0273-2289/01/94/0257/$12.00

Optimization of Inulinase Production by *Kluyveromyces marxianus* Using Factorial Design

Susana J. Kalil,[1] Rodrigo Suzan,[2] Francisco Maugeri,[2] and Maria I. Rodrigues*,[2]

[1]Department of Chemistry, Fundação Universidade do Rio Grande, 96201-900, Rio Grande, RS, Brazil; and [2]Department of Food Engineering, UNICAMP, CP 6121, 13083-970, Campinas, SP, Brazil, E-mail: bel@ceres.fea.unicamp.br

Received July 1, 2000; Revised February 1, 2001; Accepted March 1, 2001

Abstract

Factorial design and response surface techniques were used to optimize the culture medium for the production of inulinase by *Kluyveromyces marxianus*. Sucrose was used as the carbon source instead of inulin. Initially, a fractional factorial design (2^{5-1}) was used in order to determine the most relevant variables for enzyme production. Five parameters were studied (sucrose, peptone, yeast extract, pH, and K_2HPO_4), and all were shown to be significant. Sucrose concentration and pH had negative effects on inulinase production, whereas peptone, yeast extract, and K_2HPO_4 had positive ones. The pH was shown to be the most significant variable and should be preferentially maintained at 3.5. According to the results from the first factorial design, sucrose, peptone, and yeast extract concentrations were selected to be utilized in a full factorial design. The optimum conditions for a higher enzymatic activity were then determined: 14 g/L of sucrose, 10 g/L of yeast extract, 20 g/L of peptone, 1 g/L of K_2HPO_4. The enzymatic activity in the culture conditions was 127 U/mL, about six times higher than before the optimization.

Index Entries: Inulinase; *Kluyveromyces marxianus*; optimization; factorial design and response surface analysis.

Introduction

Inulin is the storage carbohydrate in the roots and tubers of plants such as Jerusalem artichoke, chicory, and dahlia. It represents good sources

*Author to whom all correspondence and reprint requests should be addressed.

of high fructose, calorie-reduced sweeteners and consists of linear β-2,1-linked polyfructose chains displaying a terminal glucose unit *(1)*. Fructose formation from complete hydrolysis of inulin is a single-step inulinase reaction and yields up to 95% of fructose. By contrast, conventional fructose production from starch needs at least three enzymatic steps, yielding only 45% of fructose solution *(2)*. Inulinases are encountered in plants and microorganisms. However, it is difficult to isolate plant inulinases in sufficient quantity. Therefore, microbial inulinases, which can be induced by growing microorganisms, have a potential for industrial use in the production of fructose from inulin *(3,4)*. Inulinase is produced by many microorganisms as *Aspergillus niger (5)*, *Penicillium* sp. *(6)*, *Kluyveromyces marxianus (7–10)*, and *Pseudomonas* sp. *(11)*. Comparison of the inulinase-yield demonstrated that some *Kluyveromyces* strains produce a high amount of inulinase *(3,7,8)*. The high enzyme levels in yeast batch culture have been obtained with media containing oligofructosides such as inulin. However, the industrial application of inulinase will be viable only when this enzyme is available in large quantities at a competitive price *(7)*.

Most works related to inulinase production have been carried out by means of one-factor-at-a-time technique. This single-dimensional search is laborious and time-consuming, especially for a large number of variables. Poorna and Kulkarni *(5)* used a more practical method, the fractional factorial Plackett-Burman design method, to study the effects of process parameters affecting inulinase production from *A. niger*.

The factorial design makes it possible to study many factors simultaneously as well as to quantify the effect of each of them and to investigate their possible interactions *(12)*. In the present work, the inulinase production by *K. marxianus* in a shake flask was optimized using two experimental design and response surface analysis.

Parameters such as pH, sucrose concentration, peptone, yeast extract, and K_2HPO_4 were previously evaluated in a fractional design (2^{5-1}) with very good resolution. In this kind of factorial design, the main effects are only confounded with interactions of fourth order, which are considered negligible *(12)*. The fractional factorial design was followed by a full factorial design, 2^3 plus axial points, to establish the optimal conditions for inulinase production.

Materials and Methods

Fermentation

K. marxianus ATCC 16045 was employed for inulinase production. The microorganism was grown on MY broth. The inoculum cultures were grown on a medium containing 2% sucrose at pH 6.5. Inulinase was produced in 500-mL agitated flasks with 100 mL of culture medium at 30°C and 150 rpm for 48 h. The fermentations were started with 10% (v/v) inoculum.

Table 1
Values of Coded Levels Used
in Fractional (Design 1) and Full (Design 2) Factorial Designs

	Coded variable level	Sucrose (g/L)	Yeast extract (g/L)	Peptone (g/L)	K_2HPO_4 (g/L)	pH
Design 1	–1	10	4	8	1	3.5
	0	20	7	14	3	4.5
	+1	30	10	20	5	5.5
Design 2	–1.68	10	4	8	—	—
	–1	14.1	5.2	10.4	—	—
	0	20	7	14	—	—
	+1	26	8.8	17.6	—	—
	+1.68	30	10	20	—	—

Experimental Design

In this work, the effects of sucrose, yeast extract, peptone, and K_2HPO_4 concentrations and pH on inulinase production were studied using a fractional design of 2^{5-1} trials plus 3 central points, which means a total of 19 trials.

A full factorial design (2^3 plus star configuration) with three replicates at the center point, which means a total of 17 trials, was utilized for the three selected variables from the fractional design, having inulinase activity as the response. Table 1 gives the values of the coded levels used in both the fractional and full factorial designs.

Inulinase Assay

Activity was assayed as follows. One milliliter of enzyme solution was mixed with 9 mL of 2% (w/v) sucrose or inulin on 0.1 *M* acetate buffer, pH 4.5. The mixture was maintained at 50°C, and the rate of appearance of fructose was determined by the dinitrosalicylic acid method *(13)*. One unit of inulinase activity is defined as the amount of enzyme that hydrolyzes 1 µmol of sucrose/min (sucrose as substrate) or the amount of enzyme catalyzing the liberation of 1 µmol of reducing sugar/min (inulin as substrate) under the aforementioned conditions. Inulinases have different hydrolytic activities on sucrose compared with the hydrolytic activities on inulin. This behavior is represented by the sucrose:inulin activities (S:I) ratio. Values of S:I lower than 50 is a characteristic behavior of an inulinase, and higher than 50 is representative of an invertase *(14)*. The inulinase from *K. marxianus* ATCC 16045, used in this work, has an S:I ratio of about 13.8, which means that this enzyme is an inulinase. The optimization work was carried out by measuring only the sucrose hydrolytic activity, since the S:I ratio does not change significantly according to the composition of the medium.

Results and Discussion

Fractional Factorial Design

Enzymatic activities following the fractional factorial design were measured at 24 and 48 h of fermentation; however, only data at 48 h were considered because at this time all trials showed higher activities.

Table 2
Effect Estimates for Inulinase Activity[a] for the Fractional Factorial Design

Factor	Effect (U/mL)	Standard error	$t(2)$[c]	p	Conf.[b] limit (–95%)	Conf.[b] limit (+95%)
Mean	20.70	0.13	159.96	0.00004	20.14	21.26
Sucrose (g/L)	–10.12	0.28	–35.88	0.0008	–11.33	–8.91
Yeast extract (g/L)	11.73	0.28	41.59	0.0006	10.52	12.94
Peptone (g/L)	13.62	0.28	48.29	0.0004	12.41	14.83
pH	–23.50	0.28	–83.31	0.0001	–24.71	–22.28
K_2HPO_4 (g/L)	2.82	0.28	9.99	0.01	1.61	4.03

[a]All numerical values are significant factors (95% confidence level).
[b]Conf., confidence.
[c]Degrees of freedom according to central points.

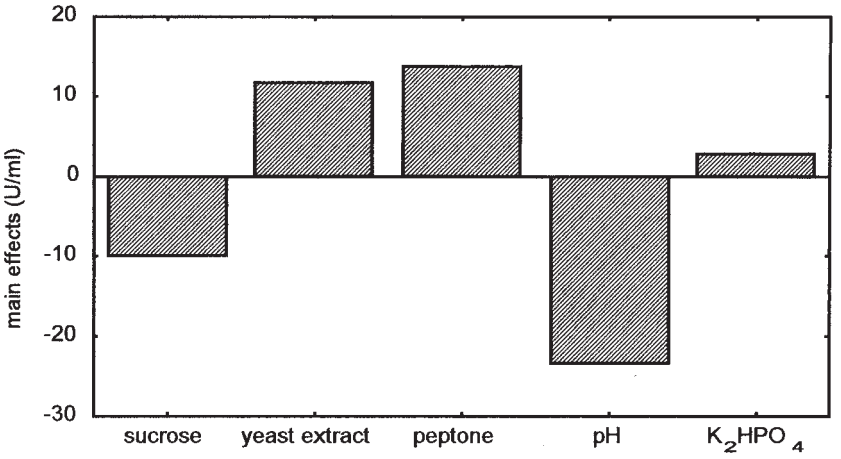

Fig. 1. Effect estimates for sucrose, yeast extract, peptone, and pH in the fractional factorial design.

Activities varied according to the fermentation conditions, from 3.88 to 61 U/mL. The best results were achieved in trials 7 and 8, with pH 3.5, 10 g/L of yeast extract and 20 g/L of peptone for both trials, and 10 g/L (trial 7) and 30 g/L of sucrose (trial 8), and 5 g/L (trial 7) and 1 g/L of K_2HPO_4 (trial 8). The activities after 48 h of fermentation were 61 and 59.5 U/mL, for trials 7 and 8, respectively.

As can be seen in Table 2, the effect of K_2HPO_4 on the enzymatic activity is weak although statistically significant. The results also showed that inulinase production was more significantly affected (significance at the 0.05 level) by sucrose, yeast extract, peptone, and pH. An increase in sucrose concentration and pH (level –1 to +1) led to a decrease in activity, while an increase in the yeast extract, peptone, and K_2HPO_4 concentrations led to higher inulinase production. The pH was the more significant effect, as can be seen in Fig. 1, which shows that it should be maintained at a low value.

Table 3
ANOVA for Inulinase Activity[a] for Full Factorial Design

Source of variation	Sum of squares	Degrees of freedom	Mean squares	F test[b]
Regression	2737.2	5	547.4	30.0
Residual	200.6	11	18.2	
Lack of fit	196.4	9		
Pure error	4.2	2		
Total	2937.8	16		

[a]Regression coefficient: $R = 0.965$.
[b]$F_{0.95;5;11} = 3.2$.

Because of the weak enzymatic stability at pHs <3.5 *(15)*, lower pH range was not studied.

Full Factorial Design

Sucrose, yeast extract, and peptone were selected and studied using the full factorial design. Concentrations for these variables are given in Table 1 under Design 2. The pH was fixed at 3.5, for the aforementioned reasons, and K_2HPO_4 concentration was fixed at 1 g/L since it has a low effect on enzymatic activity.

In this second design, enzyme production varied from 5.0 up to 75.2 U/mL. The best value for the activity occurred in trial with concentrations of sucrose, yeast extract, and peptone of 14.1, 8.8, and 17.6 g/L, respectively. In this trial the activity reached 75.2 U/mL after 48 h of fermentation.

Table 3 shows the analysis of variance (ANOVA) for activity. The correlation coefficient (0.965 for inulinase activity) and F test were very good (9.4 times higher than the listed value). Consequently, models were found to be adequate to describe the response surface of inulinase production (Eq. 1) *(12)*. An overview of Eq. 1 indicates that the activity is a first-order function for sucrose, yeast extract, and peptone concentrations, and a second-order function for sucrose and for interaction between sucrose and yeast extract:

$$\text{Activity} = 52.94 - 2.75 \cdot \text{Sucrose} - 4.49 \cdot \text{Sucrose}^2 + 4.34 \cdot \text{Yeast extract} + 11.8 \cdot \text{Peptone} - 5.19 \cdot \text{Sucrose} \cdot \text{Yeast extract} \quad (1)$$

The model for inulinase activity was used to construct the response surfaces, which can be seen in Fig. 2. Figure 2A,B indicates that when the sucrose concentration is low the result is more sensitive to changes in the yeast extract concentration. If the peptone concentration is low (level –1.68), the activity is about 17 U/mL for all the range of sucrose concentration (Fig. 2C,D). Figure 2E,F shows the effect of peptone and yeast extract on activity. The inulinase production is more sensitive to changes in peptone than yeast extract concentration. When the culture medium has 20 g/L of peptone and 10 g/L of yeast extract, inulinase production can be more than

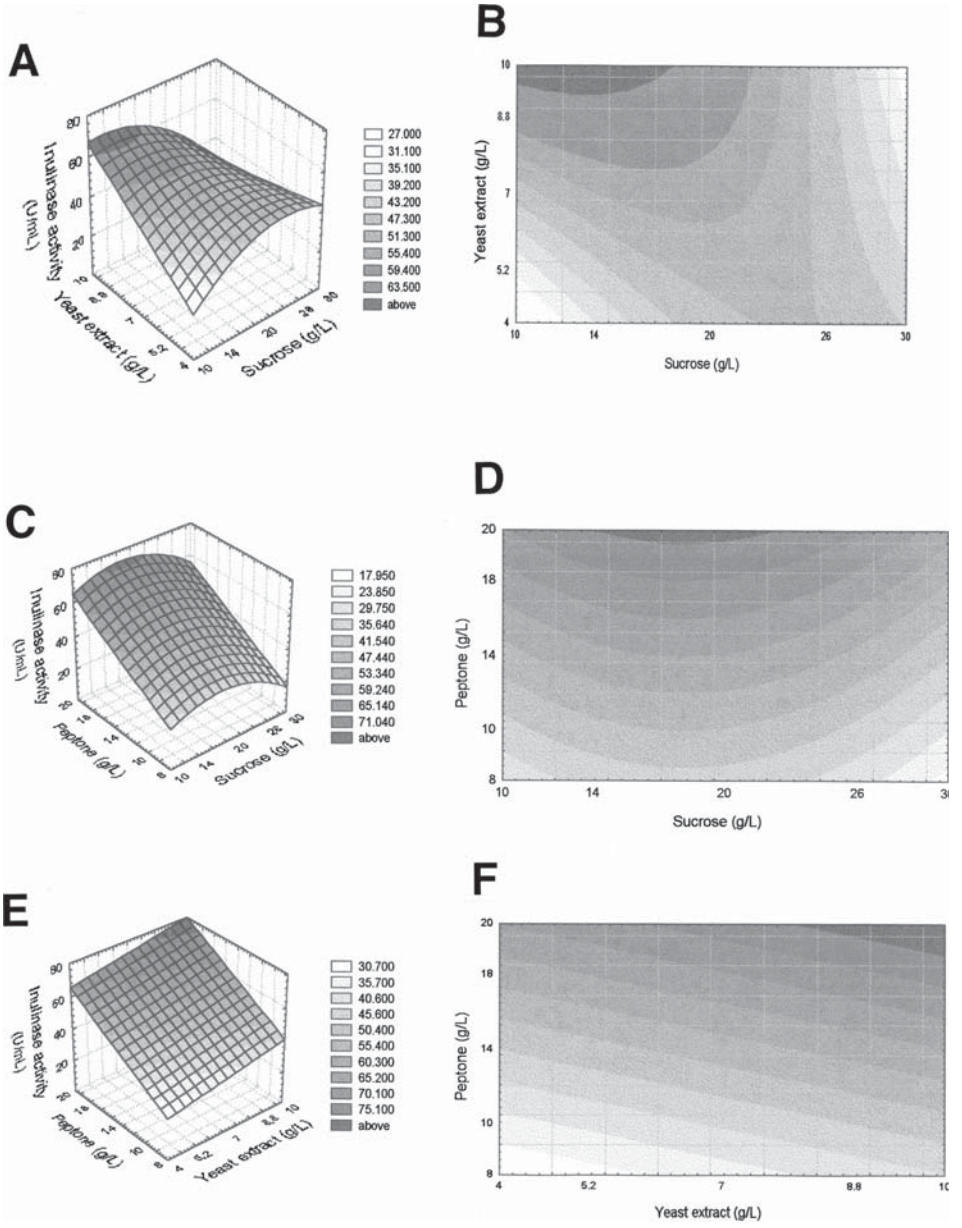

Fig. 2. Response surface and contour diagrams of inulinase activity as a function of **(A,B)** sucrose and yeast extract; **(C,D)** sucrose and peptone; and **(E,F)** yeast extract and peptone.

75 U/mL. The surfaces indicated that the optimum for the culture medium is at high peptone and yeast extract concentrations and low sucrose concentration.

On the basis of this study, the fermentation conditions were fixed at their optimal levels: 14 g/L of sucrose, 10 g/L of yeast extract, 20 g/L of peptone, 1 g/L of K_2HPO_4, and pH 3.5.

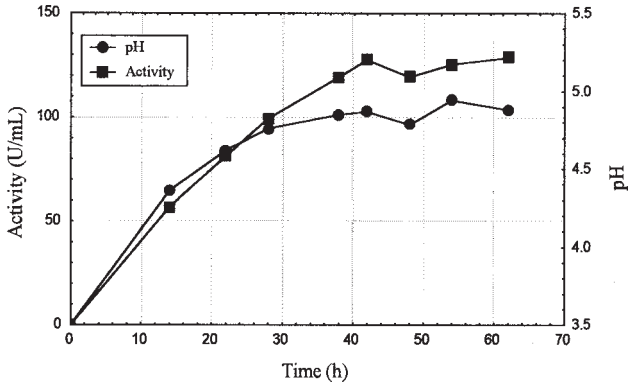

Fig. 3. Dynamics of *K. marxianus* inulinase fermentation in optimized medium.

Figure 3 shows the average results of a fermentation carried out in triplicate, using the fermentation conditions already described, at 30°C and 150 rpm. The activity and pH increased until 38 h and then became stable. The maximum activity was about 127 U/mL. Activity on sucrose and inulin of the enzyme from the optimized medium indicated an S:I ratio of 13.8. This ratio is comparable with that found by other researchers *(10)*, and the inulinase production is similar to that reported on a medium containing 1% fructan *(3)* and higher than that found by Selvakumar and Pandey *(9)* from *K. marxianus* and Guerrero *(8)* using *K. marxianus* CDBB-L-278, a hyperproducing strain.

Conclusion

The methodology of fractional factorial design was shown to be very useful for optimization purposes, mainly because many factors had to be considered. The pH was the most important parameter and was fixed at 3.5. The effect of the K_2HPO_4 concentration was low and maintained at 1 g/L (level –1). The effects of sucrose, yeast extract, and peptone concentrations were studied in a complete factorial design. An increase in the yeast extract and peptone concentrations was shown to be correlated with an increase in enzymatic activity. On the other hand, an increase in sucrose concentration led to a decrease in production of inulinase. The best fermentation condition for inulinase production was shown to be 14 g/L of sucrose, 10 g/L of yeast extract, and 20 g/L of peptone.

The main achievement of the present study is that using an experimental design, it was possible to optimize inulinase production by *K. marxianus* using sucrose as the carbon source instead of inulin or another kind of fructan. This is a quite interesting alternative since sucrose is highly soluble, cheap, and the major carbon source in molasses, which is an attractive feedstock for large-scale fermentation. On the other hand, inulin is only available in limited quantities and at high cost.

References

1. Vandamme, E. J. and Derycke, D. G. (1993), *Adv. Appl. Microbiol.* **29,** 139–176.
2. Zittan, L. (1981), *Starch* **33,** 373–377.
3. Gupta, A. K., Davinder, P. S., Kaur, N., and Singh, R. (1994), *J. Chem. Technol. Biotechnol.* **59,** 377–385.
4. Kaur, N., Kaur, M., Gupta, A. K., and Singh, R. (1992), *J. Chem. Technol. Biotechnol.* **53,** 279–284.
5. Poorna, V. and Kulkarni, P. R. (1995), *Bioresour. Technol.* **54,** 315–320.
6. Nakamura, T., Shitara, A., Matsuda, S., Matsuo, T., Suiko, M., and Ohta, K. (1997), *J. Ferment. Bioeng.* **84,** 313–318.
7. Parekh, S. and Margaritis, A. (1986), *Agric. Biol. Chem.* **50,** 1085–1087.
8. Guerrero, A. C., Pena, I. G., Barzana, E., Garibay, M. G., and Ruiz, L. G. (1995), *J. Ferment. Bioeng.* **80,** 159–163.
9. Selvakumar, P. and Pandey, A. (1999), *Bioresour. Technol.* **69,** 123–127.
10. Rouwenhorst, R. J., Hensing, M., Verbakel, J., Scheffers, W. A., and Van Dijken, J. P. (1990), *Appl. Environ. Microbiol.* **56,** 3337–3345.
11. Kim, D. H., Choi, Y. J., Song, S. K., and Yun, J. W. (1997), *Biotechnol. Lett.* **19,** 369–371.
12. Box, G. E. P., Hunter, W. G., and Hunter, J. S. (1978), *Statistics for Experimenters: An Introduction to Design, Data Analysis, and Model Building*, John Wiley & Sons, New York.
13. Miller, G. L. (1959), *Analyt. Chem.* **31,** 426–428.
14. Vandamme, E. J. and Derycke, D. G. (1983), *Adv. Appl. Microbiol.* **29,** 139–176.
15. Pessoa, A., Jr., Hartmann, R., Vitolo, M., and Hustedt, H. (1996), *J. Biotechnol.* **51,** 89–95.