's post hoc test at the 5% significance level ($p<0.05$).

RESULTS AND DISCUSSION

Five-Variable Fractional Factorial Design (FFD) for Cellulase and Xylanase Production

The production medium is one of the factors that interferes the most with the microorganism physiology and the production of compounds of interest. Thus, a five-variable FFD (Table 1) was performed with different concentrations of salts and urea to evaluate the influence of these nutrients on *P. echinulatum* enzymatic activity. Mandels and Reese (1957) found that the mineral composition of the medium has a great effect on the production of cellulases by *Trichoderma viride.*

The highest FPase activities were observed in assays 1 (0.89 $U.mL^{-1}$) and 11 (0.98 $U.mL^{-1}$) and the results for xylanase (34.3 and 34.64 $U.mL^{-1}$, respectively) were similar to that obtained in assay 10 (36.1 $U.mL^{-1}$), which yielded the highest activity for this enzyme. The highest β-glucosidase activities were obtained in assay 4 (2.5 $U.mL^{-1}$), 10 (2.52 $U.mL^{-1}$) and 11 (2.51 $U.mL^{-1}$). The highest endoglucanase activity was obtained in assay 4 (6.35 $U.mL^{-1}$) (Table 1). The effect of salt and urea concentrations on enzyme activity can be seen in Table 2. It can be observed that urea has a positive effect on all enzymes analyzed, having a significant influence on xylanase activity. For FPase, the salt concentrations had the lowest effects, which were not significant to a confidence level of 95% (Table 2). These results were similar to those obtained for β-glucosidases. Although essential, Mg and Ca salts showed negative effects on endoglucanases activities, indicating that an increase in the concentration of such salts can impair significantly the enzyme production ($p=0.0358$).

The importance of Mg and Ca salts in enzyme activity has been demonstrated by Reese and Mandels (1957). The authors observed that the levels of these minerals affect both enzyme production and glucose consumption. Slow growth and lack of cellulase production occur in the absence of Mg. Furthermore, cellulase production is increased by supplementing the medium containing $MgSO_4$ with $CaCl_2$ at concentrations of up to 0.03%. The authors suggested that calcium may act in part to compensate some inhibitory effect of magnesium, because the use of only $MgSO_4$ at 0.03% results in poor cellulase production.

Table 1: Fractional Factorial Design real and coded variables ($g.L^{-1}$) and cellulase and xylanase activities obtained at 96 h of culture using *P. echinulatum* S1M29.

Run	Experimental factors					Cellulases ($U.mL^{-1}$)			Xylanases ($U.mL^{-1}$)
	X1	X2	X3	X4	X5	FPase	β-glucosidades	Endoglucanases	
1	(-1) 1.4	(-1) 0.3	(-1) 2.0	(-1) 0.3	(+1) 0.6	0.89	2.46	5.52	34.35
2	(+1) 2.8	(-1) 0.3	(-1) 2.0	(-1) 0.3	(-1) 0.3	0.73	1.08	5.21	31.06
3	(-1) 1.4	(+1) 0.6	(-1) 2.0	(-1) 0.3	(-1) 0.3	0.7	1.38	4.63	30.6
4	(+1) 2.8	(+1) 0.6	(-1) 2.0	(-1) 0.3	(+1) 0.6	0.77	2.5	6.35	32.33
5	(-1) 1.4	(-1) 0.3	(+1) 4.0	(-1) 0.3	(-1) 0.3	0.8	1.89	5.1	30.3
6	(+1) 2.8	(-1) 0.3	(+1) 4.0	(-1) 0.3	(+1) 0.6	0.81	2.06	5.29	33.04
7	(-1) 1.4	(+1) 0.6	(+1) 4.0	(-1) 0.3	(+1) 0.6	0.69	2.04	4.71	30.17
8	(+1) 2.8	(+1) 0.6	(+1) 4.0	(-1) 0.3	(-1) 0.3	0.78	1.35	4.69	26.85
9	(-1) 1.4	(-1) 0.3	(-1) 2.0	(+1) 0.6	(-1) 0.3	0.67	1.74	4.74	32.19
10	(+1) 2.8	(-1) 0.3	(-1) 2.0	(+1) 0.6	(+1) 0.6	0.71	2.52	4.95	36.16
11	(-1) 1.4	(+1) 0.6	(-1) 2.0	(+1) 0.6	(+1) 0.6	0.98	2.51	4.37	34.64
12	(+1) 2.8	(+1) 0.6	(+1) 4.0	(+1) 0.6	(-1) 0.3	0.6	2.15	3.95	28.89
13	(-1) 1.4	(-1) 0.3	(+1) 4.0	(+1) 0.6	(+1) 0.6	0.66	2.15	4.86	30.98
14	(+1) 2.8	(-1) 0.3	(+1) 4.0	(+1) 0.6	(-1) 0.3	0.69	1.74	4.41	29.26
15	(-1) 1.4	(+1) 0.6	(+1) 4.0	(+1) 0.6	(-1) 0.3	0.68	1.73	4.54	27.76
16	(+1) 2.8	(+1) 0.6	(+1) 4.0	(+1) 0.6	(+1) 0.6	0.68	1.55	3.79	28.91
17	(0) 2.1	(0) 0.45	(0) 3.0	(0) 0.45	(0) 0.45	0.73	1.49	5.77	31.82
18	(0) 2.1	(0) 0.45	(0) 3.0	(0) 0.45	(0) 0.45	0.76	1.84	5.1	33.59
19	(0) 2.1	(0) 0.45	(0) 3.0	(0) 0.45	(0) 0.45	0.76	1.71	5.5	31.01

X1: $(NH_4)_2SO_4$; X2: $CaCl_2$; X3: KH_2PO_4; X4: $MgSO_4$; X5: Urea

Table 2: Effect of multiple regression of the five variables on *P. echinulatum* enzyme activity at 96 h of culture.

	Interception	$(NH_4)_2SO_4$	$CaCl_2$	KH_2PO_4	$MgSO_4$	Urea	p-level
FPase	0.858	-0.002	-0.004	-0.002	-0.019	0.021	0.2690
Xylanases	34.2	-0.0138	-0.59	-0.147	0.127	0.863	0.0004*
Endoglucanases	6.50	0.004	-0.11	-0.0188	-0.231	0.091	0.0358*
β-glucosidases	1.13	-0.007	-0.0114	-0.0049	0.059	0.193	0.0814

*Significant factors, P<0.05

Salts also have great influence on the measurement of enzyme activity. Bhiri *et al*. (2008) evaluated the effect of different divalent cations (Mg^{2+}, Mn^{2+},Ca^{2+}, Co^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, Cu^{2+}) on β-glucosidase activity in the hyper-cellulolytic mutant *Penicillium occitanis* Pol6. These ions were added at a concentration of 0.2-5 mM and no significant effect was observed on the enzyme activity, except for the ions Hg^{2+} and Cu^{2+}, which had an inhibitory activity at 2 mM. Sinegani and Emtiazi (2006) showed that exoglucanase and endoglucanase activities increase in the presence of Na^{+}, K^{+}, Ca^{+2}, Ba^{+2} and Mn^{+2}, but decrease with NH_4^{+} and Mg^{+2}. According to the present study, it seems that, when high concentrations of salts are employed, possible salt residues can be found in the final broth, interfering with enzymatic analysis.

Due to the importance of the concentration of salts and urea on the enzyme production, the effects from the multiple regression showed significant variations, and a CCD was carried out with $MgSO_4$, $CaCl_2$ and with urea. $(NH_4)_2SO_4$ and KH_2PO_4 were fixed at lower concentrations (1.4 and 2.0 $g.L^{-1}$, respectively) due to the lower effects observed on endoglucanase and xylanase activities (Table 2).

Three-variable Central Composite Design (CCD) for Cellulase and Xylanase Production

The data obtained from the three-variable CCD indicate that, among the enzymes evaluated, endoglucanase had the highest production levels in this study. The FPase, β-glucosidase and xylanase activities were similar or slightly superior to the fivevariable CCD (Table 3). The optimization of the concentration of urea and Ca and Mg salts on enzymatic activity by the CCD was valid and the significance of the effects of each variable is shown in Table 4.

The maximum endoglucanase activity, obtained at 96 h, was 9.6 ± 0.5 $U.mL^{-1}$ when the medium contained 0.375 $g.L^{-1}$ of $MgSO_4$, 0.375 $g.L^{-1}$ of $CaCl_2$ and 0.525 $g.L^{-1}$ of urea. Comparing assays 2 and 4, it was observed that a 40% increase in the $MgSO_4$ concentration in the medium reduced the enzyme

Table 3: Cellulase and xylanase activities at 96 h in cultures with different $MgSO_4$, $CaCl_2$ and urea concentrations employing Central Composite Design ($g.L^{-1}$).

Run	Experimental factors			Cellulases ($U.mL^{-1}$)						Xylanases ($U.mL^{-1}$)	
				FPase		β-glucosidades		Endoglucanases			
	X_1	X_2	X_3	Observed	Predicted	Observed	Predicted	Observed	Predicted	Observed	Predicted
1	(-1) 0.375	(-1) 0.375	(-1) 0.375	1.08	1.03	1.42	1.28	8.44	7.36	36.38	34.3
2	(-1) 0.375	(-1) 0.375	(+1) 0.525	1.09	0.97	1.51	1.9	9.06	8.35	36.62	33.15
3	(+1) 0.525	(-1) 0.375	(-1) 0.375	1.07	0.91	1.3	1.53	7.37	6.36	35.68	34.2
4	(+1) 0.525	(-1) 0.375	(+1) 0.525	1.01	0.87	1.57	1.23	6.59	6.64	34.67	33.28
5	(-1) 0.375	(+1) 0.525	(-1) 0.375	0.9	0.8	0.93	1.45	7.49	6.33	34.2	32.4
6	(-1) 0.375	(+1) 0.525	(+1) 0.525	1.11	1.04	2.6	1.75	6.87	6.89	37.63	35.92
7	(+1) 0.525	(+1) 0.525	(-1) 0.375	0.85	0.73	1.25	1.71	6.52	6.23	27.92	28.22
8	(+1) 0.525	(+1) 0.525	(+1) 0.525	1.17	0.99	0.74	1.06	6.13	6.19	33.02	31.93
9	(0) 0.45	(0) 0.45	(-2) 0.30	0.66	0.75	1.41	1.16	4.87	5.71	30.02	30.94
10	(0) 0.45	(0) 0.45	(+2) 0.60	0.83	0.96	1.05	1.11	6.93	6.65	31.27	33.51
11	(-2) 0.30	(0) 0.45	(0) 0.45	1.07	1.12	2.05	1.91	7.76	8.71	32.32	35.25
12	(+2) 0.60	(0) 0.45	(0) 0.45	0.77	0.94	1.57	1.52	6.98	7.01	30.95	31.18
13	(0) 0.45	(-2) 0.30	(0) 0.45	0.92	1.03	1.51	1.38	6.74	7.55	32.72	35.33
14	(0) 0.45	(+2) 0.60	(0) 0.45	0.79	0.91	1.45	1.38	5.79	5.96	31.57	32.11
15	(0) 0.45	(0) 0.45	(0) 0.45	0.74	0.82	1.82	1.72	6.88	6.42	30.43	32.56
16	(0) 0.45	(0) 0.45	(0) 0.45	0.89	0.82	1.52	1.72	5.45	6.43	33.18	32.56
17	(0) 0.45	(0) 0.45	(0) 0.45	0.75	0.82	1.86	1.72	6.48	6.42	33.04	32.56
18	(0) 0.45	(0) 0.45	(0) 0.45	0.76	0.82	1.81	1.72	6.8	6.43	30.83	32.56
19	(0) 0.45	(0) 0.45	(0) 0.45	0.86	0.82	1.62	1.72	5.35	6.43	33.11	32.56
20	(0) 0.45	(0) 0.45	(0) 0.45	0.79	0.9	1.92	1.72	5.85	6.75	32.19	32.87

X1: $MgSO_4$; X2: $CaCl_2$; X3: Urea

Table 4: Regression coefficients and significance values of variables.

	Xylanases		Endoglucanases		FPase		β-glucosidases	
Factor	Coefficient	p-level	Coefficient	p-level	Coefficient	p-level	Coefficient	p-level
Mean	33.13	0.0006*	6.75	$1.4.10^{-9}$*	0.82	2.10^{-8}*	1.52	$3.2.10^{-10}$*
X_1 (L)	-1.02	0.042*	-0.426	0.05*	-0.043	0.034*	-0.097	0.021*
X_1 (Q)	-1.02	0.03*	-0.43	0.05*	-0.04	0.035*	-0.1	0.022*
X_2 (L)	-0.8	0.0913	-0.396	0.07	-0.03	0.107	-3.10^{-7}	1
X_2 (Q)	-0.8	0.069	-0.4	0.23	-0.03	0.108	0	1
X_3 (L)	0.641	0.163	0.235	0.24	0.051	0.016*	-0.013	0.716
X_3 (Q)	0.641	0.132	0.235	0.237	0.051	0.016*	-0.01	0.717
X_1X_2	-1.03	0.094	0.228	0.406	0.013	0.603	0.008	0.877
X_1X_3	0.053	0.924	-0.15	0.577	0.005	0.834	-0.23	0.002*
X_2X_3	1.163	0.065	-0.11	0.688	0.0073	0.016*	-0.09	0.089

*Significant factors, P<0.05

activity to 6.58 ± 0.35 $U.mL^{-1}$, showing the negative effect of a salt concentration increase, which was a significant effect for all enzymes (Table 4). This same 40% increase in the $CaCl_2$ concentration reduced the endoglucanase activity by 31.8% (assays 2 and 6), but this effect was not significant. The increase in urea concentration was beneficial only with $MgSO_4$ and $CaCl_2$ concentrations lower than 0.525 $g.L^{-1}$.

Gautam *et al*. (2010) evaluated different Ca and Mg concentrations (10-80 mM) and found that higher levels of these ions impair the FPase, as well as endoglucanase and β-glucosidase activities in *T. ressei*. The authors considered 10 $mmol.L^{-1}$ as the optimal concentration for enzymatic activity.

Endoglucanase activity obtained in this study was greater than that obtained by Han *et al*. (2009) with *P. waskmanii* F10-2. The authors found that endoglucanase activity is increased by $MgSO_4$, NaCl and KH_2PO_4, with activities of 5.64 $U.mL^{-1}$ when the microorganism is grown in a medium containing 0.2, 3.3 and 2.7 $g.L^{-1}$ of the respective salts. According to Kim *et al*. (2012), the optimal concentrations of K_2HPO_4, NaCl, $MgSO_4$ and $(NH_4)_2SO_4$ for endoglucanase production are 3.00, 0.52, 0.34 and 0.45 $g.L^{-1}$, respectively, for *Psychrobacter aquimaris* LBH-10.

Saratale *et al*. (2012) studied the effect of different physicochemical parameters on the activity of cellulolytic and hemicellulolytic enzymes in *Streptomyces*

sp. MDS. The microorganism was studied under ideal growing conditions and the results indicate that supplementation of $CaCl_2$ (5 mmol.L^{-1}) significantly induced the enzyme system.

As noted in the five-variable experimental design (Table 2), the effects of urea were positive for all enzymes, indicating the importance of this organic source of nitrogen in the culture medium. Although the effects of urea were positive for enzyme activity, the effects of $CaCl_2$ and $MgSO_4$ were negative for most of the enzymes. While $MgSO_4$ concentration showed the highest negative effects for xylanases, β-glucosidases and endoglucanases, the $CaCl_2$ concentration had a more negative effect for xylanases and endoglucanases. According to Rabinovich *et al*. (2002) and Xiong *et al*. (2004), xylanases and endoglucanases have different isoforms. The presence of isoforms may explain their greater susceptibility to varying salt concentrations.

For endoglucanase activities, the explained variation was 52.5% (Fcal=1.22), with representative agreement between the experimental values and those predicted by the model (Eq. (2)).

$$Y_{Endoglucanase}(U.mL^{-1}) = 6.75 - 0.426x_1 - 0.43x_1^2 \quad (2)$$

The FPase was influenced by the concentration of urea and $CaCl_2$ and $MgSO_4$ salts, with significant effect of urea and $MgSO_4$. A decrease in concentration from 0.6 g.L^{-1} (assay 11, Table 1) to 0.525 g.L^{-1} (assay 8, Table 3) resulted in a 20% increase in enzyme activity. For the FPase, the explained variation was 49% (Fcal=1.01), with representative agreement between the experimental values and those predicted by the model (Eq. (3)).

$$Y_{FPA}(U.mL^{-1}) = 0.82 - 0.043x_1 - 0.04x_1^2 + 0.051x_3 + 0.051x_3^2 + 0.0073x_2x_3 \quad (3)$$

For β-glucosidase activities, the explained variation was 51% (Fcal=1.12), with representative agreement between the experimental values and those predicted by the model (Eq. (4)).

$$Y_{\beta-glucosidase}(U.mL^{-1}) = 1.52 - 0.097x_1 - 0.1x_1^2 - 0.23x_1x_3 \quad (4)$$

For xylanase activities, the explained variation was 47.3% (Fcal=1.75), showing representative agreement between the experimental values and those predicted by the model (Eq. (5)).

$$Y_{Xylanases}(U.mL^{-1}) = 33.13 - 1.02x_1 - 1.02x_1^2 \quad (5)$$

Dobrev *et al*. (2007) optimized the xylanase production by *Aspergillus niger* B03 by 33% using experimental design. The maximum xylanase activity was obtained in a medium containing (g.L^{-1}) 2.6 of $(NH_4)_2HPO_4$, 0.9 of urea. 6.0 of malt sprout, 24.0 of corn cobs and 14.6 of wheat bran.

The concentration of $MgSO_4$ was significant and had a negative effect on all enzymes evaluated (significance values). Figure 1 shows FPase and β-glucosidase activities as a function of $MgSO_4$ and urea concentrations, which are the most significant variables for these enzymes. Figure 2 presents the response surface showing the effects of $MgSO_4$ and $CaCl_2$ on endoglucanase and xylanase activities.

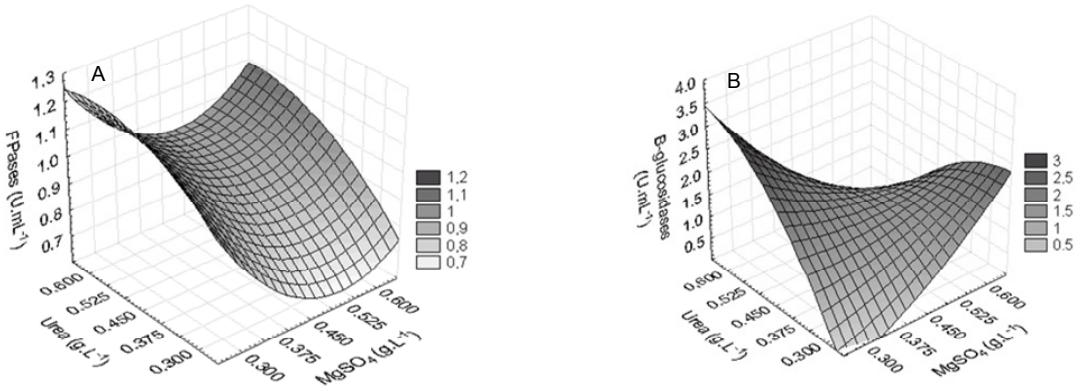

Figure 1: Surface response to the FPase (A) and β-glucosidase (B) activities in *Penicillium echinulatum* S1M29 at 96 h of culture, as a function of urea and $MgSO_4$ concentrations. $CaCl_2$ was fixed at the central point.

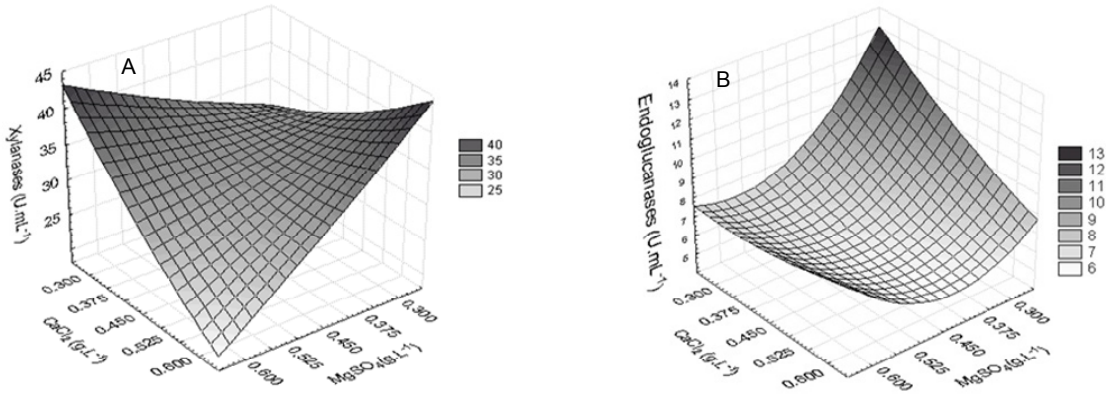

Figure 2: Surface response to the xylanase (A) and endoglucanase (B) activities in *Penicillium echinulatum* S1M29 at 96 h of culture, as a function of $MgSO_4$ and $CaCl_2$ concentrations. Urea was fixed at the central point.

From the data analysis, condition 2 of the experimental design, with three variables, was defined as ideal for the production of cellulases and xylanases because it yielded the highest endoglucanase activities, as well as xylanase and FPase activities similar to the higher values obtained under other conditions. The optimal conditions determined by the model were not tested, because the salt and urea concentrations in the medium were higher than those employed in the optimal condition obtained in the experimental design. Moreover, the enzymes activities predicted were similar to those obtained in assay 2.

The condition obtained via the experimental design (assay 2) was repeated in flasks kept under reciprocal agitation and the results were compared with the Reese and Mandels (1957) standard solution. It was found that the FPase and β-glucosidase activities were higher than those obtained in the experimental design, but the endoglucanase and xylanase activities were lower (Figures 3A-D). This discrepancy may be due to a slight variation of the experimental conditions.

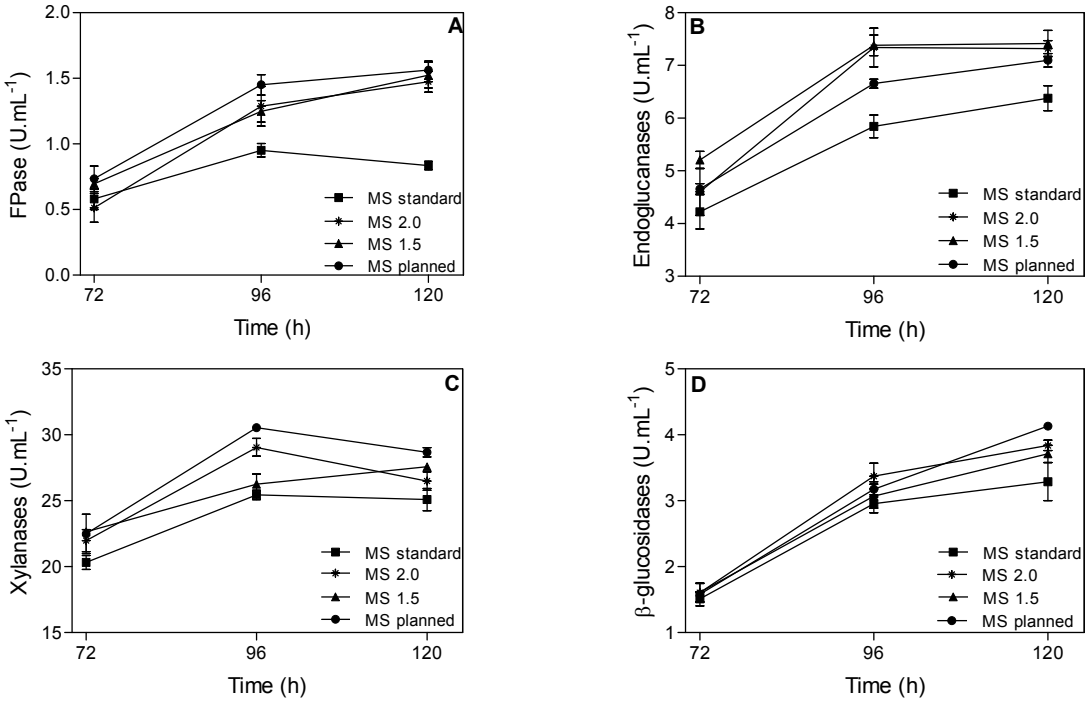

Figure 3: FPase (A), endoglucanase (B), xylanase (C) and β-glucosidase (D) activities of *Penicillium echinulatum* S1M29 during submerged culture for the optimal condition obtained in the experimental design.

Variations in the concentrations of urea, $MgSO_4$ and $CaCl_2$, in relation to the standard mineral solution resulted in significantly higher activities when compared to the standard solution at concentrations 1.5 and 2.0 times higher. This indicates that the highest levels of enzymatic activity can be obtained by increasing the concentration of some of the salts (Table 5). During the submerged culture employing the planned mineral solution, the pH values were lower than those obtained in the culture with the standard solution (Figure 4). This can explain the higher activity. According to Sternberg and Dorval (1979), the pH is an indicative parameter of metabolism intensity.

Table 5: Mineral Solution (MS) composition and enzyme activity obtained at 96 h.

	MS Standard	MS 2.0	MS 1.5	MS Planned
KH_2PO_4 (g.L^{-1})	2.0	4.0	3.0	2.0
$(NH_4)_2SO_4$ (g.L^{-1})	1.4	2.8	2.1	1.4
$MgSO_4.7H_2O$ (g.L^{-1})	0.3	0.6	0.45	0.375
$CaCl_2$ (g.L^{-1})	0.3	0.6	0.45	0.375
Urea (g.L^{-1})	0.3	0.6	0.45	0.525
FPase (U.mL^{-1})	0.83^{b}	1.28^{ab}	1.24^{ab}	1.45^{a}
Xylanase (U.mL^{-1})	25.4^{b}	29^{a}	26.2^{b}	30.5^{a}
Endoglucanase (U.mL^{-1})	5.8^{b}	7.3^{a}	7.3^{a}	6.7^{ab}
β-glucosidase (U.mL^{-1})	2.95^{a}	3.4^{a}	3.1^{a}	3.2^{a}

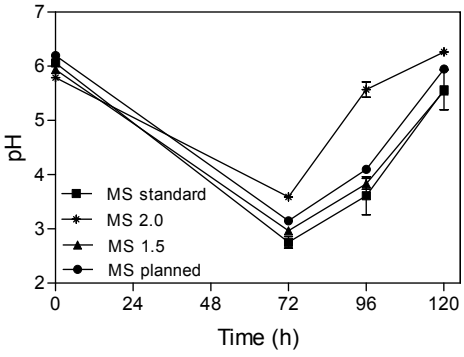

Figure 4: pH values during submerged culture of *Penicillium echinulatum* S1M29 for the optimal condition obtained in the experimental design.

Comparing the pH values at 72 h of culture using the medium formulated with the standard solution and those that were 1.5 and 2.0 times more concentrated, it was verified that, in the conditions in which all components were concentrated, the pH was below 3. These values can affect the enzymatic activity because, as reported for *T. reesei* by Ryu and Mandels (1980), cellulases are inactivated below pH 3.

CONCLUSIONS

The data obtained in this study clearly indicate the need to adjust the concentrations of minerals and urea for each microorganism used in the production of enzymes, such as cellulases and xylanases. Although the model could explain about 50% of the variability in the response of all evaluated enzymes, the FPase (1.45 U.mL^{-1}) and xylanase (30.5 U.mL^{-1}) activities obtained under the optimal conditions were significantly higher than those verified in the standard conditions. The experimental design contributed to adjust the requirements of culture medium for cellulase and xylanase production, avoiding the waste of components from the medium and contributing to reducing the costs of enzyme production.

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