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OPTIMIZATION OF BIOMASS AND ENDO-B-1,4-GLUCANASE BY WHITE ROT FUNGI NATIVE FROM ARGENTINA

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Abstract

A large number of microorganisms are capable of producing cellulases; however, fungi are considered among the most active enzymes producers. Different strains of white rot fungi (WRF) were reported to have a great potential for biotechnological applications and cellulase enzymes is much involved in this effect, especially for bioethanol production. Misiones is included in a forest region characterized by a vast biodiversity, and within this environment, a large number of microorganisms can be isolated, including numerous white rot fungi. In the present work, response surface methodology (RSM) was applied for the determination of biomass and endo-β-1,4-glucanase activity factors dependence from three Argentinean WRF in submerged cultures. RSM was carried out with a Box-Behnken design using 3 variables, temperature (25, 29 and 33°C), incubation time (7, 10.5 and 14 days) and pH (3.5, 4.5 and 5.5) with three central points and 2 replications. The best conditions for enzymatic activity were achieved at maximum level of temperature (33°C), low level of pH (3.5) and intermediate level of incubation time (10.5 h). We have presented here, preliminary studies on biomass and enzyme production of three fungi. Our data show that we have not yet reached the optimal enzyme production conditions, so further experiments are needed to reach optimal conditions.

Keywords: cellulases, response surface methodology, white rot fungi

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1. Introduction

Cellulose is a linear glucose polymer composed of β-1,4-glucose units by a β-1,4-D-glycosidic bond (Gielkens et al., 1999; Han et al., 1995). Cellulolytic enzymes degrade cellulose by cleaving the glycosidic bonds (Han et al., 1995). Many fungi and bacteria are capable of producing multiple enzymes, which are collectively known as cellulases. Endoglucanases (endo-β-1,4-glucanase, EC 3.2.1.4), is one of the major component enzymes of the cellulose complex and catalyzes the hydrolysis of cellulose by randomly splitting the sugar residues within the molecule (Sohail et al., 2009). Exoglucanases (exo-β-1,4-glucanase, EC 3.2.1.91) and β-glucosidase (EC 3.2.1.37) can synergistically convert cellulose into glucose and hence they can be used in an industrial scale (Bhat, 2000).

On a commercial scale, bioconversion of cellulosic biomass requires the use of cellulases that makes the process highly expensive (Zhang et al., 2006). Hence, cellulase production from a wide range of microorganisms has been extensively studied (Lynd et al., 2005); therefore, the screening and characterization of novel isolates is essential to make the enzyme production process feasible (Duarte and Costa-Ferreira, 1994; Sohail et al., 2009). However, the high cost of cellulases production coupled with low enzyme activities limits its industrial use. Therefore, efforts are needed to economize cellulase production by media optimization and use of supplements/additives (Kocher et al., 2008).

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Studies on fermentation parameters to obtain maximum yields of cellulase enzymes seem necessary. Temperature and pH are regarded as the most important variables beside the use of suitable inducers in this regard. Although a significant number of fungal strains were reported to have cellulolytic activities (Joni et al., 2010; Singhania et al., 2010), extensive search is still going on to isolate a hyperproducing microbial strain that could be used for bulk production of cellulases at a nominal cost.

Response Surface Methodology (RSM) is commonly used to find the optimal conditions, which is an efficient statistical technique for optimization of multiple variables with minimum number of experiments (Montgomery, 2001). With Box-Behnken experimental design approach, most of the disadvantages of conventional one-factor-at-a-time experiments can be avoided (Li et al., 2008; Xu et al., 2008). This methodology has already been successfully applied for the optimization of medium composition, production of extracellular cellulases on different growth substrates and enzymatic hydrolysis (Daroit et al., 2007; Ferreira et al., 2009).

In this work, RSM was used to evaluate the main and interaction effects of the physicochemical variables on biomass and endo-β-1,4-glucanase production by three white rot fungi (WRF) native from Misiones in submerged cultures. The factors, considered for experimentation and analysis were growth temperature, initial pH and incubation time.

2. Material and methods

2.1. Microorganisms

We used 3 strains that showed potential for cellulase production tested by solid media: *Peniophora* sp. BAFC 633, *Coriolus versicolor* f. *antarcticus* BAFC 266 and *Pycnoporus sanguineus* BAFC 2126. They were provided by the Mycological Culture Collection of Biodiversity and Experimental Biology Department, Faculty of Exact and Natural Sciences, University of Buenos Aires, Argentina. Isolates were maintained in stock by periodic sub-culturing on malt extract agar plates containing 12.7 g/L malt extract and 20 g/L agar (MEA) at 4°C.

2.2. Culture conditions

To prepare the liquid inoculums, 0.5 mm-agar plugs from each fungus were grown five to seven days in MEA, then were cut and transferred to 250 ml-Erlenmeyer flasks containing 50 mL of medium containing 12.7 g/L malt extract and 5 g/L corn steep liquor (MECL). The flasks were incubated at desired temperature in static conditions. The desired pH was achieved by adding HCl 1N.

2.3. Biomass determination

Biomass growth was determined by measuring the mycelium dry weight. Liquid media was separated from the supernatant mycelia by filtering in a Büchner funnel using fiberglass filters (GF/C) and frozen at -20°C until use. Biomass dry weight was determined by the difference between the fiberglass filters (GF/C) weight before and after filtration through a Büchner funnel and subsequent drying at 80ºC till constant weight.

2.4. Enzyme activity assay

Endo-β-1,4-glucanase (EC 3.2.1.4) activity was determined by measuring the liberation of reducing sugar with the 3,5-dinitrosalicylic acid (DNS) method using 2% (p/v) carboxy-methylcellulose (sodium salt, Sigma, USA) as substrate in 0.05 M sodium acetate buffer pH 4.8 (Miller, 1959). Reactions were incubated at 50˚C for 60 min. Absorbance was measured at 540 nm in a Shimadzu UV-3600 spectrophotometer. The carbohydrate fraction was extracted from the culture supernatant and the amount of sugar liberated was calculated using a glucose standard curve. One endo-β-1,4-glucanase activity unit was defined as the amount of enzyme that releases one μmol of reducing sugar per min at 50˚C.

2.5. Experimental design and statistical analysis

Many variables may potentially affect the production and secretion of cellulolytic enzymes into the culture medium. In this study, a Box-Behnken design was used to determine the effects of independent variables on the response and factors interactions. The variables were studied at low, middle, and high levels and were designated as −1, 0 and +1, respectively, which were given in the variable levels X_i coded as x_i according to Eq. (1):

$$x_i = \frac{x_1 - x_0}{\Delta X_i}, \qquad i = 1, 2, 3 \tag{1}$$

where: X_i and x_i are the actual value and coded value, respectively. X_0 is the value of an independent variable at center point, and ΔX_i is the step change. The selection of low, middle, and high levels of each variable were based on results of a single factor method investigation and bibliographic (Zhang et al., 2012). The experimental design consisted of 15 trials including three replications at the central point. The independent variables and levels studied are shown in Table 1.

Each experiment was carried out in duplicate and values of biomass (Y_1) and endo-β-1,4-glucanase activity (Y_2) were averaged. Statistical analysis was carried out with *Statgraphics plus* version 5.1 and involved multiple regressions and analysis of variance (ANOVA). A second-order model was used to fit the response to the independent variables as shown in Eq. (2), where Y is the predicted response, x_i and x_j are the input variables, β_0 is the intercept, β_i is the linear coefficient of x_i, β_{ij} is the interaction

coefficient between x_i and x_j and β_{ii} is the quadratic coefficient of x_i. The significance of the model equation and model terms were evaluated by F test.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i<j}^{k} \beta_{ij} x_i x_j + \sum_{i=1}^{k} \beta_{ii} x_i^2 \qquad (2)$$

Table 1. Process variables and their levels for the Box-Behnken design

Factors	***Independent variables***	***Range and levels***		
		-1	0	+1
X_1	Temperature (˚C)	25	29	33
X_2	Incubation time (days)	7	10.5	14
X_3	pH	3.5	4.5	5.5

The quality of the polynomial model equation was expressed by the coefficient R^2. The polynomial equation was expressed as three-dimension surface plots to visualize the relationship between the response and the experimental levels of each factor.

3. Results and discussion

In the present work, a Box-Behnken design was used to study the effects of temperature, incubation time and pH on biomass and endo-β-1,4-glucanase production from *Peniophora* sp. BAFC 633, *C. versicolor* f. *antarcticus* BAFC 266 and *P. sanguineus* BAFC 2126 in liquid medium with response surface methodology. The fitted model was designed to explain the relationship between the independent variables and dependent responses. The mathematical expressions of biomass and endo-β-1,4-glucanase production with variables coded as X_1, X_2 and X_3 are shown in the corresponding Figure of RSM, where Y_1 is the biomass, Y_2 is endo-β-1,4-glucanase production, X_1 is the temperature (˚C), X_2 the incubation time (days) and X_3 the pH. The mathematical expressions of biomass (Y_1) and endo-β-1,4-glucanase activity (Y_2) with coded variables X_1, X_2, and X_3 for *Peniophora* sp. BAFC 633, *C. versicolor* f. *antarcticus* BAFC 266 and *P. sanguineus* BAFC 2126 are shown in Eqs. (3-8).

Mathematical models coefficients were evaluated by regression analysis and tested for their significance. The ANOVA for each model term and its significance (*p*-values lower than 0.05 indicated that model terms were significant) and the proportion of total variation attributed to each fit model were evaluated by the R-squared value (a value of R-square > 0.75 indicates the goodness of fit of the model) (Haaland, 1989). Table 2 shows the matrix of Box-Behnken design with the three variables and the experimental and predicted responses of biomass and enzyme activity for all fungi tested. The residual values between the experimental and theoretical values were also estimated.

Peniophora sp. BAFC 633 reached the maximum fungal growth at high level of temperature and incubation time and intermediate level of pH. Maximum endo-β-1.4-glucanase activity (U/L) was obtained at high, low and intermediate levels of temperature, incubation time and pH, respectively (Table 2).

In the case of *C. versicolor* f. *antarcticus* BAFC 266 the maximum fungal growth was obtained at intermediate levels of temperature, incubation time and pH, while the maximal enzymatic activity required low temperature and pH and intermediate incubation time (Table 2). *P. sanguineus* BAFC 2126 reached the maximal biomass growth at intermediate level of temperature, high level of incubation time and low level of pH, while the highest value of enzymatic activity was related to low temperature, high incubation time and intermediate pH (Table 2).

Peniophora sp. BAFC 633

$$\text{Biomass} = 0.171167 + 0.0000875x_1 + 0.0419375x_2 - 0.022875x_3 - 0.00389583x_1^2 + 0.012825x_1x_2 + 0.01195x_1x_3 - 0.0247958x_2^2 + 0.01105x_2x_3 - 0.0155708x_3^2 \qquad (3)$$

$$\text{Enzyme Activity} = 79.57 + 12.4375x_1 - 11.2662x_2 - 20.7762x_3 - 26.821x_1^2 + 12.3325x_1x_2 - 5.2225x_1x_3 - 11.1975x_2^2 + 5.1x_2x_3 - 28.5075x_3^2 \qquad (4)$$

C. versicolor f. *antarcticus* BAFC 266

$$\text{Biomass} = 0.2896 - 0.02035x_1 + 0.041275x_2 + 0.0045x_3 - 0.0903625x_1^2 + 0.031975x_1x_2 - 0.019525x_1x_3 - 0.0746125x_2^2 - 0.021125x_2x_3 - 0.112612x_3^2 \qquad (5)$$

$$\text{Enzyme Activity} = 16.7433 - 0.3275x_1 - 2.0975x_2 - 6.2x_3 + 6.75583x_1^2 - 2.755x_1x_2 + 3.99x_1x_3 - 7.28917x_2^2 + 1.77x_2x_3 - 1.12417x_3^2 \qquad (6)$$

P. sanguineus BAFC 2126

$$\text{Biomass} = 0.150267 + 0.0065x_1 + 0.031275x_2 - 0.012125x_3 - 0.0466583x_1^2 + 0.0173x_1x_2 - 0.0064x_1x_3 - 0.0108083x_2^2 - 0.00745x_2x_3 - 0.0114583x_3^2 \qquad (7)$$

$$\text{Enzyme Activity} = 37.76 - 13.1075x_1 - 5.79625x_2 + 2.42625x_3 - 5.71375x_1^2 - 6.675x_1x_2 + 1.56x_1x_3 - 0.61625x_2^2 - 7.2375x_2x_3 - 10.2213x_3^2 \quad (8)$$

Table 2. Actual and predicted values of biomass (g) and endo-β-1,4-glucanase activity (U/L) of *Peniophora* sp. BAFC 633, *C. versicolor* f. *antarcticus* BAFC 266 and *P. sanguineus* BAFC 2126 recorded in experimental set-up of RSM

Peniophora sp. BAFC 633			***Biomass (g)*** ***Y_1***			***Enzyme Activity (U/L)*** ***Y_2***		
X1	*X2*	*X3*	***Experimental***	***Predicted***	***Residuals***	***Experimental***	***Predicted***	***Residuals***
-1	1	0	0.189	0.172	0.017	49.0	59.2	-10,20
-1	0	1	0.099	0.117	-0.018	73.0	49.9	23,10
0	-1	1	0.065	0.055	0.01	0.0	25.3	-25,30
0	1	1	0.162	0.161	0.001	0.0	12.9	-12,90
1	1	0	0.189	0.197	-0.008	106.5	108.7	-2,20
-1	-1	0	0.122	0.113	0.0089	108.5	106.4	2,10
1	0	1	0.149	0.141	0.008	79.4	64.3	15,10
-1	0	-1	0.179	0.186	-0.007	65.9	81.0	-15,10
0	1	-1	0.175	0.185	-0.01	69.5	44.3	25,20
0	-1	-1	0.122	0.123	-0.001	89.9	77.0	12,90
1	0	-1	0.181	0.163	0.018	93.2	116.3	-23,10
1	-1	0	0.070	0.088	-0.018	116.7	106.6	10,10
0	0	0	0.177	0.171	0.0059	71.5	79.6	-8,10
0	0	0	0.165	0.171	-0.006	87.6	79.6	8,00
0	0	0	0.171	0.171	0.000	79.6	79.6	0,00
C. versicolor f. *antarcticus* BAFC 266			***Biomass (g)*** ***Y_1***			***Enzyme Activity (U/L)*** ***Y_2***		
X1	*X2*	*X3*	**Experimental**	**Predicted**	**Residuals**	**Experimental**	**Predicted**	**Residuals**
-1	1	0	0.115	0.154	-0.039	15.10	17.20	-2.100
-1	0	1	0.164	0.127	0.037	16.41	12.51	3.900
0	-1	1	0.073	0.083	-0.010	0.00	2.46	-2.460
0	1	1	0.125	0.123	0.002	0.00	1.80	-1.800
1	1	0	0.205	0.178	0.027	12.47	11.03	1.440
-1	-1	0	0.108	0.136	-0.028	14.44	15.88	-1.440
1	0	1	0.018	0.047	-0.029	20.20	19.84	0.360
-1	0	-1	0.117	0.087	0.030	32.53	32.89	-0.360
0	1	-1	0.174	0.164	0.010	13.12	10.66	2.460
0	-1	-1	0.037	0.040	-0.003	20.20	18.40	1.800
1	0	-1	0.048	0.085	-0.037	20.36	24.26	-3.900
1	-1	0	0.071	0.031	0.040	22.83	20.74	2.090
0	0	0	0.238	0.290	-0.052	16.25	16.74	-0.490
0	0	0	0.341	0.290	0.051	17.24	16.74	0.500
0	0	0	0.290	0.290	0.000	16.74	16.74	0.000
P. sanguineus BAFC 2126			***Biomass (g)***			***Enzyme Activity (U/L)***		
X1	*X2*	*X3*	**Experimental**	**Predicted**	**Residuals**	**Experimental**	**Predicted**	**Residuals**
-1	1	0	0.114	0.135	-0.021	57.17	45.42	11.750
-1	0	1	0.105	0.080	0.025	26.25	35.80	-9.550
0	-1	1	0.043	0.092	-0.049	50.92	42.38	8.540
0	1	1	0.135	0.140	-0.005	14.11	16.32	-2.210
1	1	0	0.089	0.113	-0.024	4.84	5.85	-1.010
-1	-1	0	0.062	0.038	0.024	44.67	43.66	1.010
1	0	1	0.109	0.080	0.029	15.92	12.70	3.220
-1	0	-1	0.063	0.091	-0.028	30.85	34.07	-3.220
0	1	-1	0.228	0.179	0.049	17.40	25.94	-8.540
0	-1	-1	0.106	0.101	0.005	25.26	23.06	2.200
1	0	-1	0.092	0.117	-0.025	14.28	4.73	9.550
1	-1	0	0.106	0.085	0.021	19.04	30.79	-11.750
0	0	0	0.179	0.150	0.029	38.42	37.76	0.660
0	0	0	0.122	0.150	-0.028	37.10	37.76	-0.660
0	0	0	0.150	0.150	0.000	37.76	37.76	0.000

The regression curves between predicted theoretical responses and experimental values are depicted in Fig. 1 (a-f).

The analysis of variance (ANOVA) shown in Table 3 is an indication of the predictability of the model. The coefficients of regression, *F*-ratio and *p*-

values for enzyme activity were calculated and are shown in the table. P values lower than 0.05 indicate that the model was statistically significant. The predicted response fit well those of the experimentally obtained response. The results indicate that linear term of incubation time significantly affected biomass production from *Peniophora* sp. BAFC 633 ($p< 0.005$). A coefficient of determination (R^2) value of 92.63% for biomass and 79.04% for enzymatic activity (*Peniophora* sp. BAFC 633) showed the equation was highly reliable. The model was presumed to be adequate for prediction within the range of variables, because only 21% of the variance can be attributed to error, indicating a good fit of the model.

Coefficient of determination (R^2) values of 88.15% (biomass) and 93.13% (endo-β-1.4-glucanase activity) for *C. versicolor* f. *antarcticus* BAFC 266 showed that the equations are highly dependable. The p-values indicate that squared terms of temperature, incubation time and pH had a significant effect on biomass production. In this model 12% of the variance can be attributed to error, indicating a good power model.

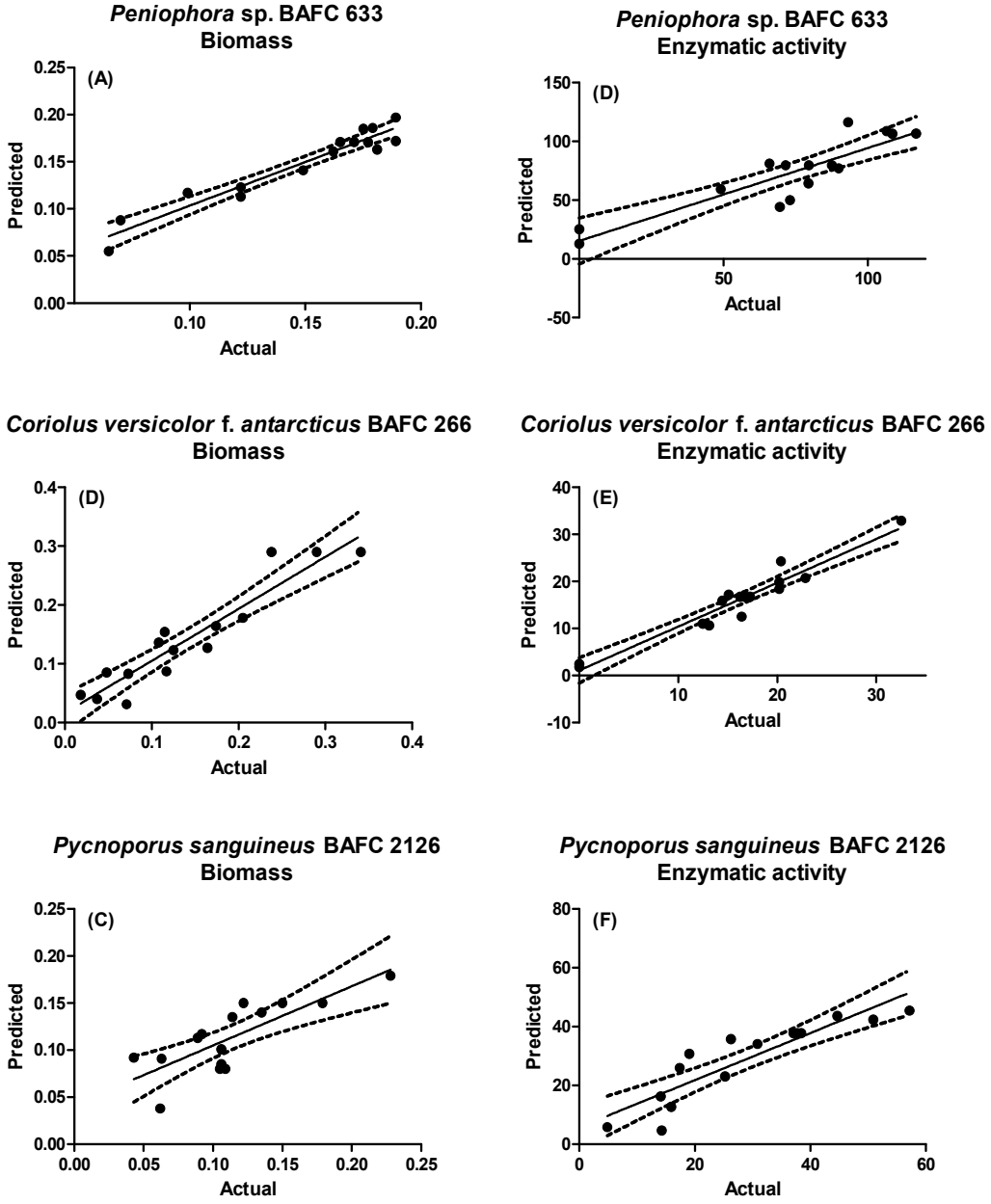

Fig. 1. Statistical studies of the model for the response Biomass (Y_1) and enzymatic activity (Y_2) of *Peniophora* sp. BAFC 633 (a-d), *Coriolus versicolor* f. *antarcticus* BAFC 266; (b-e) and *Pycnoporus sanguineus* BAFC 2126; (c-f). The regression curves were adjusted between predicted theoretical responses and experimental values

Table 3. Estimated coefficients. *F*-ratio and *p*-values corresponding of biomass (g) and endo-β-1.4-glucanase activity (U/L) of (A) *Peniophora* sp. BAFC 633. (B) *C. versicolor* f. *antarcticus* BAFC 266 and (C) *P. sanguineus* BAFC 2126. * Statistical significant differences (p<0.05)

A	*Biomass (g)*			*Enzymatic Activity (U/L)*		
Variables	*Coefficients*	*F-ratio*	*p-Value*	*Coefficients*	*F-ratio*	*p-Value*
Mean	0.171167			79.57		
X_1	0.0000875	0	0.9902	12.4375	1.78	0.2398
X_2	0.0419375	38.56	0.0016*	-11.2662	1.46	0.281
X_3	-0.022875	11.47	0.0195*	-20.7762	4.96	0.0763
X_1X_1	-0.00389583	0.15	0.7113	26.82	3.82	0.1081
X_1X_2	0.012825	1.8	0.2371	12.3325	0.87	0.3926
X_1X_3	0.01195	1.57	0.2662	-5.2225	0.16	0.7084
X_2X_2	-0.0247958	6.22	0.0549	-11.1975	0.67	0.4517
X_2X_3	0.01105	1.34	0.2995	5.1	0.15	0.7149
X_3X_3	-0.0155708	2.45	0.178	-28.5075	4.31	0.0924
R^2	92.63%			79.04%		

B	*Biomass (g)*			*Enzymatic Activity (U/L)*		
Variables	*Coefficients*	*F-ratio*	*p-Value*	*Coefficients*	*F-ratio*	*p-Value*
Mean	0.2896			16.7433		
X_1	-0.02035	1.13	0.3371	-0.3275	0.07	0.804
X_2	0.041275	4.63	0.084	-2.0975	2.81	0.1546
X_3	0.00045	0	0.9822	-6.2	24.55	0.0043*
X_1X_1	-0.0903625	10.25	0.024*	6.75583	13.45	0.0145*
X_1X_2	0.031975	1.39	0.2914	-2.755	2.42	0.1803
X_1X_3	-0.019525	0.52	0.5037	3.99	5.08	0.0738
X_2X_2	-0.0746125	6.99	0.0458*	-7.28917	15.66	0.0108*
X_2X_3	-0.021125	0.61	0.4712	1.77	1	0.3631
X_3X_3	-0.112612	15.92	0.0104*	-1.12417	0.37	0.5683
R^2	88.15%			93.13%		

C	*Biomass (g)*			*Enzymatic Activity (U/L)*		
Variables	*Coefficients*	*F-ratio*	*p-Value*	*Coefficients*	*F-ratio*	*p-Value*
Mean	0.150267			37.76		
X_1	0.0065	0.15	0.7158	-13.1075	10.78	0.0219*
X_2	0.031275	3.44	0.1229	-5.79625	2.11	0.2063
X_3	-0.012125	0.52	0.5044	2.42625	0.37	0.57
X_1X_1	-0.0466583	3.53	0.119	-5.71375	0.95	0.3756
X_1X_2	-0.0173	0.53	0.5008	-6.675	1.4	0.2903
X_1X_3	-0.0064	0.07	0.7992	1.56	0.08	0.7934
X_2X_2	-0.0108083	0.19	0.6814	-0.61625	0.01	0.9206
X_2X_3	-0.00745	0.1	0.7674	-7.2375	1.64	0.2562
X_3X_3	-0.0114583	0.21	0.6638	-10.2213	3.02	0.1425
R^2	62.98%			80.09%		

The model was presumed to be adequate for prediction within the range of the studied variables. Regarding enzymatic activity, linear terms of pH and quadratic terms of temperature and incubation time had a significant effect ($p< 0.005$). In this model less than 7% of the variance can be attributed to error, indicating a good fit model.

In the case of *P. sanguineus* BAFC 2126, only enzymatic activity showed a coefficient of determination (R^2) value of 80.09% indicating the goodness of fit. The *p*-values indicated that temperature linear terms significantly influenced the endo-β-1.4-glucanase production ($p<0.05$). In this model 20% of the variance can be attributed to error, indicating a good fit model.

Three-dimensional response surface plots were constructed by plotting the response (biomass and endo-β-1,4-glucanase activity) on the Z-axis against any two independent variables, while maintaining the other variables at their center levels (Figs. 2-4, A-B). Soni et al. (2010) conducted a Box–Behnken design of experiment to study the influence of selected process variables on cellulase and xylanase production. The applied model proved to be a good indicator and a useful tool to predict the level of beef extract, temperature and tween 80 required to achieve optimal cellulase production by *A. fumigates fresenius* (AMA).

The three-level three-factorial Box–Behnken experimental design was applied to investigate and validate adsorption process parameters affecting the removal of Acrylonitrile (AN) by agri-based adsorbent-sugarcane bagasse fly ash (BFA). The results also showed that the RSM is a very useful optimal design for experiments on the adsorption of toxics onto adsorbents (Kumar et al., 2008).

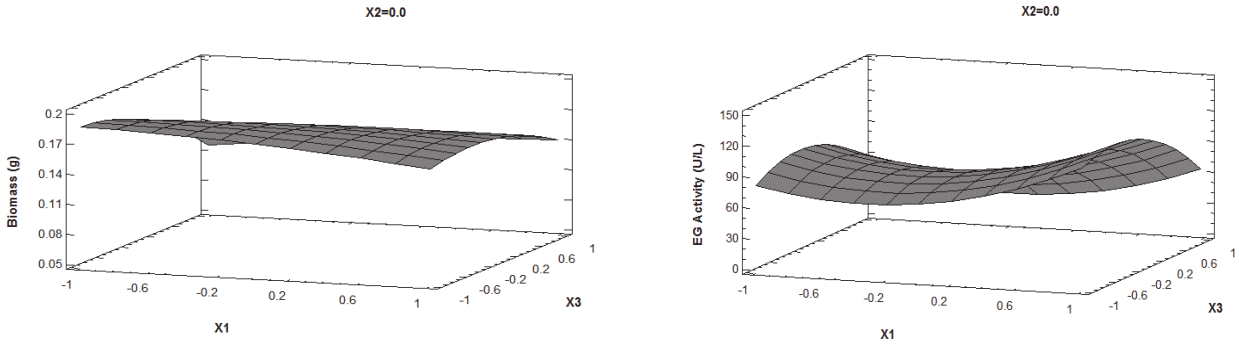

Fig. 2. Surface Plots of (A) Biomass (g) and (B) endo-β-1.4-glucanase activity (U/L) of *Peniophora* sp. BAFC 633 as a function of Temperature and pH (in coded values)

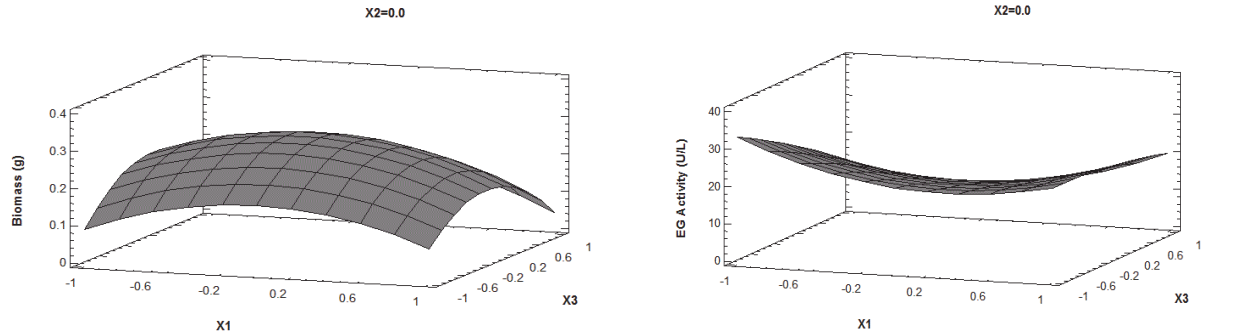

Fig. 3. Surface Plots of (A) Biomass (g) and (B) endo-β-1.4-glucanase activity (U/L) of *C. versicolor* f. *antarcticus* BAFC 266 as a function of Temperature and pH (in coded values)

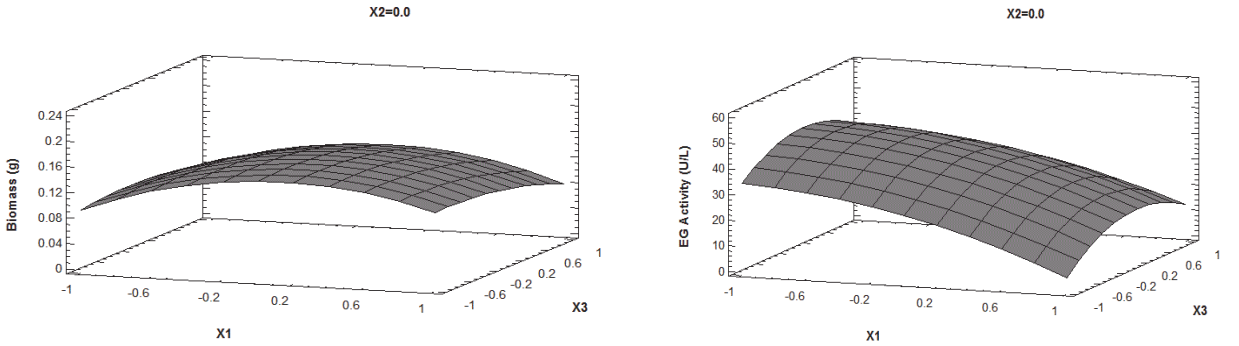

Fig. 4. Surface Plots of (A) Biomass (g) and (B) endo-β-1.4-glucanase activity (U/L) of *P. sanguineus* BAFC 2126 as a function of Temperature and pH (in coded values)

Galai et al. (2012) have successfully applied RSM for the laccase activity using two substrates: ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) and DMP (2,6-dimethoxyphenol).

The optimization of laccase production media for C. versicolor MTCC 138 using a Box-Behnken statistical experimental design method was achieved by Mishra et al. (2008). Parameters such as medium, pH, temperature, moisture content, inducers among others were considered.

When comparing the secretion ability of the three fungi, the present study revealed that the highest titers of enzymes (116.7 U/L) were secreted by *Peniophora* sp. BAFC 633 at 33ºC, 7 days of incubation time and pH 4.5. This is a worthy outcome considering biotechnological prospection since working at wide range of temperature would be an economic and easy attainable heating condition for the bioreactors operation bearing in mind the subtropical weather from our region (mean temperature of 25ºC). In addition, fungi that developed an optimal growth at higher pH are more suitable for industrial applications because the process conditions would be less aggressive for the fermentation equipment.

From the equipment design standpoint this last feature would favor oxygen transfer and rheological behavior due to the low broth viscosity. Broth rheology greatly affects transport processes in

bioreactors which, in turn, have a strong influence on the efficiency and productivity of the entire enzyme production process. Mycelial fermentation broths are initially Newtonian because of low biomass concentrations.

4. Conclusions

The present design proved to be an important tool to get information regarding the effect of the presence or absence of the main factors. We showed that favorable growth conditions of each fungus will depend on the temperature and pH conditions and should be adjusted for each particular case.

Our data showed that the optimal enzyme production conditions have not been achieved yet, so further experiments are needed to reach these conditions.

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