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Zymomonas mobilis As Catalyst for the Biotechnological Production of Sorbitol and Gluconic Acid

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Abstract

The conversion of glucose and fructose into gluconic acid (GA) and sorbitol (SOR) was conducted in a batch reactor with free (CTAB-treated or not) or immobilized cells of *Zymomonas mobilis*. High yields (more than 90%) of gluconic acid and sorbitol were attained at initial substrate concentration of 600 g/L (glucose plus fructose at 1:1 ratio), using cells with glucose-fructose-oxidoreductase activity of 75 U/L. The concentration of the products varied hyperbolically with time according to the equations $(GA) = t\,(GA)_{max}/(W_{GA} + t)$, $(SOR) = t\,(SOR)_{max}/(W_{SOR} + t)$, $v_{GA} = [W_{GA}\,(GA)_{max}]/(W_{GA} + t)^2$ and $v_{SOR} = [W_{SOR}\,(SOR)_{max}]/(W_{SOR} + t)^2$. Taking the test carried out with free CTAB-treated cells as an example, the constant parameters were $(GA)_{max}$ = 541 g/L, $(SOR)_{max}$ = 552 g/L, W_{GA} = 4.8 h, W_{SOR} = 4.9 h, v_{GA} = 112.7 g/L·h and v_{SOR} = 112.7 g/L·h.

Index Entries: *Zymomonas mobilis*; sorbitol; gluconic acid.

Introduction

Sorbitol (SOR) and gluconic acid (GA) are compounds employed in food (SOR as humectant, softener, and texturizer, whereas GA as coagulant in tofu production and in the prevention of milkstone and beerstone in dairy and brewing products, respectively), pharmaceutical (SOR as flavor enhancer and a tablet excipient, whereas GA as a replenisher of magnesium, calcium, and ferrous ions) and chemical (SOR in the synthesis of sorbose, ascorbic acid, and propylene glycol, whereas GA in galvanoplasty and in the textile printing process) industry.

Zymomonas mobilis, an anaerobic, gram-negative, and nonpathogenic bacterium, has in the periplasmic space of the cell envelope the glucose-fructose-oxidoreductase (GFOR), which converts glucose (G) and fructose

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(F) in GA and SOR, respectively (1). The GFOR has tightly linked in its structure the NADP, which is reduced to $NADPH_2$ during the glucose oxidation to glucono-δ-lactone and oxidized again by the reduction of fructose to sorbitol (1). The cyclic nature of GFOR catalysis is quite advantageous because the cofactor, generally an expensive reagent, is not consumed. Moreover, the enzymatic conversion is less polluting and health hazardous than the fructose/sorbitol nickel catalyzed hydrogenation-(carried out at 150°C and pressure of 40–50 atm) and more simple than the glucose/gluconic acid fermentative oxidation by fungi of the genus *Aspergillus*. Despite the technical advantages of the GFOR catalyzed bioconversion over the fermentation and chemical synthesis, the market prices for GA and SOR combined with the production overall costs have not been favorable to the biotechnological process worldwide (1). However, according to Silveira and Jonas (1) the biotechnological production of sorbitol is economically possible in at least some countries. This aspect stimulates further studies about G-F/GA-SOR bioconversion (2).

The conspicuous localization of GFOR in *Z. mobilis* cell, the establishment of GFOR catalytic mechanism and the large data available on the biochemistry of this bacterium has stimulated its use in the G-F/GA-SOR bioconversion carried out in reactors operated through batch, fed-batch, or continuous process. Yields greater than 90% were attained by using permeabilized or not, free or immobilized cells of *Z. mobilis* through continuous and discontinuous processes (1).

From the data published in the literature it can be seen that the formation of GA and SOR along the reaction time follows a hyperbolic pattern when the bioconversion is carried out with an initial substrate concentration (glucose + fructose) higher than 300 g/L (3–4). Based on this result a mathematical model might be developed aiming to quantify the G-F/GA-SOR bioconversion as far as nothing about this subject was found in the literature.

The present work aims to establish a quite simple mathematical model for estimating the maximum amount of GA and SOR that might be attained through the bioconversion catalyzed by free (permeabilized or not) and immobilized *Z. mobilis* cells.

Materials and Methods

Microorganism and Culture Conditions

Z. mobilis ATCC 29191 was maintained and cultivated anaerobically as described previously (5). The standard medium (SM) contained (per L): 2 g $(NH_4)_2SO_4$, 1 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4$, 3.5 g KH_2PO_4, 5 g Bacto yeast extract, 0.2 g sodium citrate, and 150 g glucose. Batch fermentation was performed in 20-L fermentor (BIOSTAT ED, B. Braun Diessel Biotech, Melsungen, Germany) containing 14 L of sterilized SM and inoculated with 1 L of inoculum (4.5 g dry matter). The culture was carried out under

anaerobic conditions at 30°C, impeller speed 400 rpm and pH 5.5 (adjusted with MES-buffer (2-[*N*-Morpholino]ethanesulfonic acid).

Cell Treatment With CTAB

The cell permeation using CTAB (cetyl trimethyl ammonium bromide) was accomplished as follows: 160 µL of CTAB solution (3 g/L) were added to a cell suspension of 9 g dry matter/L under agitation of 300 rpm at 4°C. After 10 min of stirring, the mixture was centrifuged (9000*g*; 10 min) and the supernatant discharged. The cake was suspended in 80 mL of distilled water and employed in the bioconversion test.

Cell Immobilization

The cell immobilization was carried out as described previously *(6)*. Almost 4 g of sodium alginate (Satialgine S1100X, viscosity of 550 cp and mannuronic acid/guluronic acid ratio of 1:5, purchased from SKW-Biosystems, Trenton, NJ) were dissolved in 100 mL of distilled water, followed by the addition of 100 mL of cell suspension (30 g dry matter/L). The final suspension was left under agitation of 150 rpm for 2 h. Then, the cell suspension was totally dropped into 0.3 *M* $CaCl_2$ solution for obtaining calcium alginate beads (external diameter of 2 mm) with *Z. mobilis* cells entrapped. The cell-entrapped beads were maintained immersed in distilled water at 4°C till their use in the bioconversion test. Not less than 95% of the initial cell concentration was entrapped inside the calcium alginate beads, as determined by counting the free cells present in $CaCl_2$ solution through a Neubauer chamber ($1/400\ mm^2 \times 0.1\ mm$).

Glucose-Fructose-Oxidoreductase Activity

The following assay conditions were defined: cell concentration, 30 g dry matter/L; substrate concentration, 180 g of glucose/L and 180 g of fructose/L; temperature, 39°C; pH, 6.4 (1.0 *M* phosphate/citrate buffer); reaction time, 20 min. A volume of 1.6 mL of the substrate solution (containing glucose and fructose) was mixed with 0.4 mL of the cell suspension. After 20 min the reaction was stopped by immersing the test tube in a boiling-water bath for 1 min, followed by centrifugation for 10 min at 8000*g*. The supernatant was collected and stored at 4°C for the determination of gluconic acid. The buffer solution without sugars was used as a blank. One GFOR unit (U) was defined as the amount of enzyme catalyzing the formation of 1 g gluconic acid/h at 39°C. Cells used in all tests had a GFOR activity around 75 U/L (2.5 U/g of dry matter) regarding gluconic acid.

Bioconversion Tests

In a 250-mL batch reactor were mixed the 600 g/L substrate solution (glucose and fructose, ratio 1:1, dissolved in 0.5 MES/MES.K buffer, pH 6.2)

and the suspension containing free (CTAB-permeated or not cells) or Ca-alginate entrapped cells. The reaction was carried out at 39°C, 300 rpm and the pH maintained at 6.2 by the addition of 2 *M* NaOH. In all tests the suspension had a total cell concentration of 30 g dry matter/L. All tests were made in triplicate and the whole volume of samples taken for analytical purposes was lower than 5% of the reacting medium inside the reactor.

Assuming that one molecule of glucose reacts with one molecule of fructose giving one molecule of gluconic acid and one molecule of sorbitol, respectively, then the yield regarding gluconic acid (R_{GA}) and sorbitol (R_{SOR}) formed were calculated, respectively, through the following equations:

$$R_{GA} = (Y_{G/GA} \,/\, 1.09) \times 100 \quad (1)$$

$$R_{SOR} = (Y_{F/SOR} \,/ 1.01) \times 100 \quad (2)$$

where $Y_{G/GA}$ (glucose/gluconic acid conversion factor) = $(GA_{final} - GA_{initial})/(G_{initial} - G_{final})$; $Y_{F/SOR}$(fructose/sorbitol conversion factor) = $(SOR_{final} - SOR_{initial})/(F_{initial} - F_{final})$; ($GA_{final}$, G_{final} , SOR_{final} and F_{final}) and ($GA_{initial}$, $G_{initial}$, $SOR_{initial}$ and $F_{initial}$) are, respectively, the final and initial concentrations (g/L) of gluconic acid, glucose, sorbitol and fructose.

Analytical Techniques

The cell dry matter was determined according to Neves et al. *(7)*. Gluconic acid was assayed enzymatically using the test kit of Boehringer (Mannheim, Germany) *(8)*. Glucose, fructose, and sorbitol concentrations were analysed by using a Merck (Darmstadt, Germany) HPLC with a Eurokat-Pb (KANAUER, Munich, Germany) 300 × 8 column and a software (Merck D-6000) was used for data retrieval and analysis. Ethanol was determined by gas chromatography (Hewlett-Packard HP5890-II, Palo Alto, CA) with a HP-FFAP capillary column.

Results and Discussion

From Table 1, it can be seen that the Ca-alginate entrapped and free (CTAB-treated or not) cells converted glucose and fructose, respectively, in gluconic acid and sorbitol at a yield higher than 90% as well as no ethanol formation occurred. These results are in accordance with the data already published *(1)*. Figures 1–3 show that the curves related to the variation of SOR and GA concentrations along the reaction in all tests studied had a hyperbolic-like profile. Therefore, the following generic equations can be written:

$$(GA) = t\,(GA)_{max} / (W_{GA} + t) \quad (3)$$

$$(SOR) = t\,(SOR)_{max} / (W_{SOR} + t) \quad (4)$$

Table 1
Formation of Sorbitol (SOR), Ethanol (E), and Gluconic Acid (GA) in the Batch Bioconversion Catalyzed by Nonpermeabilized Free Cells (Test 01), CTAB-Permeabilized Free Cells (Test 02), and Ca-Alginate Immobilized Cells (Test 03)

Test parameters	01	02	03
S (g/L)[a]	600	600	600
(SOR) (g/L)	290	290	290
(GA) (g/L)	288	290	299
(E) (g/L)	0	0	0
R_{GA} (%)	95	92	90
R_{SOR} (%)	95.1	93	90

[a]Initial substrate concentration constituted by 50% (w/w) of glucose and fructose.

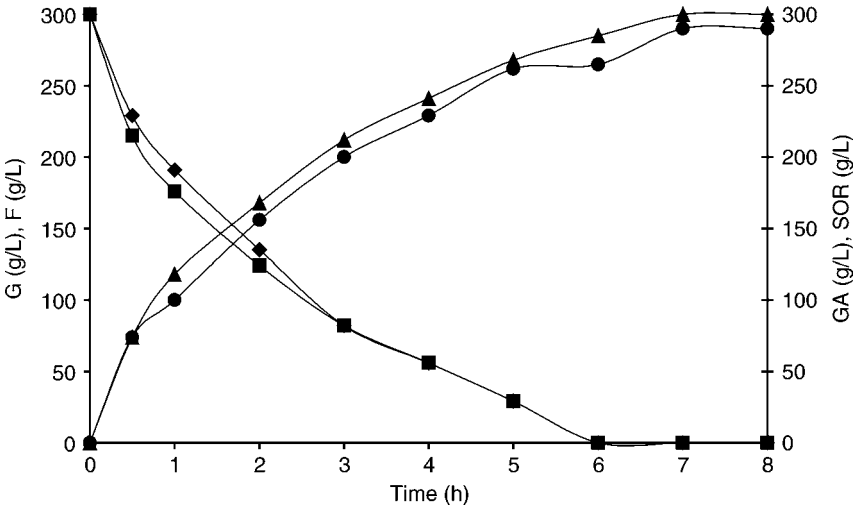

Fig. 1. Variation of glucose (G; ♦), fructose (F; ■), gluconic acid (GA; ▲) and sorbitol (SOR; •) concentrations in a biotransformation performed with whole cells of *Z. mobilis* at initial substrate concentration (glucose plus fructose at 1:1 ratio) equal to 600 g/L (Test 01). The linear equations related to (1/GA) × (1/t) and (1/SOR) × (1/t) are, respectively, $1/(GA) = 5.72 \times 10^{-3}t^{-1} + 2.69 \times 10^{-3}$ ($r = 0.998$) and $1/(SOR) = 5.65 \times 10^{-3}t^{-1} + 3.24 \times 10^{-3}$ ($r = 0.99$).

where (GA) = gluconic acid concentration (g/L) during the reaction time (h); $(GA)_{max}$= maximum gluconic acid concentration (g/L); (SOR) = sorbitol concentration (g/L) during the reaction time (h); $(SOR)_{max}$= maximum sorbitol concentration (g/L); W_{GA} and W_{SOR} are constants representing the time to which the gluconic acid and sorbitol concentration, respectively, correspond to the half of maximal concentration achieved for each of them. Moreover, an estimate of the GFOR activity regarding the production

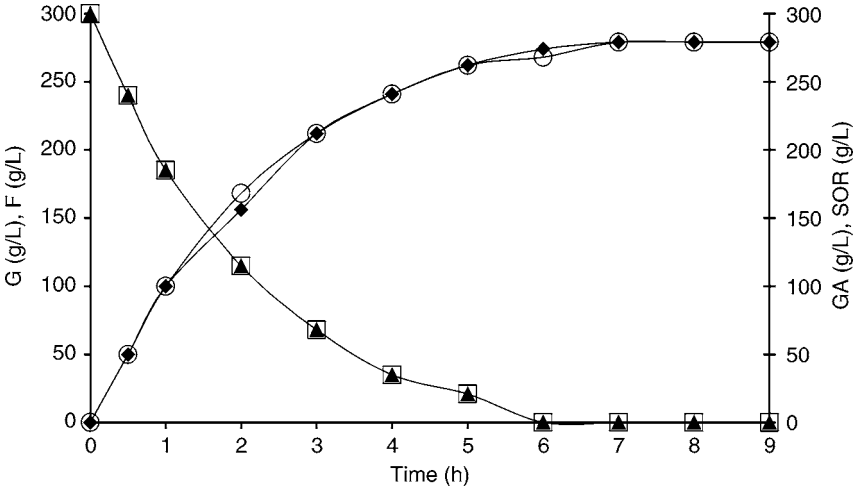

Fig. 2. Variation of glucose (G; ▲), fructose (F; O), gluconic acid (GA; ♦) and sorbitol (SOR; O) concentrations in a biotransformation performed with whole cells of *Z. mobilis* treated with CTAB. The initial substrate concentration (glucose plus fructose at 1:1 ratio)) was 600 g/L (Test 02). The linear equations related to (1/GA) × (1/t) and (1/SOR) × (1/t) are, respectively, $1/(GA) = 8.91 \times 10^{-3}t^{-1} + 1.85 \times 10^{-3}$ (r = 0.998) and $1/(SOR) = 8.91 \times 10^{-3}t^{-1} + 1.81 \times 10^{-3}$ (r = 0.997).

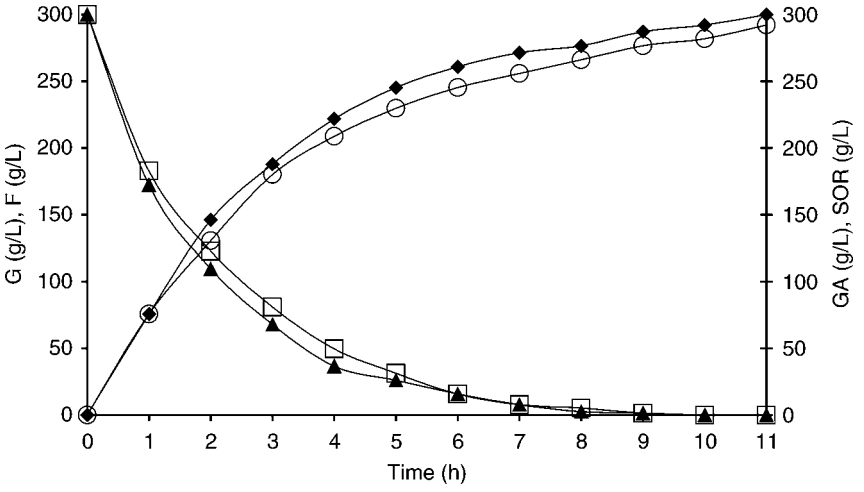

Fig. 3. Variation of glucose (G; ▲), fructose (F; O), gluconic acid (GA; ♦) and sorbitol (SOR; O) concentrations in a biotransformation performed with whole cells of *Z. mobilis* entrapped into calcium alginate beads. The initial substrate concentration (glucose plus fructose at 1:1 ratio)) was 600 g/L (Test 03). The linear equations related to (1/GA) × (1/t) and (1/SOR) × (1/t) are, respectively, $1/(GA) = 1.07 \times 10^{-2}t^{-1} + 2.09 \times 10^{-3}$ (r = 0.994) and $1/(SOR) = 1.07 \times 10^{-2}t^{-1} + 2.31 \times 10^{-3}$ (r = 0.998).

of gluconic acid and sorbitol along the reaction could be attained at each time through the derivatives of Eqs. 3 and 4, which are respectively

Table 2
Constant Parameters Related to the Formation of Gluconic Acid and Sorbitol From Glucose and Fructose, Respectively, in Bioconversions Catalyzed by Free and Immobilized *Z. mobilis* Cells

Test (no.)	$(GA)_{max}$ (g/L)	$(SOR)_{max}$ (g/L)	W_{GA} (h)	W_{SOR} (h)	$(GA)_{max}/(SOR)_{max}$	W_{GA}/W_{SOR}	v_{GA} (g/L·h)	v_{SOR} (g/L·h)
01	372	309	2.1	1.8	1.2	1.2	177.1	177.7
02[a]	541	552	4.8	4.9	0.98	0.98	112.7	112.7
03[b]	478	432	5.1	4.6	1.1	1.1	93.7	93.7

[a]The cells were treated with CTAB.
[b]The cells were entrapped into calcium alginate beads.
It were also presented the initial GFOR activity [calculated through Eqs. 5 and 6, which at t = 0 h become $v_{GA} = (GA)_{max}/W_{GA}$ and $v_{SOR} = (SOR)_{max}/W_{SOR}$, respectively] related to the production of gluconic acid (v_{GA}) and sorbitol (v_{SOR}).

$$v_{GA} = d(GA)/dt = [W_{GA} .(GA)_{max}]/(W_{GA} + t)^2 \quad (5)$$

$$v_{SOR} = d(SOR)/dt = [W_{SOR} .(SOR)_{max}]/(W_{SOR} + t)^2 \quad (6)$$

where v_{GA} and v_{SOR} are the GFOR activity related to gluconic acid and sorbitol, respectively.

Regarding Eqs. 5 and 6, two aspects might be borne out. First, the Eqs. 5 and 6 at $t = 0$ h become, respectively, $v_{GA} = (GA)_{max}/W_{GA}$ and $v_{SOR} = (SOR)_{max}/W_{SOR}$, which should represent the maximal initial GFOR activity presented by a particular biocatalyst (in the present work, CTAB non-treated, CTAB-treated and immobilized *Z. mobilis* cells) regarding the formation of both products (gluconic acid and sorbitol). So that, these ratios could allow attaining a first glance on the catalytic potential of the biocatalyst in bioconversions conducted under fixed initial substrate concentration (glucose plus fructose at 1:1 ratio), in the present case equal to 600 g/L. Accordingly, for tests 01, 02, and 03, v_{GA} is approx v_{SOR} and equal to 177.1, 112.7 and 93.7 g/L·h, respectively (Table 2). Second, coupling the v_{GA} and v_{SOR} values (calculated by Eqs. 5 and 6 and related to the GFOR activity needed) with the consumption of glucose and fructose at each time along the reaction (taken from Figs. 1–3, by subtracting G and F concentrations from 300 g/L at a desired time), it could be possible setting the suitable amount of substrate to be added into the bioreactor, when the bioconversion was planned to be conducted as a fed-batch process.

Through plots of $1/(GA) \times 1/t$ and $1/(SOR) \times 1/t$ the linear equations were established from which the parameters $(GA)_{max}$, W_{GA}, $(SOR)_{max}$ and W_{SOR} were calculated for all tests. The correspondent equations are presented in the captions of Figs. 1–3.

From Table 2 it can be seen that cells treated with CTAB (test 02) had $(GA)_{max}$, $(SOR)_{max}$, W_{GA} and W_{SOR}, as 31, 44, 56, and 64% higher than those

of nontreated cells (test 01), respectively. Undoubtedly, the CTAB treatment increases the cell wall fluidity for substrates (glucose and fructose) and products (sorbitol and gluconic acid). It could also be considered that the CTAB treatment did not affect significantly the GFOR catalysis, as the ratios $(GA)_{max}/(SOR)_{max}$ and W_{GA}/W_{SOR} for test 02 were near one (Table 2). Moreover, for test 03 the ratios $(GA)_{max}/(SOR)_{max}$ and W_{GA}/W_{SOR} both equal to 1.1 were quite similar to those of tests 01 and 02 (Table 2), an indication, even so indirect, that the immobilization did not change significantly the GFOR catalytic pattern. Thereby, an enlargement of the feasibility of the G-F/GA-SOR bioconversion might be achieved by using immobilized cells because the reaction can be conducted through continuous processes using different kinds of reactors (continuous stirred tank reactor, membrane reactor, fluidized, or packed bed reactor) *(3,9)*. In addition, an immobilized system could facilitate a future scale-up of this bioconversion.

Finally, the constants W_{GA} and W_{SOR}, which have the dimension of time (h), correspond to the moment of the reaction in which (GA) = $(GA)_{max}/2$ and (SOR) = $(SOR)_{max}/2$, respectively. The times to which a particular GA and SOR concentrations are attained, could be considered as the starting points to set the residence time, when the bioconversion is planned to be realized through a continuous process. As two products are involved, so the average between W_{GA} and W_{SOR} would be a reasonable procedure to set the residence time. Accordingly, the average time for tests 01, 02, and 03 are 1.95, 4.85, and 4.85 h, respectively (Table 2).

Conclusions

Yields greater than 90% for the G-F/GA-SOR bioconversion were attained using either free (CTAB-treated or not) or Ca-alginate entrapped *Z. mobilis* cells. The profiles of the curves (GA) × *t* and (SOR) × *t* were described by hyperbolic equations, from which the maximum amount of GA and SOR formed might be calculated, provided that the initial substrate concentration (glucose plus fructose at 1:1 ratio) was not less than 600 g/L.

References

1 Silveira, M. M. and Jonas, R. (2002), *Appl. Microbiol. Biotechnol.* **59,** 400–408.
2 Jonas, R. and Silveira, M. M. (2004), *Appl. Biochem. Biotechnol.* **118,** 321–336.
3 Ferraz, H. C., Borges, P. C., and Alves, L. M. (2000), *Appl. Biochem. Biotechnol.* **89,** 43–53.
4 Shene, C. and Bravo, S. (2001), *Appl. Microbiol. Biotechnol.* **57,** 323–328.
5 Erzinger, G. S., Silveira, M. M., Vitolo, M., and Jonas, R. (1996), *World J. Microbiol. Biotechnol.* **12,** 22–24.
6 Carvalho, W., Silva, S. S., Converti, A., and Vitolo, M. (2002), *Biotechnol. Bioeng.* **79,** 1–10.
7 Das Neves, L. C. M., Pessoa A. Jr., and Vitolo, M. (2005), *Biotechnol. Prog.* **21,** 1136–1139.
8 Rehr, B., Wilhelm, C., and Sahm, H. (1991), *Appl. Microbiol. Biotechnol.* **35,** 144–148.
9 Tomotani, E. J., Das Neves, L. C. M., and Vitolo, M. (2005), *Appl. Biochem. Biotechnol.* **121,** 149–158.