INFLUENCE OF CARBON SOURCE AND THE FERMENTATION PROCESS ON LEVAN PRODUCTION BY *Zymomonas mobilis* ANALYZED BY THE SURFACE RESPONSE METHOD[1]

Raquel Renan Jorge BORSARI[2], Maria Antonia P. Colabone CELLIGOI[2,*], João Batista BUZATO[2], Rui Sérgio dos Santos Ferreira da SILVA[3]

SUMMARY

The aim of this study is to assess sugar cane juice and sucrose as substrates, the batch and fed batch processes and their interaction in the levan production using a complete factorial design. *Zymomonas mobilis* was cultivated in different sugar cane juice and sucrose concentrations in two fermentation processes at 25 °C for 20 h. A complete factorial design (2^3) was used to analyze the effects of the type and concentration of the substrate, as well as the batch and fed batch processes. A complete second factorial design (2^2) was used to observe the importance of sugar cane juice. The results indicated that the batch process improved the levan production reaching 40.14 g/L. The addition of sugar cane juice was not statistically significant for levan formation, however sugar cane juice stimulated biomass, sorbitol and ethanol production. The best medium for levan production was 150 g/L sucrose in batch.
Keywords: batch, factorial design, fed batch, sucrose, sugar cane juice.

RESUMO

INFLUÊNCIA DA FONTE DE CARBONO E DO PROCESSO FERMENTATIVO NA PRODUÇÃO DE LEVANA POR *Zymomonas mobilis* ANALISADA PELA METODOLOGIA DE SUPERFÍCIE DE RESPOSTA . O presente estudo avaliou caldo de cana de açúcar e sacarose como substratos e os processos batelada e batelada alimentada e suas interações na produção de levana. *Zymomonas mobilis* foi cultivada em diferentes concentrações de caldo de cana de açúcar e sacarose nos dois processos fermentativos a 25 °C por 20 h. Foi utilizado um delineamento fatorial completo (23) para analisar os efeitos do tipo e concentração de substratos e processos batelada e batelada alimentada. Um segundo delineamento fatorial completo (22) foi usado para confirmar a importância do caldo de cana de açúcar. Os resultados indicam que o processo batelada foi o melhor para a produção de levana, atingindo 40,14 g/L em 150 g/L de sacarose. A adição de caldo de cana de açúcar não foi estatisticamente significativa para formação de levana, porém o caldo estimulou a produção de biomassa, sorbitol e etanol.
Palavras-chave: batelada, batelada alimentada, caldo de cana de açúcar, delineamento fatorial, sacarose.

1 - INTRODUCTION

Levan is a fructose exopolysaccharide produced by microorganisms as an energy reserve and defense. It presents low viscosity, high solubility in water, biocompatibility [15] as well as other properties that can have industrial applications such as an hypocholesterolemic agent [34], an immune modulator agent, an antitumour activity [35], an anti-inflammatory activity [32] and a blood plasma substitute and extender [21]. In the food industry, it is used as a fructose source and for the production of fructooligosaccharides [18], thickeners, stabilizers, encapsulating agents, flavors and aroma carriers as well as a color fixer [7, 3].

Zymomonas mobilis is a strictly fermentative, gram negative and ethanologenic bacteria that ferments only glucose, fructose and sucrose with by-product formation [30]. The growth in sucrose is followed by extra-cellular formation of fructo-oligomers and levan catalyzed by the levansucrase enzyme that hydrolyzes the sucrose and polymerizes the fructose in levan, significantly reducing the ethanol formation [29]. Sucrose concentration and temperature are the most important factors that regulate the activity of the levansucrase enzyme in *Zymomonas mobilis*. High sucrose concentrations and high temperatures stimulate fructo-oligosaccharide synthesis and the reduction leads to an increase in ethanol [5, 9].

Within the biotechnological processing economy, the factors: microorganism efficiency, low cost substrate exploitation and the fermentation process choice are fundamentally important [2].

The use of low cost substrates has been investigated in research concerning microbial metabolite production. Molasses and sugar cane syrup, beet juice, wheat extracts and milk serum fermentation for exopolysaccharide production including levan have been described by [14, 17]. Sorbitol and gluconic acid production by *Zymomonas mobilis* in sugar cane juice, molasses and corn steep liquor were reported by [8, 26] and molasses and beet juice for ethanol formation by [25].

The application of statistical designs for experiments and its modeling is important in a scientific study because it defines the effect of various factors and its interaction that leads to the optimization of the process. This instrument has been used in biotechnology by various authors [8, 25, 27].

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[2]*Depto. Bioquímica e Biotecnologia, Universidade Estadual de Londrina, C. P. 6001, CEP 86051-990, Londrina (Pr), Brasil*
E-mail: macelligoi@uel.br
[3]*Depto. Tecnologia de Alimentos e Medicamentos, Universidade Estadual de Londrina*
** A quem a correspondência deve ser enviada*

The aim of the present study is to assess sugar cane juice and sucrose as substrates, as well as the batch and fed batch process and their interactions in the levan production using a complete factorial design.

2 - MATERIALS AND METHODS

2.1 - Microorganism and maintenance conditions

Strains CP4 (ATCC 31821) of *Zymomonas mobilis* were maintained at 4 °C in a liquid culture medium containing (in g/L) KH_2PO_4 - 2; $(NH_4)_2SO_4$ - 1; $MgSO_4$ $7H_2O$ - 0.5; yeast extract - 10; peptone - 20; sucrose - 100 and was renewed every five weeks.

2.2 - Substrate

Sucrose was sterilized at 1 atm for 20 min and added with salts above. The sugar cane juice was filtered, sterilized at 1 atm for 20 min then diluted in distilled water to obtain the required concentrations

2.3 - Fermentation conditions

The fermentation medium consisted of the salts (in g/L): KH_2PO_4 - 2; $(NH_4)_2SO_4$ - 1; $MgSO_4$ $7H_2O$ - 0.5; yeast extract - 2 and sugar cane juice and sucrose as carbon sources according to the design *Table 1*. The batch and fed batch fermentations were carried out in 125 mL erlenmeyers with 25 mL of the different fermentation media for 20 h at 25 °C and initial pH = 5.0 without agitation. The concentration cellular was standardized in 0.2 g/L for all the conditions. In fed batch fermentation, after six hours of culture, 12.5 mL was removed from the fermentation medium and 12.5 mL of the medium was added.

2.4 - Analytical methods

At the end of fermentation, the cell growth was assessed by turbidimetry in 605 nm. The cells were centrifuged at 4300 x *g* for 20 min at 4 °C and re-suspended in 0.9 g% (m/V) saline that was correlated with a standard biomass curve. The reducing sugar was quantified by the Somogyi-Nelson method [24, 28], total sugar by Phenol-Sulfuric [11], sorbitol by HPLC column aminex HPX 87C (300 mm x 7.8 mm) ultra pure water as eluent, at 55 °C and with a refraction index as a detector [19]. Ethanol was determined by micro distillation [20]. Levan was separated by precipitation with ice-cold absolute ethanol, centrifuged at 8700 x *g* for 20 min at 4 °C washed in water and hydrolyzed with HCl 0.5% (V/V) for 60 min at 100 °C and estimated as fructose units by the Somogyi and Nelson reactions.

2.5 - Statistical analysis

A full factorial design 2^3 was used to optimize the substrate concentration and the type of fermentation process. The quantitative factors were sugar cane juice concentration (X_1) and sucrose concentration (X_2) and the qualitative factor was process type: batch and fed batch (X_3) all with two variation levels according to *Table 1*. The mathematical model proposed is shown in *Equation 1*:

$$Y_1 = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{1*2}X_1X_2 + b_{1*3}X_1X_3 + b_{2*3}X_2X_3 \quad (1)$$

Where Y_1 and Y_2 are the response (levan), b_0 the intersection, b_1, b_2 b_3 are the linear regression coefficients, b_{1*2}, b_{1*3} e b_{2*3} are the regression coefficients of the interaction and X_1, X_2, X_3 are the codified factors. After performing the 2^3 factorial designs, confirmatory experiments were used in the sub-region of the best results using complete 2^2 factorial designs with factors of sugar cane juice and sucrose at two levels (- 1 and + 1). The experiments were carried out in duplicate (*Table 3*). The proposed mathematical equation follows (*Equation 2*)

$$Y_2 = b_0 + b_1X_1 + b_2X_2 + b_{1*2}X_1X_2 \quad (2)$$

The levan, biomass, ethanol and sorbitol responses were analyzed by the STATISTICA application (1998).

3 - RESULTS AND DISCUSSION

3.1 - First design: selection of the affecting variables in levan biosynthesis

From the results obtained (*Tables 1* and *2*) the effects that most affected levan formation that were statistically significant ($p < 0.05$) were sucrose (X_2), the fermentation process (X_3) and the sugar cane juice, as well as sucrose interaction (X_1X_2). The same interpretation was obtained from the normal graph (not shown) of the values of the effects calculated by the complete 2^3 factorial design. The best levan biosynthesis condition 35.58 g/L was at levels X_1 = - 1, X_2 = + 1 and X_3 = - 1.

TABLE 1 – Full factorial design 2^3 for studies of the factors: sugar cane juice, sucrose and fermentation process.

Run	Variables			Biomass	Levan	Sorbitol	Ethanol
	X_1	X_2	X_3	g/L			
1	- 1	- 1	- 1	1.583	12.51	2.78	19.12
2	1	- 1	- 1	2.202	15.76	14.50	23.34
3	- 1	1	- 1	1.906	35.58	17.36	21.36
4	1	1	- 1	0.902	19.14	15.03	3.47
5	- 1	- 1	1	1.590	5.22	3.45	15.91
6	1	- 1	1	1.457	16.4	10.19	14.23
7	- 1	1	1	1.557	26.31	12.23	20.83
8	1	1	1	0.396	16.21	8.37	1.67

		Real levels	
		- 1	+ 1
X_1	Sugar cane juice g/L	50	150
X_2	Sucrose g/L	0	150
X_3	Fermentation process	Batch	Fed Batch

The analysis of the results obtained in *Table 1* shows that with the increase in the total sugar concentration from 200 g/L (experiment 3) to 300 g/L (experiment 4) the levan production was decreased to 46.21% and the sugar cane juice and sucrose interaction had a negative effect (*Table 2*). VIGANTS *et al.* [33] who studied culture medium osmotic pressure ascertained that the levan synthesis decreased significantly when the substrate concentration was increased. MURO *et al.* [23] reported that there were no differences in the levan formation when the sucrose concentration was increased from 200 to 300 g/L. DOELLE *et al.* [10] described that high sugar or salts concentrations caused differences in the use of fructose causing the formation of fructo-oligomers.

Table 2 shows that the X_2 variable (sucrose) presented a positive effect and X_3 variable (fermentation process) and X_1X_2 interaction (sugar cane juice - sucrose) had negative effects inhibiting levan formation.

TABLE 2 – Estimate of the effects relative to the concentration factors of sugar cane juice, sucrose and fermentation process.

Factor	Effect	p
Intercept	18.3912	0.000769
X_1	- 3.0275	0.097331
X_2	11.8375	0.007351
X_3	- 4.7125	0.043841
$X_1 X_2$	- 10.2425	0.009783
$X_1 X_3$	3.5675	0.072993

X_1 = sugar cane juice concentration; X_2 = sucrose concentration; X_3 process type; e R_2 = 0.99285.

As the mathematical model (eq. 1) obtained for the levan response (Y_1) had a qualitative variable. The X_3 variable should be substituted by its upper (fed batch) or lower (batch) level to fit it to the type of process and give estimates concerning the importance of the substrates.

$$Y_1 = 18.39 - 1.51X_1 + 5.92X_2 - 2.36X_3 - 5.12\,X_1X_2 + 1.78X_1X_3 \quad \text{(eq. 1)}$$

The predictive model confirmed the best levan production conditions: 50 g/L sugar cane juice plus 150 g/L sucrose in batch. The value observed of 35.58 g/L is very close to the predicted value of 35.08 g/L bearing in mind that the explained variation percentage is at least 97%.

From the obtained results, it can be observed that variable X_3 (fermentation process) had a negative effect on the levan production (*Table 2*) which means when the fed batch process was used there was a decrease in the levan biosynthesis (*Table 1*, experiments 1 and 5). Therefore in the next experiments, the bath process was used.

In the medium of sugar cane juice with 150 g/L total sugars, the levan formation was 15.76 g/L and 16.64 g/L in the batch and fed batch, respectively (experiments 2 and 6, *Table 1*). Similar results were obtained by RIO *et al. apud* FALCÃO DE MORAIS *et al.* [12] that used sugar cane juice with 15.7% added salts and produced 17.8 g/L levan with *Zymomonas mobilis*.

The addition of sugar cane juice to the fermentation culture medium was not statistically significant. Agro-industrial by-products present components that can inhibit product formation as reported by HAN & WATSON [14], FALCÃO DE MORAIS *et al.* [12], BEKERS *et al.* [4] e CAZETTA *et al.* [8].

Figure 1 represents the contour of the model for levan production, fixing the X_3 variable (fermentation process) at level - 1 (*Figure 1a*) and + 1 (*Figure 1b*).

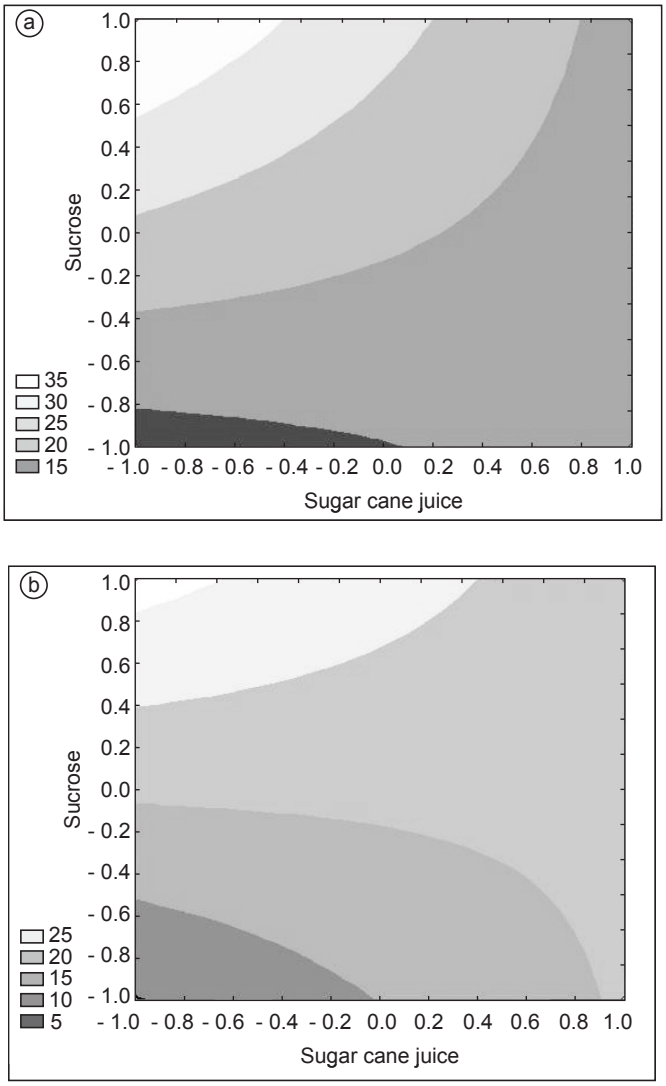

FIGURE 1 – Contour described for the levan production by *Zymomonas mobilis*, which represents the variable X_3 (fermentative process) is - 1 (a) and + 1 (b).

Regarding the cell growth, the increase in the sugar cane juice concentration from 50-150 g/L led to an increase from 1.58 g/L to 2.20 g/L in the batch process (*Table 1* experiments 1 and 2).

The sugar cane juice and sucrose interaction also had a negative effect on the ethanol and sorbitol production. The best conversion coefficient in ethanol was 73.64% and 71.48% in batch and fed batch, respectively with 50 g/L sugar cane juice. Concerning sorbitol, the best production was in experiment 3 reaching 17.36 g/L with 41.71% conversion efficiency. It is known that sorbitol is an osmoprotective agent, therefore there was more formation at the higher sugar concentrations [29].

3.2 - Second design: assessment of the importance of sugar cane juice

With the aim of confirming the substrate influence (sugar cane juice and sucrose) on the fermentation medium, defined in the first design, new experiments were performed as shown in *Table 3*.

TABLE 3 – Full factorial design (2^2) for responses: levan, biomass, ethanol, sorbitol.

Run	X_1	X_2	Sugar consume	Levan	Biomass	Ethanol	Sorbitol
			g/L				
1	+ 1	- 1	105.62	35.36	1.41	11.32	28.5
2	- 1	- 1	76.58	40.14	0.50	2.45	12.81
3	+ 1	+ 1	106.71	40.0	1.17	11.14	17.27
4	- 1	+ 1	56.56	37.50	0.47	3.58	8.25

		Real levels	
		- 1	+ 1
X_1	Sugar cane juice g/L	0	50
X_2	Sucrose g/L	150	50

The responses are means of the duplicate.

The effect of the substrates can be modeled:

$$Y_2 = 38.25 - 0.57X_1 + 0.50X_2 + 11.82\ X_1X_2 \quad \text{(eq. 2)}$$

This is statistically significant at the level of 5% according to ANOVA (*Table 4*). As $R_2 = 0.8403$, the variation percentage explained by the model is 84%. The values observed and estimated by model eq. 2 are shown in *Table 5*. The greater response values indicated the importance of the experiment interaction (2a and 2b) and experiments (3a and 3b). This corroborates with greater coefficients of the interaction in the model.

It is worth mentioning that the coefficients for sugar cane juice and sucrose are of the same order, but with opposing signs, that 150 g/L sucrose produced the best results in levan formation. To obtain a similar result, the addition of 50 g/L sugar cane juice should be compensated with 200 g/L sucrose. ANANTHALAKSHMY & GUNASEKARAN [35] reported no differences in the 150-250 g/L sucrose concentrations in the levan production. *Table 3* (experiment 2) and *Table 1* (experiment 2) show that, where the culture mediums contained 150 g/L total sugars (either sucrose or sugar cane juice) there was a 40 g/L to 15.46 g/L decrease in levan formation demonstrating that sugar cane juice and this concentration may have influenced production negatively.

TABLE 4 - Analysis of variance of the model (eq. 2).

Source of variation	Sum of square	Degrees of freedom	Mean Square	F_{cal}	$F_{tab(5\%)}$
Regression	31.0954	3	10.3651	7.0162	6.59
Residual	5.9093	4	1.4773	-	-
Total	37.0047	7	-	-	-

These experiments showed that the high level of the X_1 variable (sugar cane juice) had a positive effect on the responses: biomass (0.807), sorbitol (8.2150) and ethanol (12.355) and was statistically significant at 5% probability. The addition of sugar cane juice favored bacteria growth with a consequent increase in sorbitol and ethanol formation. The greatest sorbitol formation reached was 11.32 and 11.41 g/L in experiments 1 and 3 with a conversion efficiency of 20.17% and 19.62%, respectively.

TABLE 5 - Experimental and predicted values for model eq. 2.

Runs	X_1	X_2	Experimental	Predicted
			g/L	
1a	+ 1	- 1	36.19	35.36
2a	- 1	- 1	41.16	40.14
3a	+ 1	+ 1	40.27	40.01
4a	- 1	+ 1	38.57	37.50
1b	+ 1	- 1	34.53	35.36
2b	- 1	- 1	39.11	40.14
3b	+ 1	+ 1	39.74	40.01
4b	- 1	+ 1	36.43	37.50

X_1 = sugar cane juice; X_2 = sucrose.

4 - CONCLUSION

The present study used a complete factorial design for levan production by *Zymomonas mobilis* CP4. The fermentation conditions were the type and concentration of the substrate and fermentation processes (batch and fed batch). The highest levan production of 40.14 g/L was when both sucrose 150 g/L and batch fermentation were achieved. The results showed that the addition of sugar cane juice to the fermentation medium resulted in an increase in cell concentration, as well as ethanol and sorbitol production.

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