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PRODUCTION OF SORBITOL AND ETHANOL FROM SUCROSE BY *ZYMOMONAS MOBILIS* : SUGAR FERMENTATION

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ABSTRACT

Two Zymomonas *strains were compared with respect to their sucrose hydrolysing activity, and subsequent sorbitol and ethanol formation.* Z. mobilis *IFO 13756 has been used to study sugar fermentation since it provided a higher yield of sorbitol whereas the yield of ethanol was nearly the same. The maximum yield of sorbitol was found when* Z. mobilis *IFO 13756 used 250 g* dm^{-3} *sucrose as carbon source in culture medium pH 7.5 with 10% inoculum by fermentation at 30°C. The conditions for maximum yield of ethanol were fermentation in medium pH 6.5 with 3% inoculum. Addition of some compounds such as xylose, PEG, glucose and ethanol to the culture medium led to a lower yield of both sorbitol and ethanol. A suitable amount of oxygen in the culture medium is expected to have a limiting effect on growth and sorbitol-ethanol formation.*

INTRODUCTION

The bacterium *Zymomonas mobilis* has been known as an efficient producer of ethanol from glucose-based media.[1-5] However, an industrially important sugar alcohol, sorbitol, accumulated in quite high levels when either sucrose or a mixture of glucose and fructose was used as the carbon source during growth.[6-7]

The production of ethanol from batch fermentation of glucose or fructose is also high, 94% of theoretical yield[8], but the conversion from sucrose may be as low as 70% of theoretical yield. The yield decreases with increasing sucrose concentration[9]. This is a consequence of the formation of both sorbitol and levan as major and minor by-products respectively[6]. Although sorbitol was produced in culture medium containing both glucose and fructose, it was derived from fructose only.[7]

In this work two *Zymomonas* strains, one *Clostridium* strain and four *Saccharomyces* strains were tested in order to compare their sucrose hydrolysing capacities with respect to sorbitol production. The best source for sorbitol productivity has been selected to study the formation of sorbitol.

MATERIALS AND METHODS

Microorganism strains. The organisms used were *Zymomonas mobilis* (IFO 13756 and CM 141), *Clostridium* sp. 7M9, and *Saccharomyces cerevisiae* (bergandy, carlbergensis, elipsodeus and sake).

Media. Normal medium (NM) for the cultivation contained (per dm^{-3}) : yeast extract 10 g, peptone 10 g, KH_2PO_4 2 g, $MgSO_4.6H_2O$ 2 g, $(NH_4)_2SO_4$ 1 g, and sucrose 200 g (NM 1) or sucrose 250 g (NM 2). Each component was prepared in solution separately and the pH adjusted (for more and less than 6.5) before sterilization.

Cultivation conditions. Batch culture experiments were conducted in a flask with no shaking and in a fermentor with temperature controlled at 30°C, using a 10% inoculum. Only in the fermentor, N_2 gas was bubbled through the medium at flow rate of 120 cm^3/min.

Analysis. The samples of culture broth were centrifuged for 20 min at 6,000 rpm. The cell-free supernatant was filtered through a 0.2 μm membrane filter before analysis. The sugars and sorbitol were determined by HPLC (Waters Associates) using a carbohydrate column (3.9 mm×30 cm, Waters Associates no. 84034) with a mixture of acetonitrile : ethanol : water (90 : 5 : 5) as eluent (flow rate 1.5 cm^3 min^{-1}) at 25°C, using refractive index (LKB Pharmacia) as detector. Ethanol was analysed by GC (Perkin-Elmer) using a chromosorb 101 column (0.3 mm×200 cm) with nitrogen as carrier gas (flow rate 40 cm^3 min^{-1}) and flame ionization as detector.

RESULTS

Various microorganisms have been tested for sorbitol and ethanol production. The results are presented in Table 1 indicating that *Z. mobilis* IFO 13756 was the best source of sorbitol as well as ethanol and many organisms were good sources for ethanol production. The strains of *S. cerevisiae* did not produce sorbitol. Therefore, *Z. mobilis* IFO 23756 was chosen as a source for production of the two products.

The effect of each medium composition used in ethanol production[6-9] was studied in order to improve the productivity of sorbitol by fermentation of *Z. mobilis* IFO 23756. The results in Table 2 show that all of the nutrients were important to increase the yield of sorbitol; $MgSO_4$ provided the maximum effect. Table 3 shows that sucrose at a concentration of 250 g dm^{-3} in the cultivation medium could induce maximum yield of both sorbitol (24.5 g dm^{-3}) and ethanol (98.8 g dm^{-3}). These yields decreased with higher concentrations of sucrose (300-400 g dm^{-3}), whereas the cells could produce neither sorbitol nor ethanol from more than 300 g dm^{-3} total sugar with the mixture of glucose and fructose (1 : 1). Fermentation of glucose or fructose produced approximately equal yields of both sorbitol and ethanol. The productivities of sorbitol and ethanol from fermentation of glucose or fructose were higher than from the equimolar mixture of these sugars.

The relationship between cell growth and product formation during sucrose fermentation by *Z. mobilis* IFO 13756 is shown in Fig. 1. Sorbitol formation was parallel to cell growth which became maximum after 48 hours of incubation. The rate of ethanol production was faster than sorbitol formation and maximum on the first day of fermentation. The yield of ethanol was approximately constant at maximum level from 24 hours of incubation. Therefore, fermentation at 30°C for 48 hours was chosen as the optimum to determine the various effects on the production of sorbitol and ethanol.

TABLE 1. Sucrose fermentation of normal medium (NM 2) by various micro-organisms at 30°C, 48 hours.

Microorganisms	Sucrose left (g dm^{-3})	Fermentation products in cell-free supernatant (g dm^{-3})			
		Fructose	Glucose	Sorbitol	Ethanol
Z. mobilis IFO 13756	0	34.3	19.6	24.8	64.7
Z. mobilis CM 141	0	18.0	8.5	20.5	64.8
Cl. sp. 7M9	0	29.2	16.0	2.0	57.4
S. cerevisiae bergandy	66.0	11.3	8.7	0.0	62.2
S. cerevisiae carlbergensis	75.2	13.2	9.0	0.0	46.1
S. cerevisiae elipsodeus	89.0	10.0	6.5	0.0	48.6
S. cerevisiae sake	76.4	18.2	15.0	0.0	50.2

TABLE 2. Effect of nutrients on products formation in cell-free supernatant after fermentation with 1% inoculum at 30°C for 48 hours.

Cultivation medium	Products in cell-free supernatant (g dm^{-3})			
	Fructose	Glucose	Sorbitol	Ethanol
NM 1	1.3	0.0	21.3	82.9
NM 1 without yeast extract	14.0	0.0	17.5	59.2
NM 1 without peptone	8.3	0.0	18.5	80.6
NM 1 without KH_2PO_4	1.5	0.0	20.0	77.4
NM 1 without $MgSO_4$	1.5	0.0	15.0	72.7
NM 1 without $(NH_4)_2SO_4$	8.0	0.0	19.0	80.6

TABLE 3. Effect of nutrient sugars on productivity of sorbitol and ethanol by fermentation of *Z. mobilis* at 30°C, 48 hours.

Sugar concentration in medium (g dm^{-3})		Concentration in cell-free supernatant (g dm^{-3})			
		Fructose	Glucose	Sorbitol	Ethanol
Sucrose;	50	0	0	1.7	39.5
	100	1.0	0	5.5	51.4
	150	0.8	0	8.8	51.7
	200	1.3	0	21.3	82.9
	250	6.0	0	24.5	98.8
	300	18.5	0	20.0	72.6
	350	30.0	29.3	2.5	47.4
	400	33.3	98.0	0	23.7
Glucose:Frutose;	50: 50	0	0	2.5	32.5
	100:100	7.8	56.7	6.0	45.0
	150:150	57.6	75.0	0	0
	200:200	77.5	124.0	0	0
	250:250	12.0	0	0	0
Glucose;	50	0	0	2.8	22.2
	100	0.4	1.0	3.3	46.6
	150	0.8	45.0	8.4	56.9
	200	5.5	90.5	0	39.5
	250	21.0	145.0	0	27.6
Fructose;	50	0	1.1	2.0	24.3
	100	0	0.9	2.0	46.6
	150	5.0	1.3	2.0	60.8
	200	76.0	23.0	5.0	48.3
	250	156.0	35.0	3.8	18.2

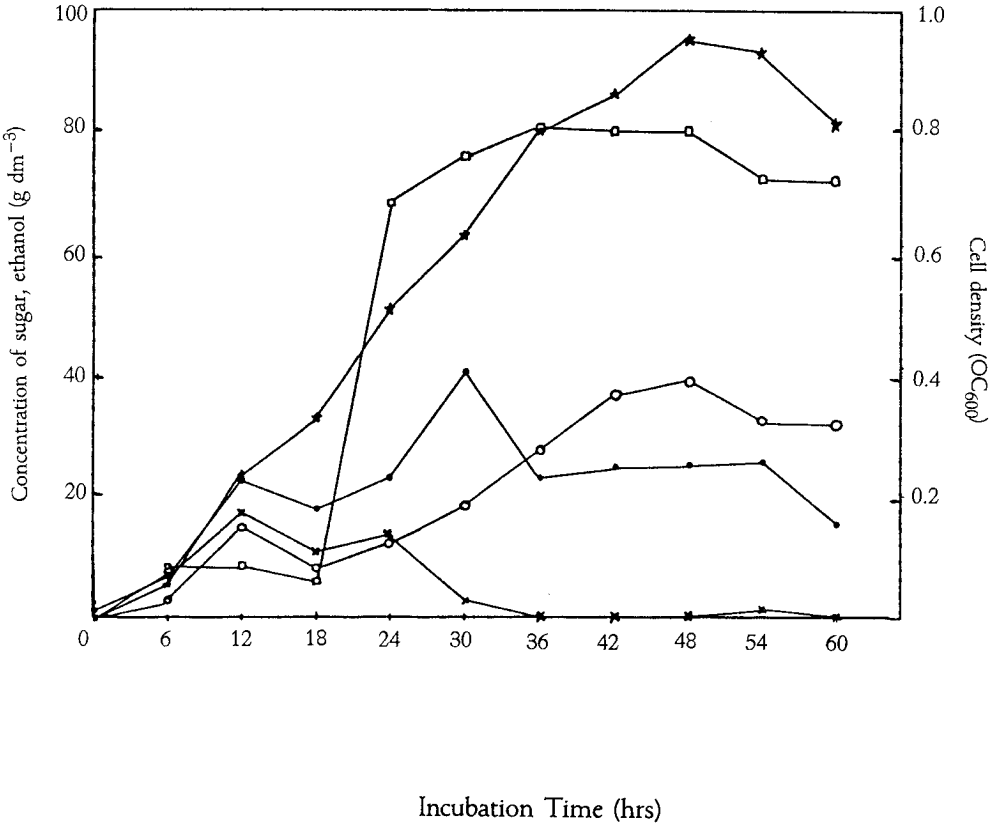

Fig. 1. Time course of sucrose fermentation by *Z. mobilis* IFO 13756, 1% inoculum at 30°C in batch without shaking ; ★_____★ growth of cell; ●_____● fructose ; x_____x glucose ; o_____o sorbitol ; □_____□ ethanol.

The effect of pH on sorbitol and ethanol production from fermentation of *Z. mobilis* IFO 13756 is shown in Table 4. The yields of sorbitol and ethanol were maximal at pH 7.5 and 6.5, respectively. Table 5 shows that the optimum temperature of fermentation for sorbitol and ethanol production was 30°C. Many nutrients have been added to the normal fermentation medium. The results in Table 6 show that none of the added substances could increase the yield of sorbitol whereas 1% methanol provided the higher productivity of ethanol. Table 7 indicates that the addition of ethanol to the nutrients of shaking or non-shaking cultures reduced the hydrolysis of sucrose, the utilization of glucose and fructose, and the formation of sorbitol and ethanol. By increasing the quantity of added ethanol from 3.95 g dm^{-3} to 18.5 g dm^{-3} the yield of sorbitol decreased from 23.6% to 18.9% of sorbitol in normal medium (without addition of ethanol) and from 46.7% to 43.8% of sorbitol in rich medium with non-shaking and shaking cultures respectively. Conversely, according to the quantity of added ethanol, the yield of ethanol was increased from 41.0 g dm^{-3} to 69.7 g dm^{-3} and from 31.6 g dm^{-3} to 55.8 g dm^{-3} in non-shaking and shaking cultures, respectively. The production of ethanol was lower than in normal medium. However, the yield of ethanol increased progressively until it became equal to that in normal medium when 31.60 g dm^{-3} ethanol was added to the non-shaking culture. The result of adding glucose to normal medium shown in Table 8 indicates that sorbitol production was completely inhibited by 50 g dm^{-3} or more of added glucose whereas ethanol production was progressively decreased compared with the normal medium.

Table 9 shows that oxygen plays an important role in ethanol production but has a lower effect on sorbitol production. The yield of ethanol was 10.4 g dm^{-3} higher in a highly oxygenated culture, and the production of sorbitol was 0.6 g dm^{-3} lower in the more oxygenated culture.

The results in Table 10 indicate that 9-10% inoculum provided the maximum yield of sorbitol (33.9 g dm^{-3}) whereas 3% inoculum induced the maximum yield of ethanol (48.8 g dm^{-3}). However, at 10% inoculum, the yield of ethanol is slightly decreased compared to the increase in sorbitol production.

The time course of sucrose fermentation in the culture flask without shaking (Fig. 1) and in the nitrogenated fermentor (Fig. 2) were compared. The cells in the fermentor (OD_{600} ~ 1.8) grew two times better than in culture flask (OD_{600} ~ 0.9). This means the growth of cells had no need of oxygen. The yield of sorbitol was higher in the culture flask, indicating that the production of sorbitol may need oxygen. The production of ethanol in both systems was nearly the same since oxygen is not necessary for the pathways of ethanol formation.

DISCUSSION

It is known that *Zymomonas mobilis* utilizes sucrose via glucose plus fructose to ethanol and sorbitol.[7] However, detailed studies on the fermentation pattern and the efficiency of this conversion by *Z. mobilis* IFO 13756 have never been carried out. The aim of this study was to improve the productivity of sorbitol at relatively high rates of ethanol production.

TABLE 4. Effect of cultivation medium pH on sucrose fermentation at 30°C, 48 hours.

pH of media NM 2	Products in cell-free supernatant (g dm^{-3})			
	Fructose	Glucose	Sorbitol	Ethanol
2.0	35.0	41.0	0.0	22.9
4.0	62.5	77.5	0.0	18.2
6.0	34.3	19.6	24.8	34.8
6.5	26.5	10.2	25.3	44.2
7.0	27.5	11.5	28.0	38.7
7.5	22.3	10.5	28.8	36.0
8.0	19.0	12.0	28.3	32.4
8.5	14.5	6.0	21.0	30.1
9.0	2.5	2.2	2.2	4.5
10.0	2.5	2.8	1.7	2.7
12.0	0.0	0.0	0.0	0.8

TABLE 5. Effect of temperature on sucrose fermentation by *Z. mobilis* IFO 13756 for 48 hours.

Temperature (°C)	Sucrose left (g dm^{-3})	Products in cell-free supernatant (g dm^{-3})			
		Fructose	Glucose	Sorbitol	Ethanol
20	16.3	25.9	60.7	18.5	24.7
30	6.2	15.4	48.0	27.6	72.8
40	0	34.9	89.3	13.2	54.4
50	0	35.2	85.2	0	12.4
60	35.5	21.2	55.3	0	2.1

TABLE 6. Effect of various substances in normal medium (NM 2) on sucrose fermentation by *Z. mobilis* IFO 13756 at 30°C for 48 hours.

Added substances	Products in cell-free supernatant (g dm^{-3})			
	Fructose	Glucose	Sorbitol	Ethanol
none	24.5	0.0	38.0	79.0
0.30% xylose	17.2	0.0	24.0	67.1
1.00% methanol	7.6	0.0	16.0	82.9
0.02% PEG 6,000	18.2	0.0	16.2	77.4
0.03% xylose + 1.00% methanol + 0.02% PEG 6,000	32.0	0.0	26.7	77.4

TABLE 7. Effect of adding ethanol to rich medium on sucrose fermentation by *Z. mobilis* at 30°C for 48 hours.

Added ethanol (g dm^{-3})	Aeration	Concentration in cell-free supernatant (g dm^{-3})				
		Sucrose	Fructose	Glucose	Sorbitol	Ethanol*
0.00	non-shaking	0.0	14.4	0.0	15.2	71.1
3.95	non-shaking	55.2	16.3	31.7	11.6	45.0
7.90	non-shaking	53.8	15.0	30.5	10.2	66.7
11.85	non-shaking	50.8	14.3	28.5	8.7	75.9
15.80	non-shaking	56.0	14.5	27.4	8.1	85.5
23.70	non-shaking	65.0	13.7	27.3	9.9	99.3
31.60	non-shaking	69.2	11.2	23.3	5.3	102.7
0.00	shaking	5.5	15.8	0.0	13.7	63.2
3.95	shaking	70.5	20.8	42.0	11.1	35.6
7.90	shaking	70.4	17.7	35.0	9.3	53.1
11.85	shaking	64.0	16.5	34.7	7.9	66.1
15.80	shaking	64.2	15.6	31.3	7.7	71.6

* Total quantity of ethanol which included the added amount.

TABLE 8. Effect of adding glucose to normal (NM 2) medium on sucrose fermentation by *Z. mobilis* IFO 13756 at 30°C for 48 hours.

Added glucose (g dm^{-3})	Concentration in cell-free supernatant (g dm^{-3})				
	Sucrose	Fructose	Glucose	Sorbitol	Ethanol
0.0	19.2	33.5	7.5	24.7	59.3
25.0	28.7	26.0	68.9	15.9	44.3
50.0	38.3	21.3	81.6	0.0	36.8
75.0	47.1	12.9	85.5	0.0	23.0
100.0	57.9	12.1	96.3	0.0	23.0
125.0	67.2	14.7	117.8	0.0	23.4

TABLE 9. Effect of oxygen on sucrose fermentation by *Z. mobilis* IFO 13756 at 30°C for 48 hours.

Oxygen in cultivation medium (NM 2)	Concentration in cell-free supernatant (g dm^{-3})				
	Sucrose	Fructose	Glucose	Sorbitol	Ethanol
Oxygen limited by tightly screw cap	2.6	31.3	87.1	33.3	57.5
Oxygen limited by loosely screw cap	1.5	27.1	54.8	33.9	47.1

TABLE 10. Concentration of sucrose and products in cell-free supernatant after fermentation in loosely screw cap flask by *Z. mobilis* IFO 13756 at 30°C, 48 hours using various volumes of inoculum.

% (v/v) inoculum	Concentration in cell-free supernatant (g dm^{-3})				
	Sucrose	Fructose	Glucose	Sorbitol	Ethanol
0.1	103.8	19.7	62.2	0.0	33.2
0.5	54.7	21.8	67.0	12.7	45.2
1.0	46.9	15.4	81.4	14.6	45.0
3.0	13.8	21.4	74.1	26.8	48.8
5.0	11.6	27.5	39.8	29.4	46.3
10.0	1.5	24.3	27.9	33.9	47.1
15.0	0.0	27.1	54.8	32.7	37.6

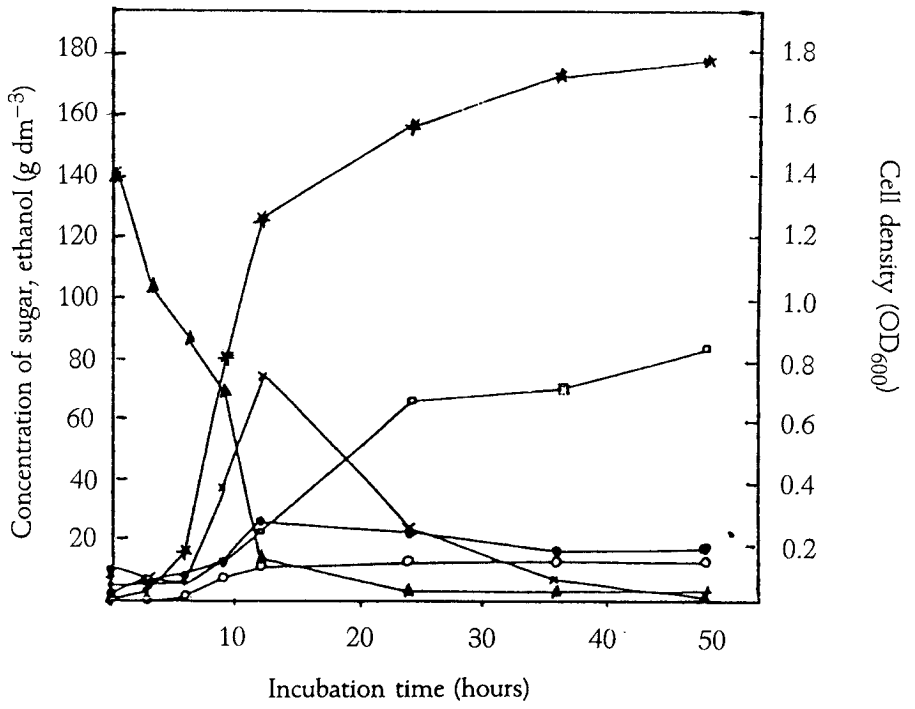

Fig. 2. Hydrolysis of sucrose and products formation during fermentation of 25% sucrose in medium by *Z. mobilis* in fermentor 2.7 dm^3 30°C, nitrogen bubbling through medium with flow rate of 120 cm^3 min^{-1}; ★_____★ growth rate ; ▲_____▲ sucrose ; ●_____● fructose ; x_____x glucose ; o_____o sorbitol ; □_____□ ethanol.

The results presented demonstrate that the most favorable conditions for sorbitol production should be fermentation of 250 g dm^{-3} sucrose in medium at pH 7.5 which was inoculated with a 10% inoculum and incubated at 30°C. Some of these conditions should be optimized such as pH (7.0) and inoculum volume (10%) to get high productivity yield of both sorbitol and ethanol. Many nutrient components have been tested for their effect on productivity of the two fermentation products. The composition of normal medium (NM 2) was the best nutrient for sorbitol and ethanol production.

In order to study the formation of sorbitol and ethanol by sugar fermentation of *Z. mobilis* IFO 13756, three sugars, glucose, fructose and sucrose, have been used in the culture medium. Since sucrose was partially hydrolysed to glucose and fructose during sterilization of cultivation medium[3], the productivity of sorbitol and ethanol from sucrose (100 and 200 g dm^{-3}) was not equal to that using an equimolar mixture of glucose and fructose (50 : 50 and 100 : 100 g dm^{-3}). When only glucose or fructose was used alone, less sorbitol was formed than using an equimolar mixture of the two sugars or sucrose. This behavior was silimar to that of other strains[6], and indicates the existence of both sorbitol dehydrogenase and aldose reductase in *Z. mobilis* IFO 13756. According to Doelle[9] the uptake mechanisms of glucose and fructose are controlled by different regulatory systems: glucose uptake is controlled by the energy balance and fructose uptake by glucose. Fructokinase is strongly inhibited by glucose, which blocks the normal metabolic pathway of fructose. The present results lead to the same suggestion as has been reported earlier[10] that when fructokinase is inhibited by glucose, fructose is utilized through reduction to sorbitol by sorbitol dehydrogenase. The reduction of fructose has to be coupled with the oxidation of NADH to NAD+. Therefore, from an energetic point of view, formation and accumulation of sorbitol are uneconomical for the cell[10]. This has been confirmed by fermentation of *Z. mobilis* IFO 13756 in a fermentor, where less sorbitol was formed than in the culture in flask but the yield of biomass was higher, whereas ethanol was produced with approximately equal yields. Therefore, high sorbitol yield could be expected to be associated with low yield of biomass. Oxygen is also another important factor in sorbitol production. Sorbitol yield in the culture flask with a loose screw cap was 0.6 g dm^{-3} lower than in the culture with a tight screw cap. Conversely, in a fermentor with nitrogen bubbling there was a lower yield of sorbitol ($\sim$13 g dm^{-3}) than in a culture flask with tight a loose screw caps ($\sim$33 g dm^{-3}).

In conclusion, it is possible to improve the yield of sorbitol at relatively high ethanol production by the *Zymomonas mobilis* strain if suitable fermentation conditions are optimized and an appropriate level of oxygenation is established in the culture medium.

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บทคัดย่อ

จากการเปรียบเทียบแบคทีเรีย *Zymomonas* สองสายพันธุ์ในแง่ประสิทธิภาพของการไฮโดรไลส์ซูโครส และการผลิตซอร์บิทอลและเอธานอล ได้เลือกแบคทีเรีย *Z. mobilis* IFO 13756 เพื่อศึกษาการหมักน้ำตาลเนื่องจากแบคทีเรียชนิดนี้สามารถผลิตซอร์บิทอลได้ในปริมาณสูงในขณะที่ผลผลิตเอธานอลยังคงมีปริมาณสูงเหมือนเดิม ผลผลิตของซอร์บิทอลจะสูงสุดเมื่อเลี้ยง *Z. mobilis* IFO 13756 ในอาหารที่มีพีเอช 7.5 ซึ่งใช้ซูโครส 250 กรัม ดม$^{-3}$ เป็นแหล่งของคาร์บอนและปริมาณหัวเชื้อ 10% ทำการเลี้ยงที่ 30°ซ ภาวะของการได้ผลผลิตเอธานอลสูงสุดทำโดยเลี้ยงจุลินทรีย์ในอาหารแบบเดียวกับที่มีพีเอช 6.5 และปริมาณหัวเชื้อ 3% การเติมสารประกอบบางชนิด อาทิ ไซโลส PEG กลูโคสและเอธานอลลงในอาหารเลี้ยงเชื้อจะทำให้ผลผลิตของทั้งซอร์บิทอลและเอธานอลลดต่ำลง ปริมาณอ๊อกซิเจนที่เหมาะสมในอาหารเลี้ยงเชื้อมีผลต่อการเจริญและการเกิดซอร์บิทอลและเอธานอล