Brazilian Journal of Microbiology (2007) 38:45-51
ISSN 1517-8283

APPLICATION OF FRACTIONAL FACTORIAL DESIGN TO LEVAN PRODUCTION BY *ZYMOMONAS MOBILIS*

I.R. Melo[1]; M.F. Pimentel[2]; C.E. Lopes[1]; G.M.T. Calazans[1]*

[1]Departamento de Antibióticos, Universidade Federal de Pernambuco-UFPE, Cidade Universitária, Recife, PE, Brasil;
[2]Departamento de Engenharia Química, Universidade Federal de Pernambuco-UFPE, Cidade Universitária, Recife, PE, Brasil

Submitted: January 12, 2006; Returned to authors for corrections: April 20, 2006; Approved: October 10, 2006

ABSTRACT

Levan is a non-toxic, biologically active, extra cellular polysaccharide composed solely by fructose units. Optimization of levan production by *Zymomonas mobilis* strain ZAG-12 employing a 2^{4-1} fractional factorial design was performed to analyze the influence of the temperature (20, 25 e 30ºC) agitation (50, 75 e 100 rpm), and the initial concentrations of both sucrose (150, 200 e 250 $g.L^{-1}$) and yeast extract (2.0, 3.5 e 5.0$g.L^{-1}$) on final levan concentration. Aerobic fermentation was performed batchwise in 500mL Pyrex flasks for 72 hours. Biomass, ethanol, levan and sucrose were determined at beginning and also at end of the fermentations. The experiments showed that the final levan concentration depended on initial sucrose concentration, temperature and agitation velocity and that the initial concentration of yeast extract did not influence levan production. However, when the production of ethanol and biomass were considered, it became evident that yeast extract was a significant variable. The best conditions for levan production occurred at 100 rpm agitation, 20ºC and 250$g.L^{-1}$ of initial sucrose resulting in 14.67$g.L^{-1}$ of levan.

Key words: factorial design, levan and *Zymomonas mobilis*

INTRODUCTION

Studies to optimize levan production are related to its applications in the food and pharmaceutical industries. This high molecular weight polysaccharide is formed by α-(2,6) linkage having α-(1,2) linkages on its branch points. It contains solely fructose units in its chemical structure (20). The production of this polymer was done under the influence of agitation; initial sucrose and yeast extract concentrations; temperature, pH and inoculum types. Different authors studied several of these parameters by the traditional univariate optimization method, which allows variation of only one factor at a time while all other variables are maintained constant (4,7,10,11,16,21). The main disadvantage of this strategy is that it fails to consider any possible interaction between the factors and, consequently, might yield misleading results. This research employed fractional design 2^{4-1} to investigate the influence of four parameters on laboratory scale levan production considering sucrose and yeast extract initial concentrations, temperature and agitation. Ethanol and biomass were also monitored.

MATERIAL AND METHODS

Microorganism

The *Zymomonas mobilis,* strain ZAG-12 (UFPEDA 241) was utilized. This strain belongs to the Culture Collection of the Antibiotics Department of the Federal University of Pernambuco - Brazil and was obtained through espheroplast fusion of the strains ZAP (UFPEDA 205) and Ag11 (UFPEDA 198).

Microorganism Culture

Maintenance Culture Medium

The *Zymomonas mobilis* strain was maintained on SSDL medium composed in $g.L^{-1}$ base by: glucose 20.0; yeast extract

*Corresponding Author. Mailing address: Dep. de Antibióticos - Univ. Federal de Pernambuco - Rua Prof. Artur de Sá s/n - Cidade Universitária. Cep: 50670-901. Recife, PE - Brasil. E-mail: calazans@ufpe.br

5.0 and pH adjusted to 6.5. (18). The *Zymomonas mobilis* was subcultured every two months and transferred to tubes containing SSDL medium. The cultures were incubated at 30ºC for 48 hours and stored at 4ºC.

Inoculum Preparation

Inoculum was prepared from activated culture using 250 mL Erlenmeyer flasks containing 150 mL of fermentation medium, maintained at 30ºC for 18 hours. After incubation, the inoculum was centrifuged for 20 minutes at 4000g. The cells were decanted, resuspended in sterile distilled water and adjusted to McFarland scale number 10 which corresponds to approximately 3.0×10^9 cells.

The medium utilized for both inoculum propagation and pre-fermentation (MPF) was composed in g. L^{-1}, by: sucrose 100.0; yeast extract 2.0; KH_2PO_4 2.0; $MgSO_4.7\ H_2O$ 0.5 and $(NH_4)_2SO_4$ 1.0.

Levan Production

The medium for levan production, called ML, had the same composition of pre-fermentation medium, differing only in some of the nutrients showed in Table 1. The aerobic fermentations were carried out batchwise in 500 mL Pyrex flasks containing 300 mL of fermentation medium, placed on orbital shakers (model MA 830) under controlled temperature during a period of 72 hours. The inoculum utilized was 10% (v/v) of the total fermented volume.

Fermentation Follow-up

Samples were collected at the beginning and the end of the fermentation process (a 72 hour period) to determine the following parameters:

Cellular Biomass

The biomass was determined in a spectrophotometer based on 660 nm a calibration curve (3).

The fermented broth was centrifuged at 4000g for 20 minutes. The sediment was suspended in distilled water and centrifuged again twise at the same conditions and the cell sediment was diluted in appropriate volumes for spectrophotometric determinations.

Purity Tests

Fresh microscopic analyses were performed, as well as inoculation on nutrient agar and simple broth medium to detect contaminants in the samples.

Sucrose Dosage

Sucrose determination was performed through dosage of total reducing sugars in each sample using the 3,5-dinitrosalicilic acid colorimetric method after acid hydrolysis (12).

For sucrose hydrolysis, 1 mL of the fermented broth was diluted to 1mL of chloride acid 1:2 and heated at 68-69ºC for 15 minutes. The samples were neutralized using a 40% sodium hydroxide concentration and diluted within the recommended sensitivity zone for the sugar determination method. The readings were taken using a spectrophotometer at 540 nm.

Table 1. Design matrix for the fractional 2^{4-1} factorial experiments, including the central point, used to study the influence of 4 factors on levan, ethanol and biomass production by *Zymomonas mobilis* (ZAG-12).

Label	Factors	Levels		
		-1	Central	+1
S	Sucrose initial concentration ($g.L^{-1}$)	150	200	250
T	Temperature (ºC)	20	25	30
E	Yeast extract initial concentration ($g.L^{-1}$)	2.0	3.5	5.0
A	Agitation (rpm)	50	75	100

Run	Factor levels			
	S	**T**	**E**	**A**
1	-1	-1	-1	-1
2	+1	-1	-1	+1
3	-1	+1	-1	+1
4	+1	+1	-1	-1
5	-1	-1	+1	+1
6	+1	-1	+1	-1
7	-1	+1	+1	-1
8	+1	+1	+1	+1
9	0	0	0	0

Determination of dry weight of levan measured in grams and expressed in $g.L^{-1}$

The produced levan was extracted from the fermented medium and its dry weight determined following the methodology proposed by (15). Absolute ethanol was added to 10 mL of fermented up to 70% concentration to precipitate levan. Later, this solution was centrifuged at 4000g for 20 minutes. The sediment was redissolved. Afterwards, this sediment was once more precipitated and centrifuged in the same conditions described previously. The precipitate was transferred to a weigh filter and dried in a heater at 110ºC, for 24 hours.

Ethanol Dosage

Ethanol concentration was determined by gas chromatography using the model CG-2000 chromatograph. A 1% ethanol solution was utilized as a standard the temperature of carborwax column 20M was 100ºC, vaporizer at 130ºC and the detector at 150ºC. The software Peak simpleII-PK-2 was used to analyze the results.

Experimental design

The design matrix is given in Table 1. We employed the 2^{4-1} design defining relation **I**=**STEA** (**I** is the identity column) because this choice of generator will result in a design of the highest possible resolution (IV). In this case, each main effect is analyzed with another three-factor interaction; that is **S**=**TEA**, **T**=**SEA**, **E**=**STA** and **A**=**STE**. Furthermore, every two-factor interaction is analyzed with another two-factor interaction (**ST**=**EA**, **SE**=**TA** and **AS**=**TE**). (13). All experiments were made in triplicate to obtain an estimate of experimental error, and another experiment was carried out in triplicate at the central point. In all 27 experiments were done. The ranges chosen for factors sucrose (**S**), yeast extract initial concentration (**E**), temperature (**T**) and agitation (**A**) were 150 g.L^{-1} - 250 g.L^{-1}, 2 g.L^{-1} - 5 g.L^{-1}, 20ºC - 30ºC and 50 rpm -100 rpm, respectively. Following the usual convention, the two extreme levels are denoted by minus one (lower level) and plus one (higher level). This leads to a convenient algorithm to analyze the experimental results.

The studied dependent variables were: produced levan (g.L^{-1}), produced ethanol (g.L^{-1}) and produced biomass (g.L^{-1}).

The calculations were made using MATLAB version 4.2 c.1 and STATISTICA version 5.1 softwares.

RESULTS AND DISCUSSION

Table 2 shows the average results of the production of biomass, levan and ethanol, using the experimental design from Table 1.

The estimates of the effects obtained from the 2^{4-1} design are shown in Table 3. All effects were calculated as differences between two averages, each average containing half of the experimental responses. Each of these responses is an average of triplicate values. The intermediate runs are ignored at this stage, and used only to investigate possible curvatures. To obtain the main effects one thus refers to the responses in Table 2, the signs of the corresponding columns in Table 1, performs the algebraic sum, and divides the result by four. To illustrate the calculations, the linear combination of observations associated with the effect **S** in levan production is l_S = (1/4) [-(4.70) + (14.67) - (1.71) + (2.80) - (8.40) + (7.15) - (1.50) + (4.20)= 3.12 g. L^{-1}] → **S** + **TEA**.

Table 2. Average values of levan, ethanol and biomass production in g. L^{-1} by *Zymomonas mobilis* (ZAG-12), according to the conditions defined in Table 1.

RUN RUN	LEVAN (g. L^{-1})	ETHANOL (g. L^{-1})	BIOMASS (g. L^{-1})
1	4.70	4.86	0.66
2	14.67	9.00	0.77
3	1.71	12.00	1.35
4	2.80	4.36	0.51
5	8.40	38.50	3.11
6	7.15	17.00	1.22
7	1.50	44.76	3.00
8	4.20	35.60	3.54
9	4.00	19.00	1.92

The interaction effects are linear combinations of the form

$$\frac{1}{4}\sum_{i}^{n} a_i y_i, \qquad [1]$$

where y_i is the average response in run *i* and the coefficient a_i is set equal to plus or minus one, depending on the sign of the product of the columns of the factors involved. For example, to calculate the **ST (=EA)** interaction

l_{ST} = (1/4) [+(4.70) - (14.67) - (1.71) + (2.80) + (8.40) - (7.15) - (1.50) + (4.20) = -1.23 g. L^{-1}] → **ST** + **EA**.

Before interpreting the physical meaning of the calculated values for the effects, it was necessary to obtain an estimate of the experimental uncertainty associated with them. The usual procedure is to pool the standard deviations of the replicate responses into a single overall estimate of experimental error, s_p. Since each effect is given by a linear combination of independent observations, the variances at each experimental setting can be combined into a single value representing the variance of an effect:

$$\hat{V}(effect) = \sum_i a_i^2 s_i^2 = \frac{s_p^2}{3}\sum_i a_i^2, \qquad [2]$$

where $a_i = \pm 1/4$ is the coefficient of the *i*th response and s_p^2 is an estimate of the pooled variance of that response. The square root of $\hat{V}$ (*effect*) is the standard error of an effect, *s*. Substituting into this equation the individual responses, standard errors of 0.45, 1.64 and 0.13 g.L^{-1} were obtained after 72 h for levan, ethanol and biomass production, respectively. At the 99% confidence level, these values imply that only effects with absolute values exceeding 1.33 g.L^{-1} for levan, 4.38 g.L^{-1} for ethanol and 0.38 g.L^{-1} for biomass production, can be considered statistically significant.

Levan production

Silva (16), studying on the influence of pH in the production of levan by *Zymomonas mobilis*, ZAG-12, in 4.5 - 6.0 band, carried out experiments in the following conditions: 200g.L^{-1} of sucrose in the 25ºC, in the batchwise, with and without of pH control. Adjusting only the initial pH, it observed that no advantage resulted in controlling the pH in the levan production.

Table 3. Estimates of effects, aliases and standard errors for the design 2^{4-1} (Table 1), calculated based on the results presented in Table 2.

Effects	Estimate ± standard error in levan production	Estimate ± standard error in ethanol production	Estimate ± standard error in biomass production
$l_S \rightarrow$ **S + ETA**	3.12 ± 0.5	-8.4 ± 1.64	-0.52 ± 0.13
$l_T \rightarrow$ **T + SEA**	-6.17 ± 0.5	6.84 ± 1.64	0.66 ± 0.13
$L_E \rightarrow$ **E + STA**	-0.65 ± 0.5	26.41 ± 1.64	1.89 ± 0.13
$l_A \rightarrow$ **A + STE**	3.20 ± 0.5	6.00 ± 1.64	0.84 ± 0.13
$l_{ST} \rightarrow$ **ST + EA**	-1.23 ± 0.5	0.14 ± 1.64	0.37 ± 0.13
$l_{SE} \rightarrow$ **SE + TA**	-2.0 ± 0.5	-6.80 ± 1.64	-0.15 ± 0.13
$l_{SA} \rightarrow$ **SA + TE**	1.5 ± 0.5	5.60 ± 1.64	0.44 ± 0.13

Based in this study pH was not selected as a relevant parameter. The fermentations had been initiated with pH 5.0 since this is the best pH for the levan production.

From Table 3, for levan production, it is reasonable to conclude that the main effects **S**, **T** and **A** have large values. Furthermore, if **S**, **T** and **A** are the most important effects, then it is logical to conclude that the interaction alias chain **SE**=**TA** has a large effect because **TA** is also significant. This is an application of Ockham´s razor, a scientific principle in which one is confronted with several different possible interpretations of a phenomenon, the simplest interpretation is usually the correct one (13). The yeast extract, an important source of nitrogen, vitamins and amino acids, is essential for *Zymomonas mobilis* growth and may be used in concentrations varying from 2 and 5 $g.L^{-1}$. In this range, this nutrient, does not interfere with levan production, which is not associated to the microorganism growth (2).

Lima (8) evidenced the influence of the initial concentration of yeast extract in the production of levan, that increased from 2.0 to 10 $g.L^{-1}$ causing an increase in the cells production and a redution in the production of levan. Although a considerable increase in the consumption of the substrat was verified only small part of the consumed sugar was converted into levan, the majority was directed to the production of biomass and possibly of other by- products of fermentation.

As factor **E** is not significant, this factorial fraction 2^{4-1} was then reduced to a complete factorial 2^3. After recalculating, now including the central point in order to test the curvature, the obtained results were presented in table 4. It was observed that the principal effects such as initial concentration of sucrose, temperature and agitation as well as the effect of interaction between **T** (temperature) and **A** (Agitation) are significant at the 99% confidence level. According to Fig. 1, the best levan production (14.67 $g.L^{-1}$) was obtained using 250 g. L^{-1} of sucrose, at temperature 20ºC and orbital agitation at 100 rpm. The interaction effect **TA** was analyzed in the diagram in Figure 2. Increasing the agitation from 50 to 100 rpm, caused an average increase of levan production of approximately 5.6 $g.L^{-1}$ when the temperature was on the lower level (20ºC). Moreover, at 30ºC there was not a significant effect of agitation on levan production. On the other hand, increasing the temperature from 20ºC to 30ºC, the production of this polymer decreases on average 3.7 $g.L^{-1}$, when agitation is on the lower level (50 rpm). This effect is higher (-8.7 $g.L^{-1}$) when the agitation is maintained at 100 rpm. Increasing the initial concentration of sucrose from 150 $g.L^{-1}$ to 250 $g.L^{-1}$, there is an improvement in levan production of 3.13 $g.L^{-1}$.

Considering only the statistically significant effects at the 99% confidence level, the model represented in Equation 3 was obtained in order to describe levan production in function of variables **E**, **T** and **A** in the studied range,

$$Y = 5.55 (\pm 0.22) + 1.48 (\pm 0.22)x_1 - 3.01 (\pm 0.22)x_2 + 1.52 (\pm 0.22)x_3 - 1.12 (\pm 0.22)x_2x_3 \quad [3]$$

Following the response surface methodology convention, the natural variables (**S**, **T**, and **A**) were transformed in coded variables, x_1, x_2, x_3, respectively (13). The transformation involved subtracting the average of the variable value and dividing the result by half the variation amplitude. The results also indicated that the curvature is not significant at the 99% confidence level.

These results were emphasized by published literature about these effects. Calazans (2) verified that temperature is an important factor for ZAG-12 polymer production, either increasing or decreasing the polymerization of this product. This is especially observed at temperatures between 30ºC and

Table 4. Results of principal effects and interactions of factorial 2^3 in levan production, with fermentation time maintained at 72 hours.

	Estimate ± standard error
Global Average	5.43 ± 0.22
Curvature	-3.71 ± 1.28
Principal Effects	
Initial Concentration of Sucrose (**S**)	3.13 ± 0.44
Temperature (**T**)	-6.18 ± 0.44
Agitation (**A**)	3.20 ± 0.44
Two-factor interaction	
ST	-1.23 ± 0.44
AS	1.26 ± 0.44
TA	-2.40 ± 0.44
Three-factor interaction	
STA	-0.66 ± 0.44

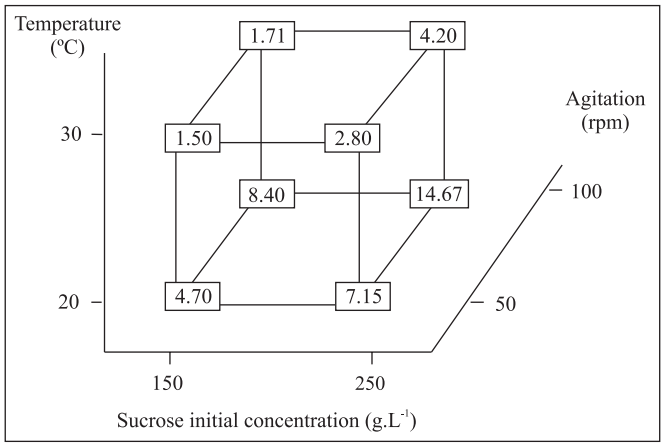

Figure 1. Geometrical representation of the results from the 2^3 design of levan production.

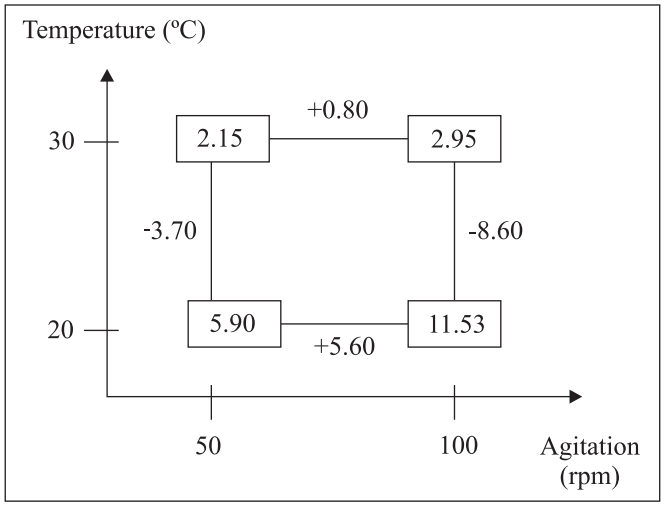

Figure 2. Geometric interpretation of the interaction effect TA on levan production.

35ºC, when there is an increase of the production of ethanol and other by-products, rather than the formation of levan. The results proved that 25ºC is the best temperature for the production of this polymer between the range of 20º and 40ºC.

Likewise, Dolle *et al.* (5) verified that 25ºC at pH 5.0 is the ideal condition for levan production. Moreover, Reiss & Hartmeier (15) demonstrated good productivity of this polymer at temperature varying between 27 - 30ºC at pH 5.0.

The temperature also affects cellular growth. Lower temperatures inhibited the growth of *Zymomonas mobilis* ZAG-12 Calazans (2), Song *et al.* (17) and Muro *et al.* (14) performed studies at temperatures between 10 and -3ºC using mutant

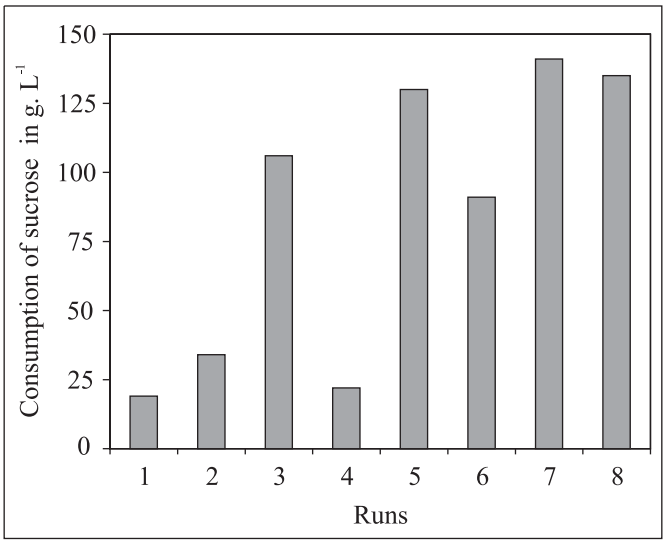

Figure 3. Consumption of sucrose in $g.L^{-1}$ by *Zymomonas mobilis* ZAG-12, obtained in different runs performed based on the fractional factorial 2^{4-1}, according to the planning described in Tables 2 and 4.

microorganisms and modified enzymes. Furthermore, low temperatures stimulate transfructolysation reactions and yet lengthen enzyme activity. On the other hand, higher temperatures stimulate sucrose hydrolysis (1,14,17,22).

It is known that *Z. mobilis* is able to survive in high sucrose concentrations. Song *et al.* (1996) published that the best sucrose concentration for levan production, using a modified levansucrase, was 20%, when the final amount of levan was approximately 56.7 $g.L^{-1}$ after 18 hours of incubation.

Magalhães (9), demonstrates that agitation may have an influence on the process, changing biochemical kinetic reaction and affecting the physiological appearance of the cell, which results in metabolic shunt and the decrease of cellular velocity.

In addition to oxygenation, agitation also facilitates medium homogeneity, allowing the access of the microorganism to the substrate. In fact, decreasing agitation (≤ 100 rpm), improves levan yielding. However, agitation up to 100 rpm may increase considerably medium oxygenation. According to literature, oxygen deviates the metabolic pathway of levan and ethanol production to acetaldehyde, which inhibits cellular growth of *Zymomonas mobilis* (6).

Low range band of agitation speed was chosen to prevent increase of aeration considering literature report that alert for the negative influence of the oxygen in the *Zymomonas mobilis* growth (5).

Biomass Production

Table 3 shows that yeast extract presented higher effect on biomass production (an average of 26.41 $g.L^{-1}$). The best

production of biomass was 3.54 $g.L^{-1}$, which corresponds to experiment 8, shown in Table 2.

It is evident that yeast extract on this level (+) improved cellular metabolism, subsequently leading to enhancement of the substrate consumption. In spite of this, experiment 3 did not present this relation (Fig. 3). Vigants *et al.* (19) observed a significant inverse correlation between the specific productivity of levan and the ATP concentration in *Zymomonas mobilis* cells, and verified that energetic metabolism reduction probably favors the production of levan.

Ethanol Production

Considering ethanol as the final product, the yeast extract concentration presented a large effect. In this case, the effects of second order interaction are not significant. The best production of ethanol was 44.76 g. L^{-1}, which corresponds to experiment 7 (Table 2).

Vigants *et al.* (19), studying osmolarity of *Z. mobilis* as catabolite regulators, observed a significant linear relation between ethanol yielding and biomass and the enhancement of ethanol and biomass. They concluded that ethanol synthesis by *Z. mobilis* is associated to growth, even though the concentration of yeast extract does not affect the levan yields, the fractionary factorial indicates that it is an important factor when desiring other products as a response.

CONCLUSIONS

The experiments showed that yeast extract does not affect levan production by *Zymomonas mobilis* ZAG-12, between 2.0 and 5.0$g.L^{-1}$. However, levan production is affected by initial concentration of sucrose, temperature and agitation in the experimental ranges studied. The best conditions for levan production (14.67 $g.L^{-1}$) were 250 $g.L^{-1}$ of sucrose, at temperature 20ºC, orbital agitation at 100rpm and yeast extract concentration between 2.0 and 5.0 $g.L^{-1}$. According to the published literature, these are the best results obtained with wild *Z . mobilis* strains.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge partial financial support from the Brazilian agencies Fundação Coordenação de Aperfeiçoamento de pessoal de Nível Superior (CAPES).

RESUMO

Aplicação do planejamento fatorial fracionário para a produção de levana por *Zymomonas mobilis*

Levana é um polissacarídeo extracelular, biologicamente ativo, não tóxico, contendo em sua estrutura apenas frutose. A maximização da produção de levana, por via fermentativa, pela linhagem de *Zymomonas mobilis* ZAG-12, foi estudada utilizando-se um planejamento fatorial de dois níveis 2^{4-1}, variando-se as concentrações iniciais de sacarose (150, 200 e 250 $g.L^{-1}$) , extrato de levedura (2.0, 3.5 e 5.0 $g.L^{-1}$), temperatura (20, 25 e 30ºC) e agitação (50, 75 e 100 rpm). As fermentações foram desenvolvidas por processos descontínuos em frascos Pyrex roscados, de 500 mL, contendo 300 mL de meio a base de sacarose, por 72 horas. No início e ao final do processo, foram dosados: biomassa, etanol, levana e sacarose como açúcares redutores totais. A análise dos dados mostra que o aumento da produção de levana depende tanto dos efeitos da concentração inicial de sacarose, temperatura e agitação, isoladamente, quanto da interação entre agitação e temperatura na faixa experimental estudada. O extrato de levedura não afeta a produção de levana, entretanto, quando a resposta é produção de etanol e biomassa, fica evidente que essa variável é significativa. Os resultados demonstraram que as melhores condições para a produção em batelada ocorreram com 250g/L de sacarose inicial, 100 rpm de agitação, a 20ºC.

Palavras chaves: planejamento fatorial, levana e *Zymomonas mobilis*

REFERENCES

1. Ananthalakshmy, V.K.; Gunasekaran, P. (1999). Optimization of levan production by *Zymomonas mobilis*. *Brasilian Archivies of Biology and Technology*, 42(3): 291-297.
2. Calazans, G.M.T. Produção de levana para uso clínico, 1997, 115p. (Tese de Doutoramento).Universidade Federal do Rio de Janeiro, UFRJ.
3. Calazans, G.M.T.; Rios, E.M.; Falcão de Morais, J.O.; Souza, M.F.V.Q.; Marçal, S.F.; Lopes, C.E. (1989). Produção de etanol e levana por *Zymomonas mobilis* amostra ZAP, em meio de sacarose. *Arquivos de Biologia e Tecnologi*a, 32, 4: 631-636.
4. Calazans, G.M.T.; Lopes, C.E.; Lima, R.M.O.C.; França, F.P. (1997). Antitumor activities of levans produced by *Zymomonas* strains. *Biotechnology Letters*, 19, 1: 19-21.
5. Dolle, W.H.; Kirk, L.; Crittenden, R.; Sientoh, H. (1993). *Zymomonas mobilis* – Science and industrial application. *Critical Reviews in Biotechnology*, 13, 1: 57-58.
6. Ishikawa, H.; Nobayashi, H.; Tanaka, H. (1990). Mechanism of fermentation performance of *Zymomonas mobilis* under oxygen supply in batch culture. *Journal of Fermentation and Bioengineering*, 70(1): 34-40.
7. Lima, N.V.R.; Melo, I.R.; Souza, I.A.; (2000). Fracionamento de levana e estudo comparativo de sua atividade antitumoral com a de outros polisssacarídeos. In: XIII SINAFERM, Teresópolis, RJ, anais, CD-Rom.
8. Lima, N.V.R. Investigações sobre bioatividade de polissacarídeos. Recife, 2001, 102p. (Tese de mestrado). Universidade Federal de Pernambuco. UFPE.
9. Magalhães, A.P. Levana como co-produto da fermentação alcoólica por *Zymomonas mobilis*. 1989, 183p. (Tese de Mestrado). Universidade Federal do Rio de Janeiro, UFRJ.
10. Melo, I.R.; Calazans, G.M.T. (2000). Efeito da concentração inicial de sacarose no inóculo para a produção de levana In: XL Congresso Brasileiro de Químicas, Recife, anais; Asssociação Brasileira de Química, p.48.

11. Melo, I.R.; Lima, N.V.R.; Lopes, C.E.; Calazans, G.M.T. (2001). Variações nos teores finais de etanol e levana em função da concentração de extrato de levedura no meio de produção. In: Congresso Brasileiro de Microbiologia, Foz do Iguaçu, anais, p.345.
12. Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, 31, 3: 426-428.
13. Montgomery, D.C. Design and Analysis of Experiments, 5ª th, John Wiley and Sons, New York, 2001, 684p.
14. Muro, A.C.; Rodríguez, E.; Abate, C.M.; Sineriz, F. (2000). Levan production using mutant strains of *Zymomonas mobilis* in different culture conditions. Biotechnology Letters 22: 1639-1642.
15. Reiss, M.; Hartmeier, W. (1990). Levan production with a flocculent strain of *Zymomonas mobilis*. *Food Biotechnology*, 4, 1: 69-75.
16. Silva, M.C. P. Influência do controle do pH sobre a produção de levana pela *Zymomonas mobilis*, ZAG-12 em meio à base de sacarose. Recife, 1996, 96p. (Tese de Mestrado). Universidade Federal de Pernambuco, UFPE.
17. Song, K.B.; Belghith, H.; Rhee, S.K. (1996). Production of levan, a fructose polymer, using over expressed recombinant levansucrase. Annals of the New York Academy of Sciences, 799: 601-607.
18. Swings J.; De Ley, J. (1977). The biology of *Zymomonas*. *Bacteriological Reviews*, 41, 1: 1-46.
19. Vigants, A.; Zikmanis, P.; Bekers, M. (1996). Sucrose medium osmolality as a regulator of anabolic and catabolic parameters in *Zymomonas* culture. *Acta Biotechonologica*, 16(4): 321-327.
20. Vina, I.; Karsakevich, A.; Karsakevich, A.; Gonta, S.; Linde, R.; Bekers, M. (1998). Influence of some physicochemical factors on the viscosity of aqueous levan solutions of *Zymomonas mobilis*. *Acta Biotechnologia*, 18:(2)- 167-174.
21. Vinhas, G.M. Estudo da produção e do fracionamento de levanas produzidas por *Zymomonas mobilis*. Recife, 1999, 66p. (Tese de mestrado). Universidade Federal de Pernambuco, UFPE.
22. Yanase, H.; Iwata, M.; Nakahigashi, R.; Kita, K.; Kato, N.; Tonomura, K. (1992). Purification, crystallization, and properties of the extra cellular levansucrase from *Zymomonas mobilis*. *Bioscience, Biotechnology, and Biochemistry*, v.56, n.8, p.1335-1337.