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Full Paper

Evaluation of Fermentative Parameters for the Production of Botryosphaeran (a (1→3;1→6)-β-D-glucan) and Mycelial Biomass by *Botryosphaeria rhodina* MAMB-05

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Abstract: Botryosphaeran is an exopolysaccharide (EPS) of the (1→3;1→6)-β-D-glucan type produced by the fungus *Botryosphaeria rhodina* MAMB-05. In attempts to enhance its extracellular production, the quantitative effects of different nitrogen sources, fermentation time, inoculum size, C/N ratio, and initial pH were evaluated on the yields of botryosphaeran. Ammonium nitrate was selected as the best nitrogen source in examining these effects using a 2^3-factorial central-composite experimental design, and analysis by the response surface method. According to a factorial design, the most important variables influencing both botryosphaeran and mycelium biomass production was inoculum concentration and the time of growth. Under these conditions, optimum botryosphaeran production occurred at 88 h of growth, 0.88 g of mycelium L^{-1} of nutrient mediumat C/N ratio of 30, and resulted in a 5.2 g L^{-1} yield of EPS in the fermentation broth. For production of the mycelium biomass, the optimum condition occurred at the same time of growth and inoculum concentration; however the best C/N ratio was 75, and resulted in a yield of 33.8 g L^{-1} of biomass. The results also indicated that the maximal mycelial growth is not associated to the yields of botryosphaeran production by *Botryosphaeria rhodina* MAMB-05.

Keywords: exopolysaccharide; mycelium biomass; shake flask optimization; response surface method

1. INTRODUCTION

Botryosphaeran, a water-soluble exopolysaccharide (EPS) produced by the ascomyceteous fungus, *Botryosphaeria rhodina* MAMB-05, consists of a linear backbone chain comprising (1→3)-β-D-glucopyranosyl groups to which are attached to the branched chains of (1→6)-linked β-glucosyl and diglucosyl (gentiobiose) residues [1]. Botryosphaeran is responsible for the increased viscosity of the culture fluid during fermentation [2], and can present more or less ramification points according to the carbohydrate substrate used as a carbon source during fungal growth [3].

Some related fungal (1→3;1→6)-β-D-glucans belong to the group of biological response modifiers (BRM's) that exhibit immune-stimulatory activities by macrophage activation through interaction with specific cell-surface receptors [4]. The (1→3)-linked β-D-glucans therefore have potential applications as anti-tumor, anti-inflammatory, and anti-oxidant activities, as anti-sense carriers, and more recently, have been employed in the formation of nano-structures in microelectronic devices [5].

Botryosphaeran was described as having strong anticlastogenic [6], hypoglycemic, and hypocholesterolemic [7] activities, and in its sulfonylated form it presented anticoagulant activity [8]. Recently, botryosphaeran was also described as possessing strong antioxidant properties [9]. The literature evidence from the production, characterization, and biological activities of botryosphaeran over the last 15 years are illustrated in

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Figure 1.

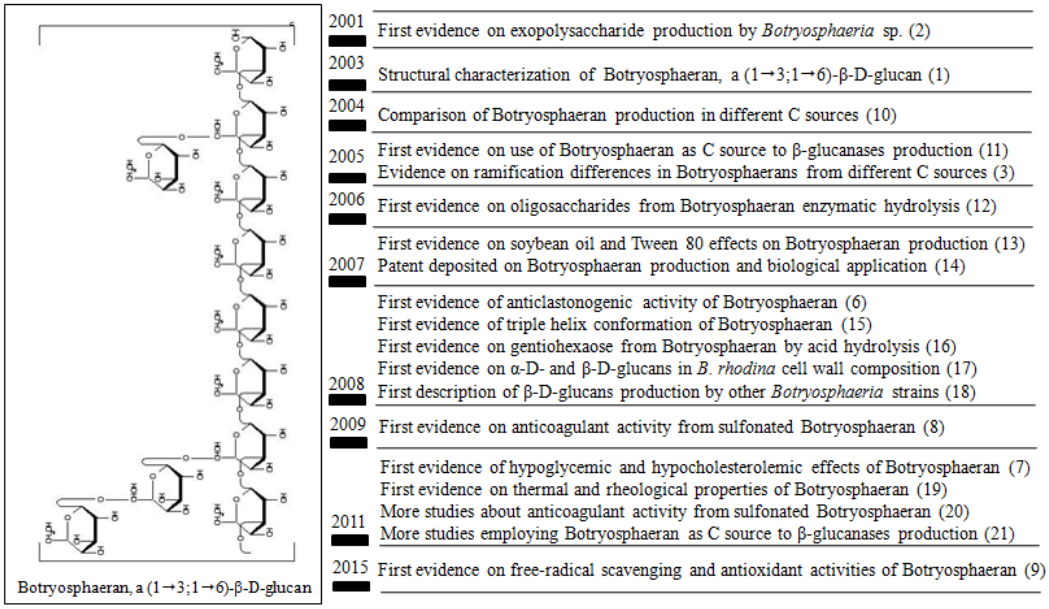


Figure 1. Chronological studies on production, chemical characterization, and biological properties of botryosphaeran reported in the last 15 years.

Several factors such as aeration rate, agitation speed, temperature, and addition of metabolic precursors (amino acids, sugar nucleotides), have been described as influencing EPS production [22-24]. Some biochemical responses can be critical and limit the production of β-D-glucan through catabolic repression, or the presence of glucan-hydrolytic enzymes [21, 25]. Some factors that influence EPS production have strong relations to fungal growth and mycelium production [26]. In the case of *B. rhodina* MAMB-05, large amounts of biomass are produced during EPS production and fermentation and the fungal mycelium so produced can be utilized as a rich source of β-D-glucans [10, 17], *e.g.*, as a carbon source for the production of β-glucanases [11].

Developments of economically feasible bioprocesses are necessary to present possibilities for the applications of these β-D-glucans, and optimization of fermentation conditions is a decisive factor. Statistical design experiments associated with response surface methodology (RSM) have been applied to optimize the composition of medium, and other parameters, to enhance the production of EPS, for example, gellan gum [26], pullulan [27], bacterial polymers [28], and botryosphaeran through membrane-modifying agents [13].

The improvement of botryosphaeran production is an essential key to the continuation of our research on potential applications of this (1→3; 1→6)-β-D-glucan, and for future development of fermentation processes based up on a β-D-glucan-rich residual biomass generated. The objective of the present paper was to evaluate the influence of different nitrogen sources on the production of botryosphaeran by *B. rhodina* MAMB-05, as well as to determine the best conditions to promote maximal production of this EPS and fungal biomass using statistical factorial design and analysis by RSM

2. MATERIAL AND METHODS

Microorganism and cultivation

B. rhodina isolate MAMB-05 [29] was maintained at 4 °C on potato dextrose agar. Inoculum was prepared by growing the fungus on agar plates containing Vogel minimal salts medium (VMSM) [30], agar (20 g L^{-1}) and glucose (10 g L^{-1}) for 120 h at 28 °C. Mycelium was transferred to baffled 125 mL Erlenmeyer flasks containing 25 mL of VMSM and glucose (5 g L^{-1}), and grown for 48 h at 28 °C under shaking conditions (180 rpm). Pre-cultures were homogenized in a sterilized chilled Blender for 0.5 min at maximum speed. The cellular homogenate was then centrifuged, the mycelium recovered and sterilized physiological saline was added to give a standard solution with an optical density between 0.4 to 0.5 (400 nm) [1, 10]. The standardized inoculum (1 mL aliquots containing 2 mg mycelium dry weight) was used to inoculate 125 mL Erlenmeyer flasks containing 25 mL of VMSM and glucose (50 g L^{-1}).

Production and determination of Botryosphaeran

B. rhodina MAMB-05 was grown in submerged liquid culture in 125 mL Erlenmeyer flasks containing VMSM modified with different nitrogen sources (inorganic: NH_4NO_3, NH_4Cl, $NaNO_3$, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, $(NH_4)H_2PO_4$; and organic: L-proline, L-asparagine, L-glutamic acid, and urea) to a final C/N ratio of 30 in the nutrient medium, and glucose (50 g L^{-1}) as the sole carbon source along with the inorganic nitrogen sources. Cultures were incubated at 28 °C on a rotary shaker at 180 rpm over a period of 72 h. Botryosphaeran was measured after first dialyzing the cell-free culture fluid against several changes of distilled water for 48 h, followed by the addition of 4 volumes of absolute ethanol and recovering the precipitate on pre-weighed filter papers, and drying to constant weight at 70 °C. All experiments were carried out in triplicate and the results represent the means ± SD.

Factorial analysis

Response surface methodology (RSM) was applied in two stages using a 2^3-factorial central-composite experimental design to evaluate some initial fermentation parameters. In the first factorial design, the cultures were developed in VMSM modified with the best nitrogen source (previously chosen as NH_4NO_3) supplemented with different concentrations of glucose: 12.5, 42.2, 86.0, 123.7 or 159.5 g L^{-1}, which conferred on the culture medium the respective C/N ratios: 8, 25, 50, 75, and 92.

The variables were coded according to Equation 1:

$$x_i = \frac{(X_i - \overline{X}_i)}{\Delta X_i} \qquad (1)$$

where: x_i = coded independent variable; X_i = real value of an independent variable; $\overline{X}_i$ = mean of an independent variable X_i; ΔX_i = increment of x_i corresponding to 1 unit of X_i.

The levels of the independent variables (x_1 = time of growth, h, x_2 = inoculum, g mycelium dry weight L^{-1} of nutrient medium and x_3 = C/N ratio), and respective levels of variation for experimental studies of botryosphaeran (Y_1 = g L^{-1}) and biomass production (Y_2 = g L^{-1}) are shown in Table 1.

Table 1. Box–Behnken 2^3-factorial central-composite design matrix defining conditions for botryosphaeran production by *Botryosphaeria rhodina* MAMB-05 together with the experimental and predicted values of the yield of botryosphaeran and mycelial biomass.

Experimental run	Variables in coded levels			EPS (g L^{-1})	Biomass (g L^{-1})
	X_1	X_2	X_3		
1	-1	-1	-1	0.21	3.67
2	-1	-1	1	0.09	2.04
3	-1	1	-1	3.19	16.28
4	-1	1	1	2.83	15.53
5	1	-1	-1	0.25	5.88
6	1	-1	1	0.35	8.86
7	1	1	-1	5.19	30.95
8	1	1	1	3.79	33.85
9	-1.68	0	0	0.94	3.14
10	1.68	0	0	1.73	16.91
11	0	-1.68	0	0.01	1.39
12	0	1.68	0	6.33	23.63
13	0	0	-1.68	1.54	14.28
14	0	0	1.68	0.99	13.41
15[a]	0	0	0	1.32	11.78
16[a]	0	0	0	1.19	12.54
17[a]	0	0	0	1.48	13.64
18[a]	0	0	0	0.95	11.32

Factors	Real levels				
	-1.68	-1	0	+1	+1.68
X_1, time of growth (h)	8	24	48	72	88
X_2, inoculum[b]	0.08	0.24	0.48	0.72	0.88
X_3, C/N ratio	8	25	50	75	92

[a] central point, [b] (g mycelium dry weight/L of nutrient medium).

A second-order polynomial equation utilized was the Equation 2:

$$\hat{Y}_i = \beta_0 + \beta_i x_1 + \beta_i x_2 + \beta_i x_3 + \beta_{ii} x_1^{\,2} + \beta_{ii} x_2^{\,2} + \beta_{ii} x_3^{\,2} + \beta_{ij} x_1 x_2 + \beta_{ij} x_1 x_3 + \beta_{ij} x_2 x_3 \quad (2)$$

where: $\hat{Y}_i$ = estimated response; β_0 = intercept term; β_i = linear coefficient; β_{ii} = quadratic coefficient; and β_{ij} = interaction effect.

Fermentation runs were conducted at an initial H of 5.80. Analysis of variance (ANOVA) and others

Analytical techniques

Reducing sugars were determined by the cupro-arsenate method [31, 32], and total sugars by the phenol-sulfuric acid method [33]. Fungal biomass was determined gravimetrically by drying to constant weight at 70 °C.

3. RESULTS AND DISCUSSION

The influence of the nitrogen source on the production of EPS has been observed in a diverse range of microorganisms such as the basidiomycetes *Sclerotium rolfsii* [34] and *Trametes versicolor* [35], and this was also demonstrated in the ascomycete *B. rhodina* MAMB-05. Figure 2 shows that highest botryosphaeran yields were obtained using the inorganic nitrogen source, NH_4NO_3 (4.39 g botryosphaeran L^{-1}), followed by the organic nitrogen sources, L-glutamic acid (3.43 g L^{-1}) and L-proline (3.16 g L^{-1}). The best inorganic nitrogen source, NH_4NO_3 (already a component of VMSM), had previously been used for botryosphaeran production by *B. rhodina* MAMB-05 [1, 10, 13]. Inorganic nitrogen sources appear to inhibit the biomass production and stimulate the EPS secretion [23, 35].

statistical tests were performed using STATISTICA Version 9 www.statsoft.com (StatSoft Inc., 2009).

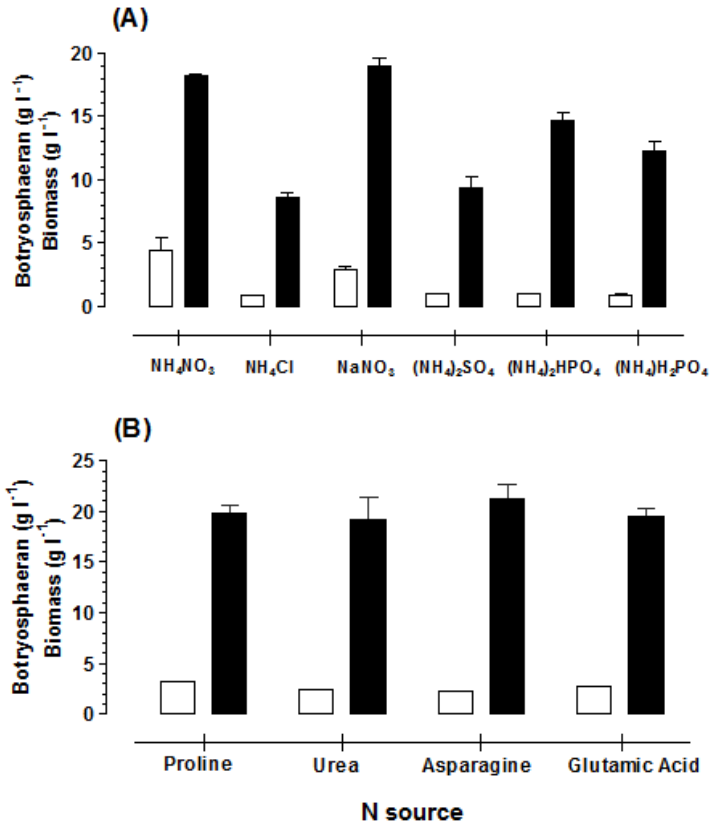


Figure 2. Production of botryosphaeran (□) and fungal biomass (■) by *Botryosphaeria rhodina* MAMB-05 grown in the presence of different nitrogen sources: (A) inorganic and (B) organic.

The amounts of fungal biomass and botryosphaeran produced do not appear to be directly related; however, the best nitrogen sources for botryosphaeran production were also those that promoted the highest mycelial growth. A similar observation was reported for *Paecilomyces japonica* [36], and *Lentinus edodes* [37]. Addition of NH_4Cl and $(NH_4)_2SO_4$ resulted in a 50-fold reduction in biomass production compared to NH_4NO_3. All of the organic nitrogen sources showed the same proportion of yields of EPS on fungal biomass ($Y_{EPS/Biomass}$=0.13), however, favored fungal growth over their inorganic nitrogen counterparts, and this observation has also been described for other microorganisms [38]. In the case of *Paecilomyces tenuipes*, the phosphorus source (KH_2PO_4) appeared be more relevant for EPS production and mycelium growth than the nitrogen source (KNO_3) [39].

The C/N ratio is an important factor in the biosynthesis of fungal metabolites. As NH_4NO_3 was chosen as the optimal nitrogen source for botryosphaeran production, the best C/N ratio was determined using this as the nitrogen source and glucose as the sole carbon source. RSM was used to determine a better C/N ratio, combined with inoculum concentration and time of growth in order to optimize the production of botryosphaeran by *B. rhodina* MAMB-05. Through multiple regression analyses of the experimental data, a second-order polynomial equation was obtained for botryosphaeran production (Equation 3), where the squared-effect terms of the variables x_1 and x_3, respectively for time of growth and C/N ratio, were discarded as being insignificant.

$$\hat{Y}_1 = 1.2824 + 0.3358x_1 + 1.8106x_2 + 0.6802x_2^{\,2} - 0.1966x_3 + 0.3328x_1x_2 - 0.2176x_2x_3 \quad (3)$$

An intercept was significant indicating that the central point had been chosen correctly. The most important variables for EPS production were inoculum concentration, followed by time of growth, and also the interaction among these variables according the *p*-values indicated in Table 2. The C/N ratio values did not appear to affect botryosphaeran production like inoculum concentration and time of growth as had also been observed for the basidiomycete, *Ganoderma applanatum* [38]. Analysis of variance (ANOVA) indicated that the regression was significant (p<0.05), and that the lack-of-fit was not significant (Table 2). The R-squared value implied that 99 % of the variability of the observed response values could be explained by the model. The pure error was low, indicating good reproducibility of the experimental data.

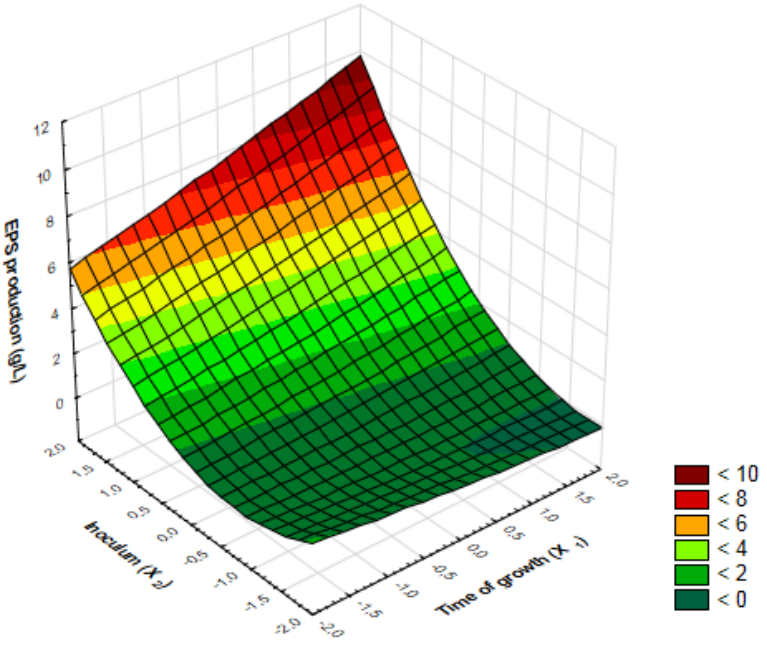
Table 2. Analysis of variance (ANOVA) for the quadratic polynomial model fitted for the optimization of the yield of botryosphaeran by *Botryosphaeria rhodina* MAMB-05 showing the effects for variables from the first 2^3-factorial central-composite design.

Source of variation	Sum of squares	Degrees of freedom	Mean square	*F*-test	p
Time of growth, X_1 (L)	1.541	1	1.54137	30.1126	0.011905
Time of growth, X_1 (Q)	0.019	1	0.01851	0.3617	0.589977
Inoculum, X_2 (L)	44.765	1	44.76565	874.5526	0.000085
Inoculum, X_2 (Q)	6.021	1	6.02105	117.6288	0.001677
C/N ratio, X_3 (L)	0.528	1	0.52811	10.3173	0.048879
C/N ratio, X_3 (Q)	0.003	1	0.00337	0.0658	0.814082
X_1X_2	0.886	1	0.88589	17.3070	0.025261
X_1X_3	0.084	1	0.08449	1.6507	0.289078
X_2X_3	0.377	1	0.37722	7.3694	0.072876
Lack of fit	0.355	5	0.07107	1.3885	0.418526
Pure Error	0.153	3	0.05119		
Total	55.023	17			

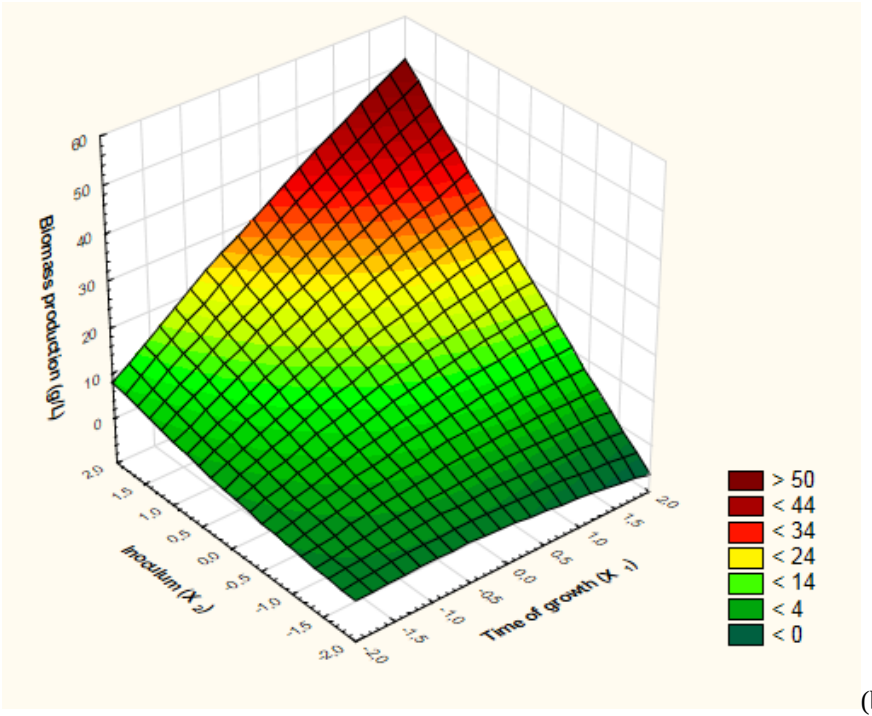
L: linear effect Q: quadratic effect. % of explained variance = 99.075. % of maximum explained variance = 98.035.

As indicated in Figure 3a, maximal EPS production (5.2 g L^{-1}) by *B. rhodina* MAMB-05 was obtained using inoculum 0.88 g myceliumL^{-1} of nutrient medium during 88 h of growth at a C/N ratio of 30.The best C/N ratio for EPS production depends upon the microorganism. In the case of *Paecilomyces*

sinclairii, EPS production did not significantly increase over C/N ratios of 12 using corn steep powder as the nitrogen source [40]. While seen in *Antrodia cinnamomea*, a C/N ratio of 40 had a beneficial effect on EPS production as well as on mycelial growth [41].



(a)



(b)

Figure 3. Response-surface plot depicted as a 3-dimensional surface for (a) botryosphaeran production (Y_1) by *B. rhodina* MAMB-05 as a function of the time of growth (X_1) and inoculum concentration (X_2) according to a first experimental design, and maintaining a C/N ratio of 30; (b) mycelium biomass production (Y_1) by *B. rhodina* MAMB-05 as a function of the time of growth (X_1) and inoculum concentration (X_2) according to a first experimental design, and maintaining a C/N ratio of 92.

Based upon the previous knowledge on the time-growth relationship on botryosphaeran formation [10], a short growing period was chosen (72 h), because when glucose was depleted in the nutrient

medium, EPS production often started to decline. The reason for this was most likely due to a combination of glucose limitation and de-repression of synthesis of glycohydrolases [21]; the latter attacking EPS, which acts as a carbon source especially during the stationary phase. Thus, longer periods of growth can unfavorably reduce the amounts of botryosphaeran formed during fermentation.

A second-order polynomial equation was obtained also for mycelium production by *B. rhodina* MAMB-05 (Equation 4) through multiple regression analysis of the experimental data, where the squared-effect terms of all variables were discarded as not being significant, as well as the linear term from variable x_3. An intercept was significant indicating that the central point was chosen correctly.

$$\hat{Y}_2 = 12.1939 + 4.775x_1 + 8.3198x_2 + 2.995x_1x_2 \quad (4)$$

The most important variables for mycelial biomass production by *B. rhodina* MAMB-05 were the same obtained for EPS production: time of growth followed by the interaction among this variable and initial pH (Table 3, Figure 3). The regression was significant (p<0.05) and the lack-of-fit was not significant according to the analysis of variance. Highest mycelial biomass production (33.85 g L^{-1}) occurred at 72 h growth, using 0.72g mycelium L^{-1} as inoculum, and at a C/N ratio of 75. For other microorganisms, an excess of carbon source can limit the cellular growth, favoring the biosynthesis of exopolysaccharide.

Table 3. Analysis of variance (ANOVA) for the fitted quadratic polynomial model for optimization of the yield of mycelium biomass by *Botryosphaeria rhodina* MAMB-05 showing the effects for variables from the second 2^3-factorial central-composite design.

Source of variation	Sum of squares	Degrees of freedom	Mean square	*F*-test	p
Time of growth, X_1 (L)	311.107	1	311.1069	43.4830	0.000170
Time of growth, X_1 (Q)	0.890	1	0.8898	0.1244	0.733462
Inoculum, X_2 (L)	944.500	1	944.5002	132.0114	0.000003
Inoculum, X_2 (Q)	4.768	1	4.7677	0.6664	0.437949
C/N ratio, X_3 (L)	0.305	1	0.3045	0.0426	0.841706
C/N ratio, X_3 (Q)	14.925	1	14.9250	2.0860	0.186649
X_1X_2	71.760	1	71.7602	10.0298	0.013255
X_1X_3	8.528	1	8.5285	1.1920	0.306711
X_2X_3	0.080	1	0.0800	0.0112	0.918390
Lack of fit					
Pure Error					
Total					

L: linear effect Q: quadratic effect. % of explained variance = 95.954. % of maximum explained variance = 91.402.

4. CONCLUSION

In conclusion, the validation of fermentation parameters in statistically designed experiments resulted in an improvement of botryosphaeran production by *B. rhodina* MAMB-05 over un-optimized conditions. Even at lower C/N ratios, *B. rhodina* MAMB-05 can produce EPS and biomass simultaneously, which is economically interesting to improve the value of the EPS production process due to the potential of using a cheaper raw material (fungal mycelium).

5. ACKNOWLEDMENTS

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