Appl Biochem Biotechnol (2010) 161:411–422
DOI 10.1007/s12010-009-8875-6

Nitrogen Source Optimization for Cellulase Production by *Penicillium funiculosum*, using a Sequential Experimental Design Methodology and the Desirability Function

Roberto Nobuyuki Maeda · Mariana Mello Pereira da Silva · Lídia Maria Melo Santa Anna · Nei Pereira Jr.

Received: 15 May 2009 / Accepted: 25 November 2009 /
Published online: 15 December 2009

Abstract The present study aimed at maximizing cellulase production by *Penicillium funiculosum* using sequential experimental design methodology for optimizing the concentrations of nitrogen sources. Three sequential experimental designs were performed. The first and the second series of experiments consisted of a 2^4 and a 2^3 factorial designs, respectively, and in the third one, a central composite rotational design was used for better visualizing the optimum conditions. The following nitrogen sources were evaluated: urea, ammonium sulfate, peptone, and yeast extract. Peptone and ammonium sulfate were removed from the medium optimization since they did not present significant statistical effect on cellulase production. The optimal concentrations of urea and yeast extract predicted by the model were 0.97 and 0.36 g/L, respectively, which were validated experimentally. By the use of the desirability function, it was possible to maximize the three main enzyme activities simultaneously, which resulted in values for FPase of 227 U/L, for CMCase of 6,917 U/L, and for β-glucosidase of 1,375 U/L. These values corresponded to increases of 3.3-, 3.2-, and 6.7-folds, respectively, when compared to those obtained in the first experimental design. The results showed that the use of sequential experimental designs associated to the use of the desirability function can be used satisfactorily to maximize cellulase production by *P. funiculosum*.

Keywords Cellulase · Sugar cane bagasse · *Penicillium funiculosum* · Response surface methodology · Nitrogen sources

Introduction

Studies indicate that the projected demand for ethanol by the year 2013 in Brazil will be 32 billion liters [1], which correspond to an additional production of 33% than the current

R. N. Maeda · M. M. P. da Silva · L. M. M. Santa Anna · N. Pereira Jr. (✉)
Center of Technology, School of Chemistry, Laboratories of Bioprocess Development,
Federal University of Rio de Janeiro, 21949-900 Rio de Janeiro, Brazil
e-mail: nei@eq.ufrj.br

production level. Several factors contribute to this, including the increase of bifuelled car selling (flex fuel); worldwide ethanol demand and, consequently, of the Brazilian exports; as well as the increasing gasoline demand, which in Brazil is mixed with 20–25% of anhydrous ethanol. However, it is forecasted that the harvested sugar cane in the same period will not be sufficient to supply such a demand, ratifying the need of producing ethanol based on other feedstocks. In this context, research and development have been intensified targeting the utilization of renewable feedstocks other than the traditional crops in substitution of fossil sources. Emphasis has been placed on the utilization of abundant agricultural and agro-industrial residues, both of lignocellulosic composition [2].

For the production of ethanol, using lignocellulosic biomass (second-generation ethanol), the *simultaneous saccharification and fermentation* process has been an interesting option since the enzyme inhibition of the cellulolytic complex can be circumvented by associating enzyme hydrolysis with ethanol fermentation [3]. However, the economical feasibility of this process is primarily dependent on the costs of the enzymes, which is considered one of the bottlenecks for the industrial implementation of this technology [4].

The biorefinery context can be incorporated by in-plant production of cellulases, which will avoid expenditures with their imports. The main challenge, however, is the development of sustainable technologies for the production of these enzymes with low cost in Brazil. For this reason, significant amount of research has been focused on the production of cellulases. For the production of theses enzymes, part of the sugarcane bagasse can be used as feedstock; nonetheless, sugarcane bagasse is a poor source in terms of nitrogen, whose main function is the synthesis of plastic and catalytic proteins, affecting significantly microbial physiology [5]. It is known that several nitrogen sources are available in different forms, i.e., ammonium, amines, amides, and nitrates [6, 7]. They can cause many effects to the microorganisms, when presented in repressive or inductive concentrations. Therefore, this work aimed at optimizing the nitrogen source concentration for the production of cellulases by a strain of *Penicillium funiculosum* using sequential experimental design methodology.

Materials and Methods

Microorganism

P. funiculosum ATCC 11797 was maintained on potato dextrose agar (PDA), and its conidia were stored in glycerol 20% at 4 °C in microtubes. This condition allows the preservation of the conidia for more than 1 year. In each experiment, the stored conidia were activated in PDA solid medium to obtain a homogeneous inoculum for the production of cellulases.

Bagasse Pretreatment

To enhance the microorganism accessibility to the substrate, a pretreatment was carried out with diluted sulfuric acid (1% *v*/*v*) with a solid:liquid ratio of 1:2 at 121 °C for 45 min, as reported by Betancur [8]. After acid pretreatment, two fractions were obtained: a hemicellulose hydrolysate liquid phase and a solid fraction, composed mainly by cellulose and lignin, which was named as cellulignin. These two fractions were separated by pressing filtration, and the cellulignin was even washed repeatedly with water until pH 5. The cellulignin was subjected to an alkaline pretreatment with sodium hydroxide (4% *w*/*v*) with a solid:liquid ratio of 1:20 at 121 °C for 30 min as established by Vasquez et al. [3]. The resulting solid material was called *cellulignin partially delignified*. For the bagasse and

cellulignin partially delignified composition determination, a chemical hydrolysis was carried out with H_2SO_4 in two steps, according to National Renewable Energy Laboratory, NREL [9] and Ververis et al. [10].

Enzyme Production

The enzyme production medium was composed of varying concentrations of urea, ammonium sulfate, peptone, and yeast extract as nitrogen sources. *Cellulignin partially delignified* was used as carbon source and inducer in a concentration of 15 g/L. The experiments were performed in 500 mL conical flasks containing 200 mL of the production medium which was inoculated with a suspension of spores (10^6 conidia/mL). The flasks were stirred in a rotary shaker (200 rpm) at a temperature of 30 °C. After 72 h of fermentation, the medium was centrifuged at 2,500×g for 10 min, and the crude enzymatic extract was used for quantifying cellulase activities (FPase, CMCase, and β-glucosidase).

Cellulase Assays

FPase, CMCase, and β-glucosidase activities were measured using filter paper, carboxymethylcellulose, and cellobiose as substrates, respectively [11]. Test tubes containing substrate and crude extract were incubated at 50 °C for 1 h for filter paper activity and 15 min for CMCase and β-glucosidase activities. The reaction systems were shaken throughout the assays. Cellulase activity was expressed in international unit (U) in which an activity unit is the amount of crude enzymatic extract that releases 1 μmol of sugars (glucose equivalent) per minute.

Optimization of Nitrogen Sources for Cellulase Production

For the optimization of the nitrogen sources, the strategy of sequential experimental design was adopted. In the first experimental design, a complete 2^4 factorial experiment was performed with four nitrogen sources (urea, ammonium sulfate, peptone, and yeast extract) in two levels, minimum and maximum, coded as "−1" and "+1", respectively, and three replicates of the center point, coded as "0" (Table 1). The center points for each nitrogen source were the same used by Mandels and Weber [12]. A second experimental 2^3 factorial design with two levels and three replicates of the center point (Table 2) was performed, eliminating peptone, since it did not present a significative effect as it is going to be shown further on. Finally, a third experimental central composite rotational design (CCRD) was carried out (Table 3). For this sequence, the concentration of ammonium sulfate was kept constant (4 g/L), since it presented significant effect only for CMCase activity, remaining urea, and yeast extract as the only factors to be analyzed. The response variables in all experimental designs were FPase, CMCases, and β-glucosidase activities.

To maximize the three response variables simultaneously, an optimization using the *global desirability function* (D) was performed, which consists in converting each response into a single *desirability function* (d_i), that ranges from 0 to 1 ($0 \leq d_i \leq 1$) [13]. For a function with three independent variables, the *global desirability function* is expressed as follows:

$$D = (d_1 \cdot d_2 \cdot d_3)^{1/3} \tag{1}$$

The predicted values were validated experimentally in batchwise submerged fermentation (four replicates), carried out in 500 mL conical flasks, containing 200 mL of the

Table 1 Factors and levels used in the experimental 2^4 factorial design for nitrogen source optimization.

Factor	Min	CP	Max
	−1	0	+1
Urea (g/L)	0.15	0.30	0.45
Ammonium sulfate (g/L)	0.70	1.40	2.10
Peptone (g/L)	0.40	0.75	1.10
Yeast extract (g/L)	0.13	0.26	0.38

Min minimum value, *CP* center point, *Max* maximum value.

production medium, at 30 °C and 200 rpm for 72 h. The activity measurements were assayed in triplicates. The statistical treatment of the results was performed by the software STATISTICA version 6.0 (StatSoft, Inc).

Results and Discussion

Sugar Cane Bagasse and Cellulignin Composition

Table 4 shows the composition of sugar cane bagasse and cellulignin partially delignified. Acid and alkaline pretreatments were efficient in reducing both hemicellulose (29.6% to 12.2%) and lignin (19.4% to 9.3%) contents and simultaneously resulted in an increase in cellulose content (34.1% to 68.0%). It is widely known that lignin hinders the direct utilization of lignocellulosic materials. This polyphenolic macromolecule offers a physical barrier for the use of native cellulose [14]. Besides disorganizing the lignocellulosic complex, the aim of the acid and alkaline pretreatments is to remove part of hemicellulose and lignin, reducing the cellulose crystallinity and enhancing the remaining solid fraction porosity [15]. Pretreatments of lignocellulosic materials are essential as for making easier the accessibility to the cellulosic substrate by microorganisms or enzymes. Additionally, if cellulose enzymatic hydrolysis is aimed, lignin can adsorb cellulases, and thus, making them unproductive requiring higher enzyme load when high lignin-containing feedstocks are used [16].

Sequential Experimental Designs

Table 5 shows the results of the 2^4 factorial design. The enzymatic activities ranged from 41 to 170 U/L for FPase, from 1,200 to 2,260 U/L for CMCase, and from 71 to 510 U/L for β-

Table 2 Factors and levels used in the experimental 2^3 factorial design for nitrogen source optimization.

Factor	Min	CP	Max
	−1	0	+1
Urea (g/L)	0.30	0.55	0.80
Ammonium sulfate (g/L)	1.20	2.60	4.00
Yeast extract (g/L)	0.08	0.16	0.25

Min minimum value, *CP* center point, *Max* maximum value.

Table 3 Factors and levels used in the experimental central composite rotational design (CCRD) for nitrogen source optimization.

Factor	Axial −1.41	Min −1	CP 0	Max +1	Axial +1.41
Urea (g/L)	0.07	0.40	1.20	2.00	2.33
Yeast extract (g/L)	0.00	0.09	0.29	0.50	0.59

Min minimum value, *CP* center point, *Max* maximum value.

glucosidase. These results allowed plotting the Pareto chart (Fig. 1), which provided important data about the statistical relevance of the factors as well as of their interactions. From these charts, one can see that urea had significant relevance to express enzymes with FPase and CMCase activities, and a negative effect on β-glucosidase activity. On the contrary, ammonium sulfate presented significant positive effect for β-glucosidase production, yet no significative effect was observed for the other activities. Yeast extract caused a significant effect only on the FPase activity. The correlation coefficient (R^2) showed a good fit to the experimental results with values higher than 83%. Peptone was eliminated from the medium composition since it did not present a significative effect for FPase and CMCase, and a negative effect of low importance for β-glucosidase activity.

According to the results of the second experimental design, an increase in cellulase activities, especially CMCase, was achieved, rising from a maximum of 2,260 (first experimental design) to 5,143 U/L (second experimental design), an increase of above 100%. Concerning FPase activity, the increase corresponded to 46% (172 to 250 U/L) related to the first experimental design (Table 6). Nonetheless, these enzymatic activities can still be enhanced, as the center points are still lower than the factorial points, showing an increasing tendency in the direction of the maximum point.

Based on the effects of parameters using the Pareto chart (Fig. 2), urea appears as the most important nitrogen source for all cellulase activities. Also, this nitrogen source showed a positive effect, indicating that in the next planning, its concentration should still be increased. The yeast extract indicated a significative effect for CMCase and β-glucosidase activities and of low importance for FPase activity. The concentration of this component was maintained for the third experimental design sequence.

The analysis of the Pareto chart also depicted significative curvature for FPase and β-glucosidase activities denoting that there exists a point of maximum activity. Moreover, the values of the center points are close to the maximum obtained in the studied range, indicating the need of adding axial points to the following experiment to optimize the cellulase production, as in the factorial design, only linear models can be evaluated. Thus,

Table 4 Composition of sugar cane bagasse and cellulignin partially delignified.

Components	Sugar cane bagasse (% *w*/*w*)	Cellulignin partially delignified (% *w*/*w*)
Cellulose	34.09±1.20	67.97±1.31
Hemicellulose	29.61±1.44	12.2±0.94
Lignin	19.41±0.44	9.30±0.62
Ash	7.94±1.05	3.50±0.42
Moisture	4.41±0.12	3.98±0.15

Table 5 Matrix of experimental 2^4 factorial design for nitrogen sources optimization and corresponding results of cellulase activities.

Run	Nitrogen sources				Activities (U/L)		
	Urea	Ammonium sulfate	Peptone	Yeast extract	FPase R^2=0.836	CMCase R^2=0.873	β-glucosidase R^2=0.910
1	−1	−1	−1	−1	39	1,328	170
2	+1	−1	−1	−1	87	1,699	122
3	−1	+1	−1	−1	48	1,332	473
4	+1	+1	−1	−1	71	1,979	511
5	−1	−1	+1	−1	43	1,458	156
6	+1	−1	+1	−1	84	2,189	204
7	−1	+1	+1	−1	45	1,343	385
8	+1	+1	+1	−1	112	1,707	288
9	−1	−1	−1	+1	19	1,257	114
10	+1	−1	−1	+1	146	2,148	116
11	−1	+1	−1	+1	50	1,592	244
12	+1	+1	−1	+1	92	1,726	126
13	−1	−1	+1	+1	107	1,203	72
14	+1	−1	+1	+1	172	2,261	210
15	−1	+1	+1	+1	62	1,434	234
16	+1	+1	+1	+1	82	1,848	154
17 (C)	0	0	0	0	75	1,726	223
18 (C)	0	0	0	0	70	1,782	219
19 (C)	0	0	0	0	89	1,753	226

the next step was to evaluate the enzyme production using the CCRD technique, since it allowed predicting response surface with curvature plots and visualizing the maximum values of the response variables.

The results of CCRD are presented in Table 7. Comparing the optimized results with those obtained by Mandels and Weber [12] media, there was an improvement in all enzymatic activities. Increases of 3.3-, 3.2-, and 6.7-folds were achieved for FPase, CMCase, and β-glucosidase activities, respectively, at the end of this study.

Using the analysis of variance (ANOVA; Tables 8, 9, and 10), significant linear effects ($p \leq 0.05$) of both nitrogen sources (i.e., urea and yeast extract) were observed. Also, no significant interactions between urea and yeast extract were verified for any group of cellulases produced. This means that each nitrogen source alone presented linear effect, without mutual interference. Since the first experimental design, interaction between urea and yeast extract has not been observed.

Corroborating with the second experimental design, which presented significant curvature, the CCRD showed quadratic effect for FPase and β-glucosidase ($p \leq 0.05$), thus denoting curvilinear plan, as can be observed in ANOVA tables (Tables 8, 9, and 10). The lack of fit was not significative ($p > 0.05$); this, combined with the F values of the parameters and the determination coefficient (R^2) values (91.98 to FPase and 88.85 to β-glucosidase), shows that the model adequately adjusts to the experimental points, representing the confidence of the results.

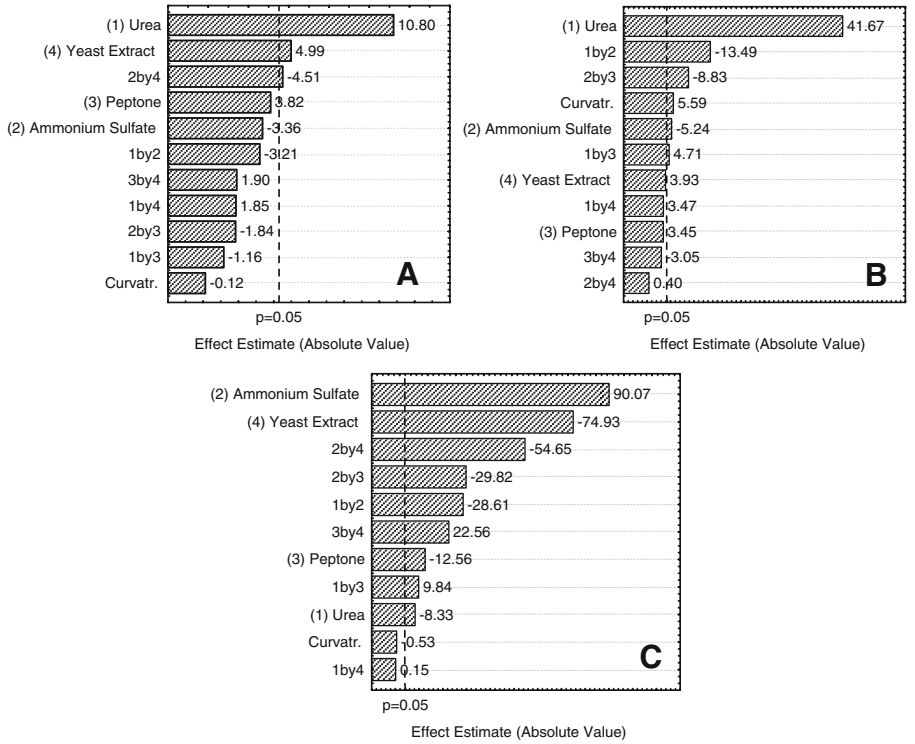

Fig. 1 Pareto chart for FPase (**a**), CMCase (**b**), and β-glucosidase (**c**) activities in 2^4 factorial design

Table 6 Matrix of experimental 2^3 factorial design for nitrogen sources optimization and their corresponding results of cellulase activities.

Run	Nitrogen sources			Activities (U/L)		
	Urea	Ammonium sulfate	Yeast extract	FPase	CMCase	β-glucosidase
1	−1	−1	−1	158	3,995	835
2	+1	−1	−1	202	4,291	1,540
3	−1	+1	−1	137	3,642	1,021
4	+1	+1	−1	240	4,895	1,702
5	−1	−1	+1	191	4,311	1,311
6	+1	−1	+1	221	4,520	1,717
7	−1	+1	+1	141	4,726	1,345
8	+1	+1	+1	175	5,144	1,728
9 (CP)	0	0	0	225	4,715	1,623
10 (CP)	0	0	0	234	4,565	1,687
11 (CP)	0	0	0	250	4,743	1,713

CP center point

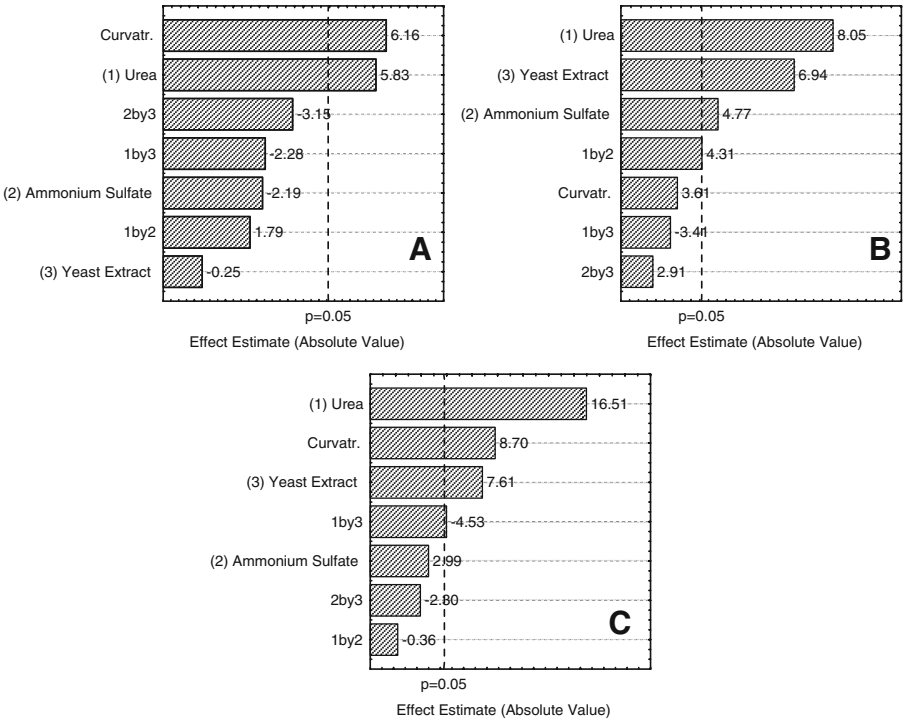

Fig. 2 Pareto chart for FPase (**a**), CMCase (**b**), and β-glucosidase (**c**) activities in the 2^3 factorial planning

Table 7 Matrix of experimental central composite rotational design (CCRD) for nitrogen sources optimization and their corresponding results of cellulase activities.

Experiment	Nitrogen sources		Activities (U/L)		
	Urea	Yeast extract	FPase	CMCase	β-glucosidase
1	−1	−1	158	4,029	727
2	−1	+1	171	4,354	1,119
3	+1	−1	166	5,302	1,080
4	+1	+1	244	5,513	1,101
5	−1.41	0	148	4,481	743
6	+1.41	0	263	6,529	1,213
7	0	−1.41	208	5,460	1,085
8	0	+1.41	255	7,105	1,435
9 (CP)	0	0	250	5,364	1,390
10 (CP)	0	0	269	5,524	1,499
11 (CP)	0	0	261	5,793	1,420

CP center point

Table 8 Analysis of variance (ANOVA) of nitrogen sources for FPase activities.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F	p_{value}
Urea (L)	8,133	1	8,133	88	0.011
Urea (Q)	7,210	1	7,210	78	0.012
Yeast extract (L)	2,693	1	2,693	29	0.032
Yeast extract (Q)	2,899	1	2,899	32	0.030
Interaction 1×2 L	1,048	1	1,048	11	0.078
Lack of fit	2,962	3	987	11	0.086
Pure error	184	2	92		
Total	23,142	10			

The response surface plots of the third experimental design are presented in Fig. 3. FPase and β-glucosidase activities presented a point of maximum relatively similar. The analysis of Fig. 3b shows that CMCase activity could be increase. However, the increase of urea and yeast extract concentrations can act in an opposite way for FPase and β-glucosidases activities. By means of ANOVA, it was found that both nitrogen sources (i.e., urea and yeast extract) presented significant quadratic effect, except for CMCase activity, confirming that for this group of enzymes, the range of nitrogen source could be even higher, since the lack of quadratic effect and positive linear effect indicates a linear model, with a trend of increasing towards higher concentrations of the nitrogen sources investigated.

The mathematical models of cellulase production under the current conditions are represented by the following equation:

$$Y = \beta_0 + \beta_1 \cdot \mathrm{Ur}^2 + \beta_2 \cdot \mathrm{El}^2 + \beta_3 \cdot \mathrm{Ur} + \beta_4 \cdot \mathrm{El} + \beta_5 \cdot \mathrm{Ur} \cdot \mathrm{El} + \varepsilon \quad (2)$$

where:

Ur = Urea
El = Yeast extract
ε = Pure error
β = Coefficients

Table 9 Analysis of variance (ANOVA) of nitrogen sources for CMCase activities.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F	p_{value}
Urea (L)	1,473,886	1	1,473,886	31	0.030
Urea (Q)	512,272	1	512,272	11	0.081
Yeast extract (L)	2,831,711	1	2,831,711	60	0.016
Yeast extract (Q)	43,303	1	43,303	11	0.438
Interaction 1×2 L	3,186	1	3,186	0.1	0.819
Lack of fit	3,093,423	3	1,031,141	22	0.044
Pure error	93,986	2	46,993		
Total	8,200,284	10			

Table 10 Analysis of variance (ANOVA) of nitrogen sources for β-glucosidase activities.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F	p_{value}
Urea (L)	145,154	1	145,154	46	0.021
Urea (Q)	373,623	1	373,623	118	0.008
Yeast extract (L)	86,185.8	1	86,186	27	0.035
Yeast extract (Q)	76,366.9	1	76,367	24	0.039
Interaction 1×2 L	34,504.9	1	34,505	11	0.080
Lack of fit	36,382.9	3	12,128	4	0.213
Pure error	6,306.2	2	3,153		
Total	692,365	10			

As it was noticed in the ANOVA table that there was no interaction between urea and yeast extract, the combined effect of both nitrogen sources was removed from the model. The equations representing each enzymatic group are provided as follows:

$$\text{FPase activity}(\text{U/L}) = 259.97 - 35.73\text{Ur}^2 - 22.66\text{El}^2 + 31.88\text{Ur} + 18.35\text{El} + 2.77 \quad (3)$$

$$\text{CMCase activities}(\text{U/L}) = 5560.19 - 301.19\text{Ur}^2 + 87.57\text{El}^2 + 429.23\text{Ur} + 594.95\text{El} + 62.58 \quad (4)$$

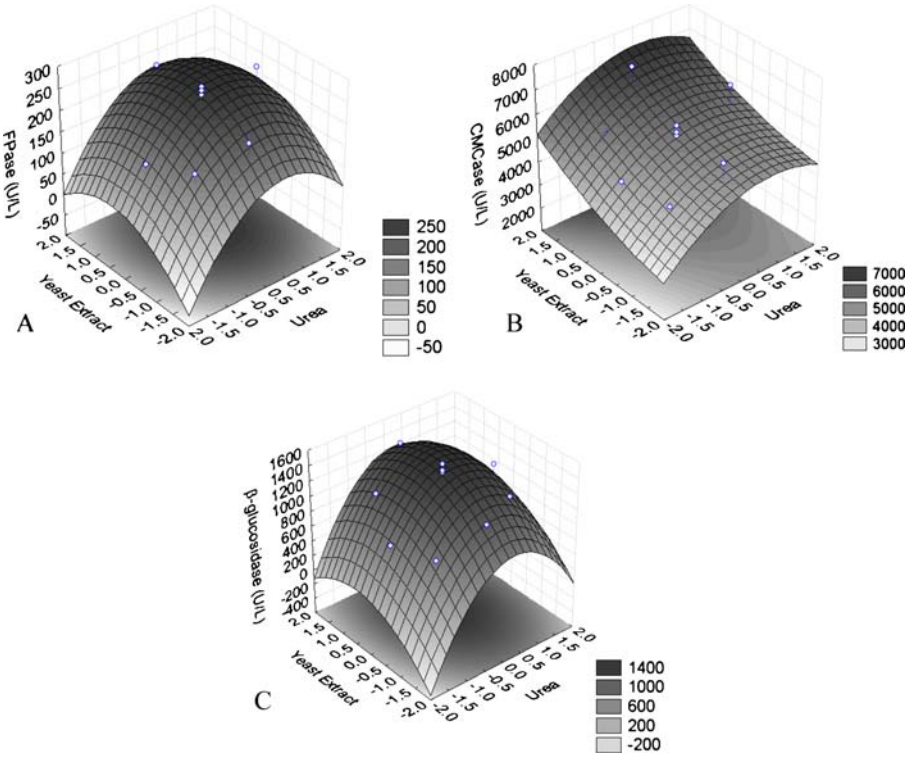

Fig. 3 Response surface of nitrogen source optimization for FPase (**a**), CMCase (**b**), and β-glucosidase (**c**) activities

Table 11 Validation of the nitrogen sources optimization.

Activities (U/L)	Limits		Predicted value	Experimental result
	−95%	+95%		
FPase (U/L)	220	287	254	227±6
CMCase (U/L)	5,805	6,959	6,382	6,917±241
β-glucosidase (U/L)	1,325	1,572	1,448	1,375±59

Predicted and experimental values of cellulase activities.

$$\beta\text{−Gase activity(U/L)} = 1436.47 - 257.22\text{Ur}^2 - 116.29\text{El}^2 + 134.70\text{Ur} + 103.79\text{El} + 16.21 \quad (5)$$

where:

β-Gase:β-glucosidase

Optimization of Nitrogen Sources using Desirability Function and Experimental Validation

The global desirability value to reach the optimum for the three enzymatic activities simultaneously was 0.872, meaning that the optimization by this function fulfills 87.2% of the maximum obtainable for each dependent variable (response variable). The optimum concentration for urea was 0.97 g/L and for yeast extract was 0.36 g/L.

The predicted values for cellulase activities determined by using the global desirability function and cellulase activities from the experimental validation of the results in optimized conditions are presented in Table 11.

It is observed that the experimental values are within the confidence limits −95% and +95%, showing that the optimized values of urea and yeast extract were experimentally validated. The deviations of experimental and predicted values were 18.6, 377.8, and 52.0 U/L for FPase, CMCase, and β-glucosidase activities, respectively, corresponding to values of the coefficient of variation of only 7.4%, 5.9%, and 3.6%.

Conclusions

The sequential experimental design strategy combined with the use of the desirability function for the optimization of nitrogen source showed to be an important tool for maximizing cellulase production by *P. funiculosum*. Apart from maximizing the three cellulase activities separately, an optimum condition was successfully found considering all of them simultaneously, which resulted in an increase of 3.3-, 3.2-, and 6.7-folds to FPase, CMCase, and β-glucosidase activities, respectively, compared with the initial condition (first experimental design). Among the evaluated nitrogen sources, urea and yeast extract are of greater importance to cellulase production by this filamentous fungus.

Acknowledgements The authors acknowledge the Brazilian Council for Research (CNPq), the Rio de Janeiro State Foundation for Science and Technology (FAPERJ), and the Brazilian Petroleum Company (PETROBRAS) for scholarship and other financial supports.

References

1. Pereira, R. E. (2006). MSc Thesis. Escola de Química. Universidade Federal do Rio de Janeiro
2. Pereira, N., Jr., Couto, M. A. P. G., & Anna, L. M. M. S. (2008). *Series on biotechnology* (2nd ed., p. 45). Rio de Janeiro: Biblioteca Nacional.
3. Vasquez, M. P., Silva, J. N. C., Souza, M. B., Jr., & Pereira, N., Jr. (2007). *Applied Biochemistry and Biotechnology, 137/140*(12), 141–154.
4. Schlitter, L. A. F. (2006). Msc Thesis. Escola de Química. Universidade Federal do Rio de Janeiro
5. Zabel, R. A., & Morrell, J. J. (1992). *Wood microbiology, decay and its prevention*. San Diego: Academic. 476p.
6. Marzluf, G. (1997). *Microbiology and Molecular Biology Reviews, 61*(1), 17–32.
7. Merrick, M. J., & Edwards, R. A. (1995). *Microbiological Reviews, 59*, 604–622.
8. Betancur, G. J. V. (2005). Msc Thesis. Escola de Química. Universidade Federal do Rio de Janeiro
9. Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., & Templeton, D. (2005). NREL, 14 p.
10. Ververis, C., Georghiou, K., Danielidis, D., Hatzinikolaou, D. G., Santas, P., Santas, R., et al. (2007). *Bioresource Technology, 98*, 296–301.
11. Ghose, T. K. (1987). *Pure and Applied Chemistry, 59*(2), 257–268.
12. Mandels, M., & Weber, J. (1969). *Advances in Chemistry Series, 95*, 391–414.
13. Derringer, G., & Suich, R. (1980). *Journal of Quality Technology, 12*, 214–219.
14. Reyes, J., Peralta-Zamora, P., & Duran, N. (1998). *Química Nova, 21*(2), 140–143.
15. Sun, Y., & Cheng, J. (2002). *Bioresource Technology, 83*(1), 1–11.
16. Jorgensen, H., & Olsson, L. (2006). *Enzyme and Microbial Technology, 38*, 381–390.