

# Evaluation of supplementation of sucrose medium on the synthesis of *Zymomonas mobilis* bio-products

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**ABSTRACT.** The effect of the variables pantothenic acid, yeast extract and sodium chloride, as well as the cell permeabilization technique, were investigated on the formation of levan, ethanol, sorbitol and biomass of *Zymomonas mobilis*, using a  $2^{4+1}$  fraction factorial design. Cell growth was determined by turbidimetry at 605 nm, relating it to a biomass with a dry weight calibration curve. Reducing sugars were quantified according to Somogyi and Nelson. Total sugars were quantified by the phenol-sulfuric acid method, sorbitol by HPLC and ethanol. The levan produced was precipitated by the addition of absolute ethanol and quantified in fructose units. In levan biosynthesis, the variable that had the largest contribution was cell condition. The results suggested that the factors that most affected biomass and ethanol formation were sodium chloride concentration and cell condition that affected negatively on production. For sorbitol, the variable that had a significant effect was permeabilization, which decreased its synthesis. Studies to amplify the range of established factors would be important.

**Key words:** fermentation, biomass, substrate, sorbitol, levan.

**RESUMO. Avaliação da suplementação do meio de sacarose na formação de bio-produtos por *Zymomonas mobilis*.** A influência das variáveis: ácido pantotênico, extrato de levedura, cloreto de sódio, e a técnica de permeabilização celular foram investigadas na formação de levana, sorbitol, etanol e biomassa de *Zymomonas mobilis* utilizando um delineamento estatístico fatorial fracionado  $2^{4+1}$ . A biomassa foi determinada por turbidimetria. Os açúcares redutores foram quantificados por Somogy e Nelson, açúcar total por Fenol Sulfúrico, sorbitol por HPLC e etanol por micro-destilação. A levana produzida foi precipitada com etanol absoluto e determinada como unidade de frutose. Na biossíntese de levana, a variável que mais contribuiu foi a condição celular. Os resultados sugerem que, para a formação da biomassa e etanol, os fatores que mais interferiram foram a concentração de cloreto de sódio e a condição celular que influencia negativamente a produção. Para o sorbitol, a variável que teve efeito significativo foi a permeabilização celular que atuou diminuindo a sua síntese. Estudos que ampliam a faixa de variação dos fatores estabelecidos são interessantes.

**Palavras-chave:** fermentação, biomassa, substrato, sorbitol, levana.

## Introduction

*Zymomonas mobilis* is of main commercial interest for producing ethanol as a biofuel. However, in sucrose medium, it synthesizes several products such as sorbitol, gluconic acid, levan and fructo-oligosaccharide (VIKARI, 1984). The biosynthesis of such bio-technological products may be affected by the concentration of nutrients in culture medium, by environmental conditions (YOO et al., 2004), and by the new biotechnological processes, cell permeabilization and immobilization (RERH et al., 1991; PARK et al., 1994).

The permeabilization process has been a tool used by different authors to improve the production of sorbitol and gluconic acid (SILVEIRA; JONAS, 2002). Jang et al. (2001) reported that using toluene-permeabilized whole cells produced low molecular weight levan.

The fermentation medium may also be supplemented with vitamins, amino acids, and other substances that have biological activities particular to the microorganism, stimulating the synthesis of a compound of interest. Bekers et al. (2002) used beet syrup silage in levan synthesis and verified that, in that medium, the composition included vitamins

that could be applied to replace the yeast extract. Muro et al. (2000) used several *Zymomonas mobilis* strains and evaluated different media with pantothenic acid and biotin in levan formation and showed a wide variation in the production of such polymer in different media.

The study of factors that may stimulate levan, sorbitol and ethanol synthesis is relevant, since levan may have potential applications in the field of food, cosmetics, pharmaceutical products, as an antitumoral, immunomodulator and hypocholesterolemic agent (CALAZANS et al., 1997; YAMAMOTO et al., 1999) and sorbitol has applications in confectionery, chewing gums, candy, desserts, ice cream, diabetic foods, and a wide range of food products, and also as a humectants, textures, and softener (SILVEIRA; JONAS, 2002). Its caloric value is similar to glucose, but it is less capable of causing hyperglycemia because it is converted to fructose in the liver (CAZETTA et al., 2005). Since the depletion of fossil fuel reserves and increasing environmental and political pressures has increased industrial focus toward alternative fuel sources (CAZETTA et al., 2007), it is interesting to study a microorganism that could produce ethanol.

The objective of the present study was to evaluate the effect of cell permeabilization and the addition of nutritional factors (pantothenic acid, yeast extract, and NaCl) in the formation of levan and other bio-products by *Zymomonas mobilis*.

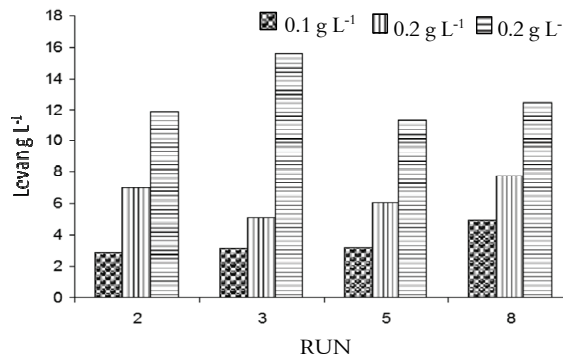
## Material and methods

### Microorganism and culture conditions

The *Zymomonas mobilis* CP4 (ATCC 31821) strain used was kept at 4°C in liquid culture medium containing (in g L<sup>-1</sup>) KH<sub>2</sub>PO<sub>4</sub> (Merck) - 2; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (Merck) - 1; MgSO<sub>4</sub> 7H<sub>2</sub>O (Merck) - 0.5; yeast extract (Biobrás) - 10; peptone (Biobrás) - 20; sucrose (Merck) - 100 and was renewed every five weeks.

### Inoculum preparation

The inoculum culture contained sucrose at 150 g L<sup>-1</sup> and the component mentioned previously. After incubation (48 hours) the culture was centrifuged and the biomass re-suspended in NaCl (Merck) 0.9%. The cell concentration was calculated by turbidity at 400 nm. The cell concentration was standardized to 0.2 g L<sup>-1</sup> for the assays without permeabilization and 0.8 g L<sup>-1</sup> for assays with permeabilized cells after the test Figure 1. The batch process was carried out in duplicate in eight different media, according to the experimental design (Table 1).



**Figure 1.** The influence of inoculum concentration on levan production in permeabilized *Zymomonas mobilis* cells in different media.

### Preparation of permeabilized cells (REHR et al., 1991)

The cells harvested in 20 hours after inoculation by centrifugation (4300 xg for 20 minutes) were slowly agitated with 0.1 M saline solution plus CTAB (Sigma) 0.2% (m V<sup>-1</sup>) at 4°C and pH 6.2 for ten minutes. Later, they were centrifuged and washed twice with the 0.1 M saline solution (JANG et al., 1992).

### Analytical methods

After fermentation, the culture was centrifuged at 4300 g for 20 minutes and the cells re-suspended in saline 0.9 g% (m V<sup>-1</sup>). The biomass concentration was determined by measuring the turbidity of diluted samples at 605 nm (Fento 700S) using a standard absorbance curve against dry cell mass (OLIVEIRA et al., 2007). For the calibration curve, an aliquot of the biomass suspension was dried to constant weight, and another aliquot of the same suspension was diluted to obtain spectrophotometric measure. Next, the corresponding values of dry weight (mg mL<sup>-1</sup>) and absorbance were plotted (CASOTTI et al., 2007). Reducing sugar was quantified according to the Somogyi and Nelson reaction (NELSON, 1945) using glucose as standard; total reducing sugars (TRS) were quantified by Phenol-Sulfuric (DUBOIS et al., 1956). The sugar consumption was determined by the difference between the initial TRS and final TRS after fermentation. Sorbitol was quantified by HPLC according to Kannan et al. (1997), with alterations in the temperature 55°C and 1 mL min<sup>-1</sup> flow. Ethanol was determined by micro-distillation (KAYE; HAAG, 1954). Levan was separated by precipitation with cold absolute ethanol, centrifuged at 8700 g for 20 min., washed in ethanol and hydrolyzed in HCl 0.5% (v v<sup>-1</sup>) for 60 min. at 100°C and estimated as units of fructose by the Somogyi and Nelson reaction.

### Statistical analysis

In order to select the factors that stimulated the production of sorbitol, ethanol, biomass and levan, was used a factorial fractional design of resolution IV ( $\frac{1}{2}$  fraction factorial). The quantitative variables were pantothenic acid ( $X_1$ ), sodium chloride ( $X_2$ ), yeast extract ( $X_3$ ), and the qualitative variable was the cell condition ( $X_4$ ). The factors were investigated at two levels of variation (Table 1).

### Results and discussion

#### Inoculum choice for the assays with permeabilized cells

For the assays with cell permeabilization (2, 3, 5, and 8 in Table 1), inoculum concentrations of 0.1, 0.2, and 0.8 g L<sup>-1</sup> were tested. From the data obtained and presented in Figure 1, it may be observed that levan production was proportional to the inoculum concentration. The largest levan biosynthesis was with cell inoculum of 0.8 g L<sup>-1</sup>. For the cultures without permeabilization, a 0.2 g L<sup>-1</sup> inoculum was used and at the end of the fermentation the maximum growth was 0.8 g L<sup>-1</sup>. Thus a 0.8 g L<sup>-1</sup> inoculum was chosen for the permeabilized cultures so that the results could be compared. Regularly such behavior is not very common with *Zymomonas mobilis* free cells because the production of metabolites is separated from cell growth.

**Table 1.** 2<sup>4-1</sup> Planning to investigate the effect of factors  $X_1$ ,  $X_2$ ,  $X_3$ , and  $X_4$  on levan formation.

Run	Variables in coded levels				Measured responses				
	$X_1$	$X_2$	$X_3$	$X_4$	Levan (g L <sup>-1</sup> )	Biomass (g L <sup>-1</sup> )	Ethanol (g L <sup>-1</sup> )	Sorbitol (g L <sup>-1</sup> )	Sugar Consumption (g L <sup>-1</sup> )
1	-1	-1	-1	-1	12.28	0.694	8.50	3.99	34.51
2	1	-1	-1	1	11.96	0.942	0.00	1.95	25.84
3	-1	1	-1	1	15.65	0.817	0.61	2.54	36.77
4	1	1	-1	-1	9.54	0.223	1.53	3.19	15.12
5	-1	-1	1	1	11.36	0.920	0.92	2.14	23.36
6	1	-1	1	-1	13.37	0.972	14.04	4.66	44.93
7	-1	1	1	-1	8.74	0.232	0.91	3.59	22.0
8	1	1	1	1	12.50	0.851	0.00	2.09	19.12

Factors		Real levels	
		-1	+1
$X_1$	Pantothenic acid (g L <sup>-1</sup> )	0.005	0.01
$X_2$	NaCl M	0.2	0.6
$X_3$	Yeast extract (g L <sup>-1</sup> )	4	8
$X_4$	Cell condition	Not permeabilized	Permeabilized

#### Fractioned factorial outlining for the selection of variables

The influence of the variables pantothenic acid, sodium chloride, yeast extract, and cell condition was studied in the responses: biomass, ethanol, sorbitol, and levan using a 2<sup>4-1</sup> design.

Analysis of the result showed that the largest levan production was found on Table 1 in run 3

(15.65 g L<sup>-1</sup>) at levels  $X_1 = -1$ ,  $X_2 = 1$ ,  $X_3 = -1$ , and  $X_4 = 1$ , with a sugar consumption of 36.77 g L<sup>-1</sup>. Nevertheless, the effect of the main variables studied, considering the variation regions established for assessment, were not statistically significant at 5% level.

The variable effect that contributed the most to increase levan formation was cell condition. Table 2 indicates that the cell condition had a positive effect (1.885) ( $p < 0.1$ ). The most important contribution ( $p < 0.05$ ) was interaction  $X_2X_4 + X_1X_3$ , with a 3.05 effect. As those effects are mistaken, it cannot be affirmed which was operating under the response (levan), as the maximum percentage of variation explained was 78.93%. Data that were not presented show that the largest levan biosynthesis was with permeabilized cells without supplementation.

**Table 2.** Estimate of the effects related to the factors sodium chloride and cell condition.

	Effect	p	R <sup>2</sup>	R <sup>2</sup> adjustment
Intercept Biomass	1.876	0.00042	0.9352	0.8866
$X_2$	-1.578	0.01055		
$X_4$	-1.548	0.01127		
$X_2X_4$ *	1.453	0.01398		
Intercept Ethanol	3.3138	0.00955	0.9132	0.848
$X_2$	-5.1025	0.02293		
$X_4$	-5.8625	0.01454		
$X_2X_4$ *	4.9475	0.02530		
Intercept Sorbitol	3.0188	0.000002	0.9079	0.8710
$X_4$	-1.6775	0.0012		
$X_2X_4$ *	0.6025	0.6375		
Intercept Levana	11.925	0.0000	0.7893	0.7051
$X_4$	1.885	0.0719		
$X_2X_4$ *	3.05	0.0143		

\*The interaction  $X_2X_4$  was mistaken with  $X_1X_3$ .

There is no report in the literature on the use of cell permeabilization techniques to stimulate levan biosynthesis, except that such permeabilization leads to the formation of levan with low molecular weight (JANG et al., 2001). Muro et al. (2000) argued that, for the wild strain of *Zymomonas*, the medium added with pantothenic acid and biotin (10 mg L<sup>-1</sup>) in 200 g L<sup>-1</sup> sucrose presented the best levan production. Studies on alternative substrate to the yeast extract revealed that beet molasses silage, which contained a larger concentration of pantothenic acid and other vitamins, did not stimulate levan biosynthesis, but that of ethanol (BEKERS et al., 2002). Melo et al. (2007) reported that yeast extract did not influence levan production. However, Oliveira et al. (2007) described that yeast extract was significant for levan production.

Analyzing Table 2 for cell growth and ethanol formation shows that factors  $X_2$  and  $X_4$  were statistically significant, with a negative effect

( $p < 0.05$ ). It is interesting to observe that  $X_2 \cdot X_4$  is significant even though it is mistaken with  $X_1 \cdot X_3$  interaction.

It is suggested that higher levels of such factors ( $X_2 \cdot X_4$ ) caused a decrease in biomass and ethanol responses. As may be observed in Table 1, the largest production of biomass and ethanol (run 6) was at the lowest sodium chloride concentration studied and in the non-permeabilized cells. An increase in the sodium chloride or potassium concentration in the culture medium has been reported as acting to inhibit cell growth or decreasing ethanol production (VIGANTS et al., 1998). Vriesekoop et al. (2002) reported that cell growth inhibition and decrease in ethanol formation were due to the sodium ion, while the chloride ion was responsible for filament formation in *Zymomonas mobilis*. Several studies have described that in cell permeabilization the essential soluble co-factors for the conversion of gluconic acid into ethanol are liberated, ceasing the formation of ethanol (JANG et al., 1992).

The largest sorbitol biosynthesis was also in run 6 (Table 1). The factor that affected the most was the cell condition ( $X_4$ ), which was negatively significant (Table 2). This factor, at its superior level (permeabilization) operates decreasing the production of sorbitol. However, studies are found in the literature with other strains of *Zymomonas* that report the largest sorbitol synthesis with permeabilized cells (REHR et al., 1991; WILBERG et al., 1997). Regarding sodium chloride ( $X_2$ ), it was reported that high concentrations inhibit sorbitol formation (BEKERS et al., 2000).

## Conclusion

In this study, the experiments showed that the cell permeabilization technique offered advantages over the free cell technique for levan production by *Zymomonas mobilis* ATCC 31821. For the responses biomass and ethanol, it was evident that higher sodium chloride decreased production. Sorbitol production decreased with permeabilization, but this *Z. mobilis* line was a better levan producer. For other factors (pantothenic acid, sodium chloride and yeast extract) more studies amplifying the selected area would be interesting.

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