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Influence of High Osmotic Pressure on Sorbitol Production by *Zymomonas mobilis*

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ABSTRACT

The objective of the present work was to study the variation on the sorbitol production in relation to the concentration of sugars, (metabolizable or not) and the cultivation time. A full factorial design was used considering the factors such as sucrose and maltose concentration and cultivation time. The addition of sugars caused increases on the sorbitol production up to the concentration of 300g/L however, decreases on the sorbitol production were observed when the concentration reached values above this. Increasing the time of fermentation was statistically significant to sorbitol production, however, little increase the production was noticed after 36h.

Key words: *Zymomonas mobilis*, sorbitol, high osmotic stress, maltose

INTRODUCTION

Zymomonas mobilis produces ethanol through the Entner-Doudoroff route in conjunction with pyruvate decarboxylase and alcohol dehydrogenase enzymes. It catabolizes only D-glucose, D-fructose and sucrose as sources of carbon and energy (Swings and De Ley, 1977). Ethanol and carbon dioxide are the main products obtained from the catabolism when cells grow anaerobically in glucose medium. However, in sucrose-based substrates, ethanol production may be reduced up to 70% due to the formation of by-products such as levan and sorbitol (Viikari, 1984; Viikari, 1986; Kannan, et al, 1998). Sorbitol is also formed and appears in the medium when cells grow in mixtures composed of glucose and fructose exceeding 5% of sugarcane (Sprenger, 1996).

When growing in sucrose medium, *Z. mobilis* converts the disaccharide into glucose and fructose with the aid of three enzymes: extracellular levansucrase (Lev U), forming glucose and fructose that are converted into levan, a extracellular invertase or sucrase (Inv B) that releases glucose and fructose (Preziosi and Barati, 1990; O'Mullan et al, 1991; Yanase, et al, 1992) and a second invertase (Inv A) whose exact function and location in *Z. mobilis* are not fully understand. After the fructose release, sorbitol may be produced by the action of the GFOR enzyme, which is an enzyme located at the *Z. mobilis* periplasmatic region (Aldrich, et al, 1992; Loos et al, 1991; Loos et al, 1993; Loos et al,

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1994) and capable of converting the mixtures of glucose and fructose into gluconate-δ-lactone and sorbitol, respectively. Sorbitol is mainly produced under high sucrose concentration conditions, where fructose accumulates favorably at high levels (Doelle et al, 1990; Rogers and Lee, 1982), requiring a sorbitol cell osmoprotection. A stressing condition that may be due to the high concentration of sugar in the medium or due to the presence of sugars not transported or metabolized such as maltose, makes the cells to accumulate intracellular sorbitol at concentration above 1 M in order to neutralize the dehydration effects of the high external osmolality (Loos et al, 1994).

In order to corroborate the osmoprotective effect of sorbitol, Wiegert et al. (1996) worked with a non sorbitol-producing glucose-fructose-oxidoreductase-deficient mutant strain and observed that this strain was not able to grow in the presence of 1M of sucrose unless sorbitol was added to the medium. Savvides et al (2000) studied mutant strains capable of growing in high concentrations of sugars (40% of sucrose) and considered that such capacity could be also attributed to a differentiated fatty acids and phospholipids composition in the membrane.

However, studies on the effect of the medium osmolality on the *Z. mobilis* metabolism are scarcely found in literature (Lin et al, 1991). In this context, changes on the medium osmolality must be considered as a promising way to induce the controlled metabolic readjustments in order to provide higher knowledge on the *Z. mobilis* physiological control in the attempt of reaching more satisfactory production levels (Vigants et al, 1996). Thus, the objective of this work was to study the effect of the medium osmolality in the presence of maltose on the sorbitol production by *Z. mobilis* ATCC 29191.

MATERIAL AND METHODS

Microorganism and fermentation medium

Zymomonas mobilis ATCC 29191 was cultivated in sucrose medium (g/L): 100, yeast extract: 10; $(NH_4)_2SO_4$: 1; KH_2PO_4: 2; $MgSO_4.7H_2O$: 0.5; $FeSO_4$: 0.5; peptone: 5. After a 20 h (exponential phase) the cells were centrifuged at 7000 rpm. Sucrose and maltose were used in the experiment at concentrations stipulated in the experimental design (Table 1) with inoculum of 2g/L at a constant temperature of 30°C under batch condition. The times of fermentation evaluated were 12 and 36 h.

Statistical analysis

The conditions tested to verify the effect of maltose on the sorbitol production are presented in Table 1. The design matrix building according Barros Neto et al. (1996), presented the experiments in the so-called standard order (Table 1).

Table 1 - Experimental design for full factorial 2^3.

Tests	Coded Variables			Decoded Variables		
	X_1	X_2	X_3	Maltose (g/L)	Sucrose (g/L)	Time (h)
1	-1	-1	-1	0	200	12
2	1	-1	-1	100	200	12
3	-1	1	-1	0	300	12
4	1	1	-1	100	300	12
5	-1	-1	1	0	200	36
6	1	-1	1	100	200	36
7	-1	1	1	0	300	36
8	1	1	1	100	300	36

The results were analyzed by means of the STATISTICA software version 5.1 (*Experimental Design*) for microinformatics. The objective was to verify which contrasts were statistically significant, particularly those concerning the main effects.

Analytical procedures

Biomass determinations were performed by means of turbidimetry at 605 nm and related to dry weight through a standard curve. Sorbitol was determined by HPLC using CLC-NH2 (Shim-Pack) (9 um, 30 cm x 1.6 cm; at 55 ^oC) and water

as eluent (1 ml/min). Detector Refraction Index was used to detect sorbitol. Reducing sugars (RS) and total reducing sugars (TRS) were determined by Somogy (1945) and Nelson (1944).

RESULTS AND DISCUSSION

Sorbitol production by *Zymomonas mobilis* is attributed to the osmoprotective effect of sorbitol on the microorganism under osmotic stress conditions due to the high concentration of sugars and other compounds present in the culture medium. The results obtained by the work on the osmotic stress caused by the high sucrose concentration and the addition of maltose in the sorbitol production are presented in Table 2, and the effects related to factors time and concentration are described in Table 3.

Table 2 showed that in total sugar concentrations from 200 to 400 g/L, there was no positive correlation with the sorbitol production. A considerable drop in the sorbitol formation was rather observed with the increase in the concentration of sugars in the medium, thus decreasing its production from 38.60 to 20.61 g/L when the sugar concentration increased from 300 g/L of sucrose to 300 g/L of sucrose + 100 g/L of maltose in 36 h cultivation, probably due to the excessive increase in osmotic pressure.

Table 2 - Biomass and sorbitol production in relation to changes on substrate concentration and time according to experimental design 2^3.

Tests	Sucrose (g/L)	Maltose (g/L)	Time (h)	Biomass (g/L)	Sorbitol (g/L)
1	200	0	12	2.27	34.66
2	200	100	12	2.66	34.84
3	300	0	12	2.7	31.915
4	300	100	12	2.74	15.925
5	200	0	36	2.32	34.03
6	200	100	36	2.65	38.07
7	300	0	36	2.69	38.68
8	300	100	36	2.54	20.61

Responses are averages between two true replications, R^2= 95.35, X_1= sucrose (g/L), X_2= maltose (g/L) X_3= time (h).

Table 3 - Estimation of the effects related to factors sucrose, maltose and time on the experimental design 2^3.

Factor	Effect	P
Average/Intercession	31.09*	0**
Block	-2.10 (ns)	0.12(ns)
X_1	-7.46*	0.0003**
X_2	-8.62*	0.0001**
X_3	3.51*	0.02*
$X_1.X_2$	-9.57*	0.00005**
$X_1.X_3$	0.44 (ns)	0.73(ns)
$X_2.X_3$	2.21 (ns)	0.11(ns)

X_1 = maltose concentration; X_2 = sucrose concentration; X_3 = time; R^2 = 0.

The effects of sucrose, maltose and cultivation time are showed in Table 3. Evidently the three factors (X_1, X_2 and X_3) as well as the interaction $X_1.X_2$ present significant effects on the sorbitol production. However, X_1 (sucrose concentration) and X_2 (maltose concentration) presented negative effects and their respective increases caused reduction on the sorbitol levels. The determination coefficient (R^2) indicated that 95.35% of the variation could be attributed to the model. Figure 1 represents the model for sorbitol when the cultivation time was fixed in 36 h. The highest sorbitol productions were at regions with levels below X_1 (sucrose) and X_2 (maltose). However, since concentrations higher than 300g/L of sucrose was not studied, it was not possible to confirm if the decrease in the production occurred due the addition of maltose or by high osmotic pressure.

Besides, it was not possible to conclude that sucrose would have the same effect considering that it was metabolizable by *Z. mobilis* and maltose not.

Table 3 showed that the effect of factor X_3 (time) was significant and positive; therefore, increases on this factor would cause increases on the sorbitol levels.

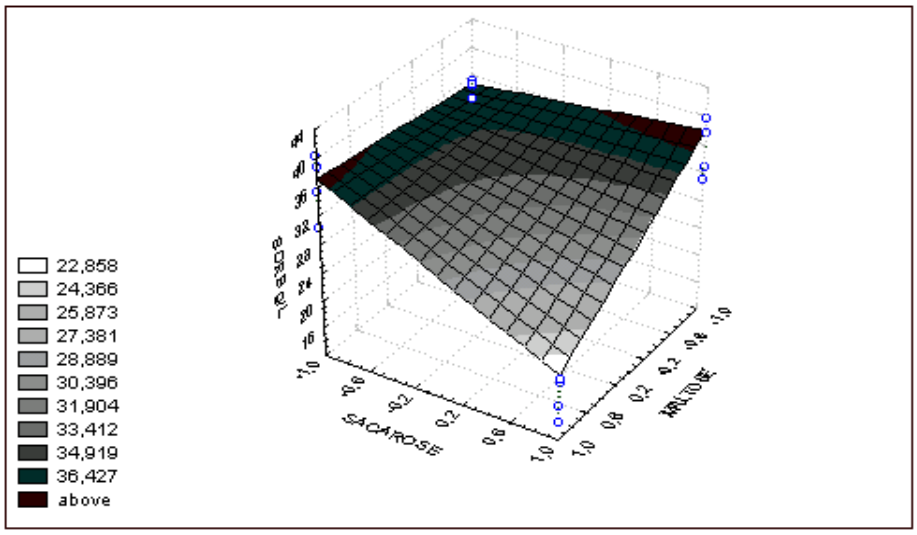

Figure 1 - Sorbitol production as a result of the *Z. mobilis* ATCC 29191 fermentation in 36 h of cultivation.

However observing the production on Table 2, it was possible to conclude that the time contribution was not superior to 10% considering the cultivation with 200g/L of sucrose + 100g/L of maltose and 21% considering 300g/L of sucrose, conditions where the time effect was more pronounced. Barros et al. (2006) found an optimum production in 36 h culture with 200g/L of sucrose. Vignoli et al. (2005) observed that the sucrose concentration did not influence sorbitol production by the immobilized culture but increase in the culture time increased sorbitol levels. Permeabilized cells of *Zymomonas mobilis* also achieved maximum production sorbitol in 36 h of fermentation (Vignoli et al., 2006). Probably, due the high concentration of sugars, the microorganism needed a longer time to metabolize it.

Although the general effect of the high sugar concentration was negative, the sorbitol production remained unchanged at 300 g/L, regardless if the sugar was sucrose or sucrose associated with maltose. Considering that the osmotic pressure in the media with 200g/L of sucrose + 100 g/L of maltose and 300 g/L sucrose was practically the same, it was expected that the sorbitol production was similar (Table 2), thus corroborating the osmoprotective effect of sorbitol at high sugar concentrations, as mentioned by Bekers et al. (2000). Furthermore, the increase in the substrate concentration from 200 to 300 g/L increased the sorbitol production by 12%, regardless if the sugar was sucrose or sucrose associated with maltose. Cazetta et al.(2005) reported a maximum sorbitol production for medium containing molasses at 300 g/L of sugars by *Z. mobilis* ATCC 29191; there was a sharp fall the in production when the sugar concentration was below or above 300 g/L. Barros and Celligoi (2006) also obtained highest sorbitol production (60.46 g/L) by *Z. mobilis* ATCC 20191 on sucrose at 300 g/L after 36h; there was significant decrease in sorbitol production when the medium was previously hydrolysed with invertase, probably due to the excessive increase in osmotic pressure. This showed that the high osmotic pressure caused a decrease on sorbitol production independent of sugar nature, metabolizable or not.

The effects of the osmolality on the culture medium are scarcely found in literature; however,

some works using high sugar concentrations are available. Silveira et al. (1999) investigated the influence of the initial substrate concentration on equimolar glucose and fructose concentrations and found optimum sorbitol production at 650 g/L of sugars. Although the high sugar concentration was optimum, a very high inoculum (30 g/L) was also used, while an inoculum of 2 g/L was used in the present work. Moreover, glucose and fructose are substrates that can be directly used by the GFOR enzyme, unlike sucrose that requires a pre-hydrolysis stage. Sasahara and Izumori (2005) also observed that the conversion rate of L-sorbitol from fructose by *Aureobasidium pullulans* was accelerated when erythritol or L-arabitol was added to the reaction mixture. Their results indicated that L-sorbitol production was facilitated in high concentrations of the substrate.

In relation to the biomass production, observed no increase from 12 to 36 h and that the increase on the substrate concentration did not produce changes on the biomass production either (Table 2). This could have occurred due to the high initial inoculum (2 g/L) or due to the growth inhibition in function of the high sugar concentration employed. The inhibition of *Z. mobilis* growth due to the high glucose concentration is widely known. Erzinger (1996) reported a significant reduction on the growth and ethanol production when initial glucose concentrations above 150 g/L were used.

The kinetic parameters obtained from the sorbitol production are presented in Table 4. Although the fermentation time led an increase on the sorbitol production, a decrease in specific productivity was observed after long periods, as expected. The highest productivity rates were approximately 2.8 and 2.9 $g/L.h^{-1}$ at 200g/L of sucrose and 200 g/L of sucrose +100g/L of maltose, respectively in a 12-h cultivation. However, within 36 h, these productivities dropped to 0.95 and 1.06 $g/L.h^{-1}$, respectively. Silveira et al. (1999) reported higher GFOR stability in the processes using shorter times, where a crescent production was observed up to 4 h of process. After this period, the production became stable and decreased, thus productivity was less influenced by the loss of activity of GFOR when fast processes were employed. Besides, the total reducing sugar consumption was more efficient with lower fermentation times [89.18% (12h) to 91.86% (36h)] in medium with 200g/L of sucrose. It was observed that the parameters productivity (Ypr) and product yield (Yps) showed more dependence on sugar concentration. A increase in sucrose and/or maltose in fermentation medium caused higher Ypr and Yps values.

Table 4 - Kinetic parameters obtained from the experimental design 2^3 for the sorbitol production.

Tests	Sucrose (g/L)	Maltose (g/L)	Time (h)	TRS Consumption (%)	Ypr (%)	Yp/s (g/g)	Yp ($g/L.h^{-1}$)
1	200	0	12	89.18	32.28	0.172	2.88
2	200	100	12	50.38	61.18	0.33	2.9
3	300	0	12	52.41	39.99	0.213	2.66
4	300	100	12	18.61	55.19	0.29	1.33
5	200	0	36	91.86	30.77	0.164	0.95
6	200	100	36	70.11	48.04	0.253	1.06
7	300	0	36	51.02	50.52	0.27	1.07
8	300	100	36	19.7	67.46	0.36	0.57

TRS: Total Reducing Sugars; **Ypr**: productivity; **Yp/s**: Product yield; **Yp**: specific productivity.

CONCLUSION

Considering the range of sugar concentration studied, a decrease in the sorbitol formation level was observed when the sugar concentration in the culture medium increased. However, within a pre-established range (from 200 to 300 g/L), the increase in the sugar concentration was followed by increases in the sorbitol, production probably as response to the necessity of an osmotic protection. On the other hand, cultivation time maintained positive correlation with sorbitol production along the entire time interval studied, despite low increasing within the range studied.

RESUMO

Zymomonas mobilis produz o poliálcool sorbitol como principal subproduto. Sua formação é atribuída principalmente a sua função A produção

de sorbitol foi avaliada através de um planejamento fatorial completo utilizando as variáveis concentração de sacarose, concentração de maltose e tempo de cultivo. A adição de açúcares causou um aumento na produção de sorbitol até a concentração de 300g/L, porém decréscimos na produção de sorbitol foram observados a concentrações superiores a esta. Aumento no tempo de fermentação foi estatisticamente significativo para aumentos da produção de sorbitol, porém pequeno aumento foi observado de 12 para 36 horas de cultivo.

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