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Original

Application of Doehlert experimental design in the optimization of experimental variables for the *Pseudozyma* sp. (CCMB 306) and *Pseudozyma* sp. (CCMB 300) cell lysis

Aplicação do modelo Doehlert na otimização das variáveis experimentais para a lise de Pseudozyma sp. (CCMB 306) e Pseudozyma sp. (CCMB 300)

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Abstract

This study aimed to verify the influence of pH and temperature on the lysis of yeast using experimental design. In this study, the enzymatic extract containing β-1,3-glucanase and chitinase, obtained from the micro-organism *Moniliophthora perniciosa,* was used. The experiment showed that the best conditions for lysis of *Pseudozyma* sp. (CCMB 306) and *Pseudozyma* sp. (CCMB 300) by lytic enzyme were pH 4.9 at 37 °C and pH 3.9 at 26.7 °C, respectively. The lytic enzyme may be used for obtaining various biotechnology products from yeast.
Keywords: *lytic enzymes; multivariate optimization; doehlert matrix design.*

Resumo

O presente trabalho visou verificar a influência do pH e temperatura na lise de leveduras utilizando planejamento experimental. No estudo, foi utilizado o extrato enzimático, contendo β-1,3-glucanase e quitinase líticas, obtidas do micro-organismo *Moniliophthora perniciosa*. O delineamento experimental mostrou que as melhores condições para a lise de *Pseudozyma* sp. (CCMB 306) e *Pseudozyma* sp. (CCMB 300), pelas enzimas líticas, foram pH 4,9 a 37 °C, pH 3,9 a 26,7 °C, respectivamente. As enzimas líticas podem ser utilizadas para a obtenção de vários produtos biotecnológicos a partir de leveduras.
Palavras-chave: *enzimas líticas; otimização multivariada; matriz doehlert.*

1 Introduction

The cell wall of yeast is basically made of glucan and mannan-protein. Lytic enzymes such as chitinases, proteases and β-1,3-glucanases are usually necessary. There are numerous applications for biotechnologies: preparation of protoplasts, extraction of pigments, mass treatment of residual yeast cell fermentation industries for preparation of animal feed, in studies of the mechanism of cell wall synthesis for control of pathogenic yeast, etc. (HUNTER; ASENJO, 1988; FLEURI; SATO, 2005; SALAZAR; ASENJO, 2007).

These enzymes are capable of lysing the cell wall of *Saccharomyces cereviseae*, *Candida* sp. and other genera of yeasts, which extends their use allowing the selective manufacture of products, regardless of scale, and can be performed under temperature and pH conditions that do not involve the denaturation of cellular products of interest (FLEURI; SATO, 2005). Mathematical models have been increasingly used to help explain responses of biochemical reactions. This technique is often used for optimization and/or verification of the influence of medium components and cultivation parameters for enzyme production (FLEURI; SATO, 2008).

The production and optimization of the parameters that affect the enzymatic synthesis should always be investigated because the optimal conditions vary among different organisms and different enzymes (BRAVO et al., 2000). In many cases, the interaction of parameters that influence fermentation processes can be evaluated with a reduced number of tests using an experimental design (THÉODORE; PANDA, 1995). Several experimental design models could be used to reduce the number of experiments under different conditions. Among them are the Plackett-Burman, Doehlert, Central Composite, and Box-Behnken (LI et al., 2007).

The choice of Doehlert design is justified by a number of advantages such as (1) its spherical experimental domain with an uniformity in space filling, (2) its ability to explore the whole of the domain, and (3) its potential for sequentially where the experiments can be reused when the boundaries have not been well chosen at first (BENSALAH et al., 2010). Doehlert designs are easily applied to optimized variables and offer advantages over the Central Composite (CCD) and Box–Behnken designs, used in response surface analysis. They need fewer experiments,

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which are more efficient and can move through the experimental domain (KIRAN et al., 2010). Other advantage of the Doehlert design over the CCD is the possibility of adding another factor or displacing the design towards a new experimental domain (SAUTOUR et al., 2001). The Plackett-Burman statistical experimental design is very useful in screening the most important factors, however, it does not consider the interaction effects between the variables (KIRAN et al., 2010).

Basically, this optimization process involves three major steps: performing the statistically designed experiments, estimating the coefficients in a mathematical model, and predicting the response and checking the adequacy of the model. Several researchers have applied these techniques to optimize different process parameters (VANOT et al., 2002; DUTRA; MALTEZ; CARASEK, 2006; IMANDI et al., 2007).

The aim of this study was to investigate the effects of pH and temperature on lysis of *Pseudozyma* sp. (CCMB 300 and 306) cells (dependent variable or response). The crude enzyme extract containing lytic enzymes was obtained after submerged fermentation by the fungus *Moniliophthora perniciosa*. The methodologies of the experimental design and response surface analysis were used to check the influence of the parameters (independent variables). In order to reduce the number of experiments, a Doehlert design was used.

2 Materials and methods

2.1 Microorganisms and maintenance

Samples of Moniliophthora perniciosa and Pseudozyma sp.

Samples of *M. perniciosa* (CCMB 0257) and *Pseudozyma* sp. (CCMB 300 and 306) were obtained from the Culture Collection of Microorganisms of Bahia (CCMB), Feira de Santana, Brazil.

Moniliophthora perniciosa and Pseudozyma sp. culture medium

Moniliophthora perniciosa was grown on potato dextrose agar for 10 days at 28 °C. Yeast cells were grown in YM medium composed of yeast extract 3 $g.L^{-1}$, malt extract 3 $g.L^{-1}$, peptone 5 $g.L^{-1}$, glucose 10 $g.L^{-1}$, and agar 20 $g.L^{-1}$ dissolved in distilled water, pH 6.8, at 28 °C in an incubator (IGO 150 Cell Life – Jouan).

2.2 Chemicals

Laminarin and chitin were obtained from Sigma (Sigma Chemical Co., St. Louis, MO, USA). All the other chemicals used were of high-quality analytical grade.

2.3 Inoculation and fermentation

The medium used for the fermentation of *Moniliophthora perniciosa* was composed of ($g.L^{-1}$): wheat bran (40 g), magnesium sulfate (0.2 g), potassium chloride (0.2 g), phosphate potassium monohydrate (1.0 g), and 1000 mL of distilled water. Yeast extract (6 $g.L^{-1}$) was added as inducer in this culture medium. The pH was adjusted to 6.8.

Next, discs of fungal mycelium, 1 cm in diameter, were taken from the solid culture and transferred into 250 mL erlenmeyer flasks containing 100 mL of liquid culture (base medium). Incubation was carried out at 28 °C for 14 days, as described in Sena et al. (2011). The liquid culture medium, on which *M. perniciosa* was grown, was filtered and centrifuged (Centrifuge 5804R – Eppendorf, São Paulo, Brazil) at 8.000 g for 15 minutes at 4 °C, and the supernatant containing the secretes proteins from *M. perniciosa* was used as a crude enzymatic extract (HUA et al., 2007).

2.4 Preparation of suspension of Pseudozyma sp. (CCMB 300 and 306)

In order to obtain yeast cells, a loop of yeast grown on plates of YM medium was transferred into 250 mL erlenmeyer flasks containing 50 mL of liquid YM medium. The vials were incubated in a Shaker (TECNAL TE-424) for 36 hours at 28 °C under agitation (98 rpm). The cells were collected by centrifugation at 10.000 g for 10 minutes at 4 °C and then resuspended in the citrate-phosphate buffer at pH 0.1 M according to the experimental design to obtain a suspension with optical density (OD) of 0.60 at 660 nm. The suspensions of yeast cells were used to test the enzymatic lysis of the cell wall.

2.5 Doehlert experimental design – yeast cells lysis

A Doehlert matrix (DOEHLERT, 1970) was applied to find the best conditions for the lysis of yeast. The number of experiments required (N) is given by $N = n^2 + n + n_0$, where n is the number of variables and n_0 is the number of center points. In our case, the n_0 value was fixed at 3; thus, with two factors (pH and temperature), the total of points of Doehlert matrix was 9. Replicates at the central level of the variables are performed in order to validate the model by means of an estimate of experimental variance. The pH was studied at five levels (3, 4, 5, 6, and 7) and temperature at three levels (20, 40, and 60 °C). Aliquots of 0.71 $U.mL^{-1}$ of β-1,3-glucanase and 1.25 $U.mL^{-1}$ chitinase were used. For statistical calculation, the experimental variables Xi have been coded as xi according to the following transformation Equation 1:

$$x_i = ((X_i - X_{0i}) / \Delta X_i) * \alpha_i \quad (1)$$

where x_i is the coded value of the ith variable, X_i the natural value, X_{oi} the value at the center point, ΔX_i the step change value, and α_i is the maximum value of the coded variable (i.e. 1.0 and 0.866 for five levels and three levels, respectively) (KANARAM; MEDICHERLA, 2010).

The original values and the corresponding coded values (Table 1) are used for setting up the experiments and the model, respectively.

The experimental data were processed using the software STATISTICA 7.0 (STATSOFT, 2005). All experiments at this stage were conducted in a random order.

Table 1. Original values and the corresponding coded values for the application in the lysis *Pseudozyma* sp. (CCMB 300 and 306).

Independent variables		Coded and natural values						
		–1	–0.866	–0.5	0	0.5	0.866	1
pH	X_1	3		4	5	6		7
Temperature (°C)	X_2		20		40		60	

2.6 Enzyme activity

Determination of β-1,3-glucanase activity

For the determination of enzyme activity, the laminarin was dissolved in citrate buffer (0.05 mol.L^{-1}, pH 6.2). The reaction medium was composed of 100 µL of 0.1% laminarin and 100 µL of enzyme solution. The samples were incubated for 15 minutes at 50 °C, and the reaction was stopped by adding 200 µL of 3.5-Dinitrosalicylic acid (DNS) 1% and heating to 98 °C for 15 minutes. The reducing sugars released in the reaction were determined spectrophotometrically (Spectrophotometer - Cary Varian, São Paulo, Brazil) at 540 nm by the Miller method (MILLER, 1959) using glucose solution as standard. One unit (AU) of β-1,3-glucanase activity was defined as the amount of enzyme capable of releasing reducing sugars equivalent to 1 µmol of glucose per minute under the conditions mentioned above. All tests for the activity assay were performed in triplicate.

Determination of chitinase activity

This step was performed using an adaptation of the method described by Nawani and Kapadnis (2005). Chitinase was determined by quantification of reducing sugars released during the reaction (MILLER, 1959). An aliquot of 250 µL of enzyme extract was added to 250 µL of 1% solution of chitin in sodium phosphate buffer 0.05 M, pH 7.0, and incubated for 30 minutes at 50 °C. Next, 500 µL of DNS were added, and it was incubated at 95 °C for 15 minutes. After cooling down to room temperature, 5 mL of distilled water were added, and absorbance was read at the wavelength of 540 nm.

One unit of chitinase activity was defined as the amount of enzyme capable of releasing reducing sugars equivalent to 1 µmol of glucose per minute under the conditions mentioned above. All tests for the activity assay were performed in triplicate.

2.7 Determination of lytic activity

The lytic activity was determined according to method described by Ventom and Asenjo (1991), modified. The enzyme extract (0.71 U.mL^{-1} β-1,3-glucanase and 1.25 U.mL^{-1} chitinase), 1 mL, and the suspension (1 mL) were kept for 1 hours under stirring at temperatures that followed the experimental design. The suspension was prepared with citrate-phosphate buffer 0.1 M. One lytic unit is defined as a 0.1 decrease in absorbance under test conditions (OBATA; IWATA; NAMBA, 1977).

3 Results and discussion

Purification of glucanase and chitinase from *M. perniciosa* was performed according to previous studies by the authors (SENA et al., 2011, GALANTE et al., 2012). On the other hand, in this study, an experimental design was used to evaluate the influence of independent variables on lysis of *Pseudozyma* sp. (CCMB 300 and CCMB 306) cells. Enzyme activity depends mainly on pH and temperature, and a novel approach was used to control the combined effect of pH and temperature on β-1,3-glucanase and chitinase using Response Surface Methodology (RANA et al., 2003; NAWANI; KAPADNIS, 2005).

The nine experiments performed under different conditions provide important information related to lytic activity and the importance knowledge, as well as the establishment of the optimum condition for enzymatic lysis. Multivariate techniques are important because they are able to study several variables and their interactions. They allow the simultaneous optimization of variables and therefore are more economical and effective.

The effects of two factors were modeled, and the lytic activity obtained is listed in Table 2 (see *Pseudozyma* sp. CCMB 306). Table 2 indicates that the highest lytic activity was obtained at the midpoint.

The effect of pH and temperature is not strictly linear since the equation contains both a positive individual effect (first order term) and a negative quadratic coefficient. The effect of independent variables, pH, and the quadratic effects were positive (19.85) and negative (–1.95), respectively. The variable temperature showed its positive linear term (2.58) and negative quadratic term (–0.033). Therefore, an increase in the pH and temperature in the medium determines an increase in lytic activity; however, the negative quadratic terms indicate that there is a critical pH and temperature, thus reducing the lysis. The interaction of the variables caused a positive effect (0.021) on the lysis of the yeast enzyme extract. The positive values for effect of the interaction suggested a synergistic influence of both variables on the lytic activity. The temperature and pH (quadratic terms) were found to be significant ($p \leq 0.05$), and the temperature had a great effect. The remaining terms were found to be insignificant ($p \leq 0.05$).

Multiple regression analysis based on the least square method was performed using software STATISTICA 7.0 (StatSoft, USA). Therefore, according to the Equation 2, the lytic activity (CCMB 306) was a second-order polynomial model with 6 coefficients. Equation 2 illustrates the relationship between these two variables (original values) and the response (lytic activity).

$$\begin{aligned} \textit{Lytic activity} &= -26.15(\pm 7.8549) + 1.295 * X_1 (\pm 0.1775) - \\ &-0.01675 * X_1^2 (\pm 0.0016) + 9.975 * X_2 (2.3796) - 0.975 * \\ &* X_2^2 (\pm 0.2165) - 0.01125 X_1 * X_2 (\pm 0.0237) \end{aligned} \qquad (2)$$

The results of the second order response surface model generated by fitting in the form of Analysis of Variance (ANOVA) are shown in Table 3.

Table 2. Experimental design in the study of the lysis of the *Pseudozyma* sp. (CCMB 306 and CCMB 300) using β-1,3-glucanase and chitinase from *M. perniciosa*.

Run order	Temperature (°C)	pH	Lytic activity on CCMB 306 (U.mL^{-1})	Lytic activity on CCMB 300 (U.mL^{-1})
1	60	4	13.5	13.6
2	60	6	11.3	3.9
3	40	3	18.0	15.9
4	40	5	23.09	14.0
5	40	5	21.2	14.0
6	40	5	22.0	13.7
7	40	7	18.4	2.9
8	20	4	17.1	16.7
9	20	6	15.8	12.5

Table 3. Analysis of variance for the data presented in Table 2 (*Pseudozyma* sp. CCMB 306).

Source of variation	SS	df	MS	Fcal	Ftab
Regression	118.475	5	23.695	16.39	9.01
Residual	4.3351	3	1.4450		
Lack-of-Fit	2.5350	1	2.5350	2.82	18.51
Pure Error	1.8001	2	0.9000		
Total SS	122.8101	8			

SS – sum of squares; df – degree of freedom; MS – mean square; Fcal – calculated F value; Ftab – tabulated F value.

A test based on the Fisher distribution (F-test) indicated that the fitted equation is statistically significant (F = 16.39 > 9.01) and has a very low probability value ($P_{model} > F = 0.00009$). A lack-of-fit sum of squares (F = 2.82 < 18.51) indicates that there is good agreement between the predicted model response and the experimental values studied for each variable.

The goodness of fit of the model was measured by the coefficient of determination (R^2). The R^2 value was found to be 0.9647 indicating that 96.47 % of the total variation in the residual activity was explained by the fitted model. In addition, the value of the adjusted coefficient of determination (Adj $R^2 = 0.91$) is also very high to advocate for a high significance of the model.

For quadratic models, the optimal point can be characterized as maximum, minimum, or saddle. It is possible to calculate the coordinates of the optimal point through the first derivate of the mathematical function, which describes the response surface and equates it to zero (KANARAM; MEDICHERLA, 2010).

From the regression equation, the optimized values are calculated by partially differentiating the above equation with respect to X_1 and X_2 and equating to zero. The final optimum lytic activity was 4.9 at 37.0 °C for pH and temperature, respectively, yielding a unit cell lytic average of 22.3 U.mL^{-1}(Figure 1).

The analysis of lysis of *Pseudozyma* sp. (CCMB 300) cells is also shown in Table 2. The highest lytic activity was obtained in test 8 (pH 4 at 20 °C). The same results were obtained in the analysis of the effects. The pH linear and quadratic effects were positive (1.82) and negative (–0.41), respectively. The variable temperature showed its positive linear term (1.94) and quadratic negative (–0.019). The interaction of the variables caused a positive effect on the lysis of the yeast enzyme extract. However, all variables had a significant effect on lytic activity.

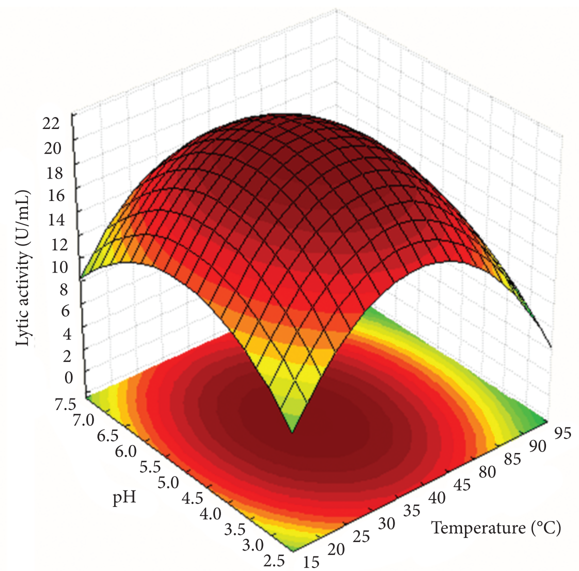

Figure 1. Response surface and contour plot of pH vs. temperature on lysis of *Pseudozyma* sp. (CCMB 306).

From the validation of the studied parameters, the quadratic model that represents the behavior of the lytic activity of β-1,3-glucanase on yeast *Pseudozyma* sp. (CCMB 300) was obtained, and it describes the response surface and contour lines (Equation 3).

$$\begin{aligned} Lytic\ activity &= -9.899(\pm 1.4341) + 0.4175 * X_1 (\pm 0.0324) - \\ &-0.00275 * X_1^2 (\pm 0.0003) + 10.675 * X_2 (\pm 0.4345) - 1{,}125 * \\ &* X_2^2 (\pm 0.0395) - 0.06875 X_1 * X_2 (0.0043) \end{aligned} \quad (3)$$

The validity of the model was determined by ANOVA. The coefficient of determination obtained was 0.99, which indicates a good agreement between the experimental and predicted values. The value of the F-test indicated that the second order model was statistically significant (612.78 > 9.01).

The predicted optimum values of pH and temperature obtained by differentiating Equation 3 were 3.92 at 26.86 °C, respectively (Figure 2).

Comparing the results obtained with both yeasts, it was found that the temperature was the most significant variable. However, only the quadratic term for CCMB 306 was significant.

Smaller amount were found in the literature, mainly because of the importance in biological control and acquisition of biotechnological products, but this application of β-1,3-glucanase from *M. perniciosa* in another organism was showed for the first time.

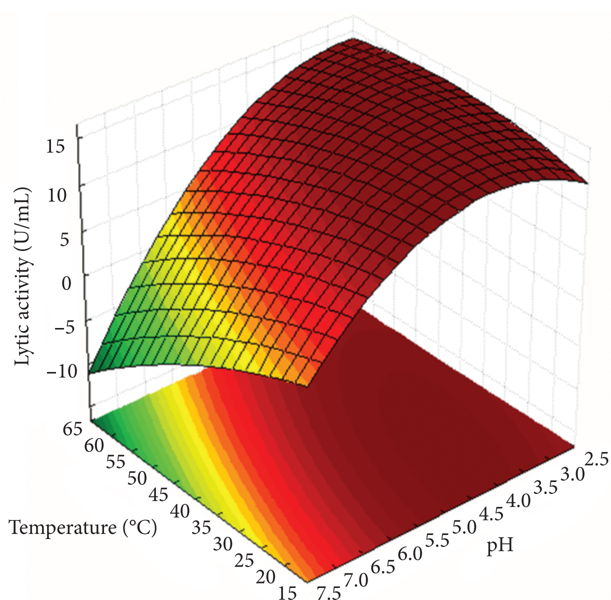

Figure 2. Response surface and contour plot of pH vs. temperature on lysis of *Pseudozyma* sp. (CCMB 300).

Yeast *Oerskovia xanthineolytica* was used against *Saccharomyces cerevisiae* X2180-1B under pH and temperature of 7.5 at 30 °C, respectively. The organism was only able to lyse the wall in the presence of alkaline protease (SCOTT; SCHEKMAN, 1980).

Fleuri and Sato (2008) produced β-1,3-glucanase from *Cellulomonas cellulans* 191 and used it in the analysis of *Saccharomyces cerevisiae* KL-88 and the extraction of intracellular enzymes. In the experimental design, these authors used temperature and pH of lysis as independent variables and used time and agitation to grow the yeasts. The highest lytic activity (7.08 $U.mL^{-1}$) was found at pH 6.5 and 35 °C. The pH and temperature (linear) had positive and negative effects, respectively. Comparing the present results with those above, the lytic activity was three times higher, and a few variables were analyzed. Fleuri and Sato (2010) studied the application of lytic β-1,3-glucanase (0.1 $U.mL^{-1}$) obtained from *Cellulosimicrobium cellulans* strain 191 in lysis of yeast cell walls. The crude extract demonstrated lysis activity against several yeasts.

The results of the present study showed that lytic enzymes from *M. perniciosa* acted differently in the two strains of lysis *Pseudozyma* sp., proved more effective against *Pseudozyma* sp. (CCMB 306), probably due to differences in cell wall composition of the yeasts

4 Conclusion

The use of Response Surface Methodology allowed studying the simultaneous effect of pH and temperature on enzymatic lysis. From the experiment, it was observed that the yeasts were sensitive to crude enzyme preparation containing lytic enzymes. The maximum lytic activity of β-1,3-glucanase and quitinase from *M. perniciosa* on *Pseudozyma* sp. (CCMB 306) cells was predicted to be 22.3 $U.mL^{-1}$, when the optimized reaction conditions were set as follows: pH 4.9 at 37.0 °C. The predicted optimum values in lysis *Pseudozyma* sp. (CCMB 300) cells was 16.7 $U.mL^{-1}$ at pH 3.9 at 26.7 °C.

It can be concluded that the lytic enzymes may be used to obtain various biotechnological products, among which are the pigments found in the yeasts studied.

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