L-asparaginase production by *Zymomonas mobilis* during molasses fermentation: optimization of culture conditions using factorial design

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ABSTRACT. L-asparaginase is an enzyme with anti-leukemia activity produced by microorganisms especially gram-negative bacteria. *Zymomonas mobilis* is a gram-negative bacteria that presents potential for L-asparaginase production. This study sought to optimize L-asparaginase production by *Zymomonas mobilis*, during sugarcane molasses fermentation, using an incomplete 3^3 factorial design. A model obtained by the response surface methodology was optimized by Otplex software. Good fit of the experimental data to the model was obtained, and the total determination coefficient (R^2) was 95%. Maximal enzyme activity (16.55 IU L^{-1}) was detected under the following conditions, of molasses concentration 100.0 g L^{-1} of total reducing sugars, yeast extract concentration 2.0 g L^{-1} and fermentation time 21 hours. The experimental validation confirmed the predictive capacity of the model, and the difference between the estimated response ($\hat{Y}_1$) and the observed response (Y_1) was only 1%.

Key words: L-asparaginase, *Zymomonas mobilis,* factorial design, sugarcane molasses.

RESUMO. Produção de L-asparaginase por *Zymomonas mobilis* durante a fermentação do melaço: otimização das condições de cultivo utilizando delineamento fatorial. A L-asparaginase é uma enzima com atividade anti-leucêmica produzida por microrganismos, principalmente bactérias gram-negativas. *Zymomonas mobilis* é uma bactéria gram-negativa, que apresenta potencial para produção de L-asparaginase. Esse estudo buscou a otimização da produção de L-asparaginase por *Zymomonas mobilis,* durante a fermentação do melaço de cana-de-açúcar, utilizando um delineamento fatorial incompleto 3^3. O modelo obtido através da metodologia de superfície de resposta foi otimizado pelo software Otplex. Obteve-se bom ajuste do modelo aos dados experimentais, sendo o coeficiente de determinação total (R^2), 95%. A máxima atividade enzimática (16,55 UI L^{-1}) foi obtida com uma concentração de açúcares redutores totais no melaço de 100,0 g L^{-1}, 2,0 g L^{-1} de extrato de levedura e com 21 horas de fermentação. A validação experimental confirmou a capacidade preditiva do modelo, sendo a diferença entre resposta estimada($\hat{Y}_1$) e resposta observada (Y_1) apenas de 1%.

Palavras-chave: L-asparaginase, *Zymomonas mobilis,* delineamento fatorial, melaço de cana-de-açúcar.

Introduction

L-asparaginase is a type of intracellular enzyme produced by microorganisms that can be used as an effective anti-leukemia agent (Zhao and Yu, 2001). The enzyme restricts the availability of L-asparaginase to the leukemia cells inducing efficacious and selective inhibition of the protein synthesis (Maladkar *et al.*, 1993). This activity has encouraged the search for new sources of L-asparaginase that present potential for therapeutic use (Borkotaky and Bezbaruah, 2002). L-asparaginase activity is frequently found in gram-negative bacteria (Bascomb *et al.*, 1975). *Zymomonas mobilis* is a gram-negative bacteria that uses glucose, fructose and sucrose as carbon sources (Swings and De Ley, 1977) and presents high potential for industrial ethanol (Doelle, 1993), sorbitol and levan production (Viikari and Gisler, 1986). Its potential to produce L-asparaginase has been reported recently (Abud *et al.*, 2003). Response surface methodology (RSM) is currently the most popular set of techniques for optimization, because of its complete

theory, efficiency and simplicity (Box and Draper, 1987). The Box-Behnken factorial design is a model used for optimization procedures. The design consists of replicated center point to measure the experimental variability, and the set of points lying at the midpoints of each edge of the multidimensional cube that defines the region of interest (Box and Behnken, 1960). This design is satisfactory to explore quadratic response surfaces and to construct second order polynomial models, thus simplifying the optimization process because of the few experiments needed (Karnachi and Khan, 1996). The objective of this study was to present a factorial plan to optimize L-asparaginase production by *Z. mobilis* during sugarcane molasses fermentation.

Materials and methods

Microorganism

Zymomonas mobilis CP4 was used, and kept at 4°C on plates containing sucrose (20.0 g L^{-1}), yeast extract (2.5 g L^{-1}), $(NH_4)_2SO_4$ (1.0 g L^{-1}), KH_2PO_4 (1.0 g L^{-1}), $MgSO_4.7H_2O$ (0.5 g L^{-1}) and agar (20.0 g L^{-1}) and replicated every 30 days.

Culture medium

Molasses (concentration in g L^{-1} of total reducing sugars) (Table 1); yeast extract (g L^{-1}) (Table 1); KH_2PO_4 1.0 (g L^{-1}) and $MgSO_4.7H_2O$ 0.5 (g L^{-1}).

Fermentation process

The experiments were carried out according to the principles of the response surface statistical methodology (3^3) incomplete factorial design with two replications at the central point) Table 1. The fermentation in batch used 125 mL Erlenmeyer flasks with 25 mL culture medium without agitation, at 30°C. A 10% inoculum (v/v) was used at the 2.0 g L^{-1} cell concentration.

Analytical methods

The total reducing sugars were quantified according to Dubois (Dubois *et al.*, 1956). The L-asparaginase activity was determined according to Peterson and Ciegler (1969) considering one L-asparaginase unit (IU) as the quantity of enzyme necessary to release one μmol ammonia per minute at 37°C in 8.5 pH (0.1 m borate buffer), and the L-asparaginase activity was presented in IU L^{-1}.

Experimental design

Optimization of L-asparaginase production by *Z. mobilis* was attempted by Box- Behnken experimental design (3^3 incomplete factorial design with two replications at the central point) (Box and Behnken 1960). Three factors selected were used in designing the experiment: sugarcane molasses concentration (X_1), yeast extract concentration (X_2) and fermentation time (X_3). The ranges of the variables investigated in this study are given in Table 1. For creating response surface, the experimental data obtained based on the above design was fitted to second order polynomial equation of the form:

$$\hat{Y} = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_4X_1^2 + A_5X_2^2 + A_6X_3^2 + A_7X_1X_2 + A_8X_1X_3 + A_9X_2X_3$$

Where $\hat{Y}$ represents the expected L-asparaginase activity; X_1 sugarcane molasses concentration in g L^{-1} of TRS; X_2 yeast extract concentration in g L^{-1}; and X_3 fermentation time in hours; A_0 is a intercept term; A_1, A_2, A_3 are the linear terms; A_4, A_5, A_6 are the quadratic terms; A_5, A_8, A_9 are the interaction terms. The regression analyses, statistical significances and response surfaces were obtained using STATISTICA (data analysis software system), version 5.0. The optimal levels of the three components as obtained from the maximum point of the polynomial model were estimated using the Otplex software (Bona *et al.*, 2002), that uses the constrained Simplex method with incorporation of the Derringer and Suich functions (Derringer and Suich, 1980).

Table 1. Box-Behnken factorial experimental design.

Trials	Molasses Concentration[c]		Yeast extract Concentration[c]		Fermentation Time in hours		L-asparaginase activity[b]
1	-1	(10.0)[a]	-1	(2.0)[a]	0	(15)[a]	2.86
2	1	(100.0)	-1	(2.0)	0	(15)	13.67
3	-1	(10.0)	1	(20.0)	0	(15)	12.74
4	1	(100.0)	1	(20.0)	0	(15)	10.52
5	-1	(10.0)	0	(11.0)	-1	(6)	1.98
6	1	(100.0)	0	(11.0)	-1	(6)	4.11
7	-1	(10.0)	0	(11.0)	1	(24)	6.73
8	1	(100.0)	0	(11.0)	1	(24)	12.74
9	0	(55.0)	-1	(2.0)	-1	(6)	1.72
10	0	(55.0)	1	(20.0)	-1	(6)	1.50
11	0	(55.0)	-1	(2.0)	1	(24)	13.46
12	0	(55.0)	1	(20.0)	1	(24)	13.46
13	0	(55.0)	0	(11.0)	0	(15)	12.21
14	0	(55.0)	0	(11.0)	0	(15)	12.40
15	0	(55.0)	0	(11.0)	0	(15)	12.31

[a]Natural units of variables are presented between brackets; [b]L-asparaginase activity in UI L^{-1}; [c]Concentration in g L^{-1}.

Results and discussion

The fitted equation, using an incomplete 3^3 factorial design, is represented by:

$$\hat{Y}_1 = 12.3067 + 2.0913X_1 + 0.8138X_2 + 4.6350X_3 - 1.7521X_1^2 - 0.6071X_2^2 - 4.1646X_3^2 - 3.2575X_1X_2 + 0.9700X_1X_3 + 0.0550X_2X_3$$

$\hat{Y}_1$ represents the expected L-asparaginase activity;

X_1 sugarcane molasses concentration in g L^{-1} of total reducing sugars; X_2 yeast extract concentration in g L^{-1} and X_3 fermentation time in hours.

The value of the coefficient of total determination (R^2), observed by the $\hat{Y}_1$ response (L-asparaginase activity in IU L^{-1}), was 95%, that suggests a good fit of the model to the experimental data. The analysis of variance of the quadratic model indicated that the intercept, the linear term of the sugarcane molasses concentration, the linear and quadratic term and the interaction between the molasses concentration and the fermentation time were the factors that most influenced ($p<0.05$) the L-asparaginase activity. However, the linear and quadratic terms of the yeast extract concentration were not significant ($p>0.05$), indicating that the concentration of yeast extract established for the lower limit of the design of 2.0 g L^{-1} was sufficient for the process. The interactions between X_1X_3 and X_2X_3 were also not significant that does not invalidate the model for predictive purposes.

The optimization, using the complex method, as shown in Table 2, presented as optimum molasses concentration (X_1) 100.0 g L^{-1} of total reducing sugars, yeast extract concentration (X_2) 2.0 g L^{-1} and fermentation time (X_3) 21 hours, with expected response of $\hat{Y}_1$ =16.37 IU L^{-1}.

Table 2. Values of the original independent variables and the expected and observed responses in the optimization of the culture conditions.

	Independent variables			Expected	Observed
Optimization	X_1	X_2	X_3	$\hat{Y}_1$ [a]	Y_1 [a]
L-asparaginase	100.0 g L^{-1}	2.0 g L^{-1}	21h	16.37	16.55

Conclusion

The validation of the model in the conditions studied confirmed its great predictive ability, and the difference between the estimated response ($\hat{Y}_1$) and the observed response (Y_1) was only 1%. Thus the equation obtained can be used in the prediction of the culture conditions in sugarcane molasses fermentation by *Z. mobilis* for L-asparaginase production. The application of the Box-Behnken factorial design in L-asparaginase production by *Z. mobilis* presented progress in the prediction of culture conditions. Optimization using the predictive equation derived from the surface response was efficient for the optimization of the selected factors obtaining the desired response, adding data that can, in the future, testify the possible use, for therapeutic purposes, of L-asparaginase from *Z. mobilis*.

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