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ACTIVITY OF GLUCOSE-FRUCTOSE OXIDOREDUCTASE IN FRESH AND PERMEABILISED CELLS OF *ZYMOMONAS MOBILIS* GROWN IN DIFFERENT GLUCOSE CONCENTRATIONS

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ABSTRACT

Previously grown cells of the ethanologenic bacterium *Zymomonas mobilis* produce sorbitol and gluconic acid, in reactions catalysed by the periplasmic enzymes glucose-fructose oxidoreductase (GFOR) and glucono-δ-lactonase (GL). The GFOR/GL system activity, in cells to be used in this bioconversion, depends on growth conditions. In batch runs, with initial glucose concentrations (S_0) from 42 to 230 g/L, the highest specific and total GFOR/GL activities were obtained with S_0 = 153 g/L (12.6 U/g cells and 62 U/L). Higher S_0 led to decreasing activities in fresh cells. With S_0 = 209 g/L, the final specific activity was only 7.0 U/g. After disruption of cells, however, an activity over 15 U/g was revealed. Since growth inhibition with S_0 over 153 g/L was observed in batch mode, fed-batch runs, equivalent to a batch of 230 g/L, were done. Although no growth inhibition occurred in fed-batch cultivation, enzyme activity remained low (5.2 U/g). A further fed-batch experiment, carried out under low pressure to remove ethanol from the medium, resulted in a specific activity of 9.8 U/g and a total activity of 68.7 U/L. These results indicate that the low GFOR/GL activities in *Z. mobilis* cells grown on higher S_0 were due to changes in the cell wall, caused by high concentration of ethanol, that hindered the transport of the substrate to the intracellular enzymes in biconversion runs. This conclusion was confirmed by bioconversion runs with cells cultivated under different conditions.

Key words: *Zymomonas mobilis*, glucose-fructose oxidoreductase, glucose.

INTRODUCTION

Sorbitol is a polyol of increasing interest as sweetener, humectant, texturizer and softener in the food industry. Furthermore, this substance is used for the production of vitamin C, and also sorbose, propylene glycol, synthetic plasticizers and alkyd resins, among other products (1,10). The worldwide production of sorbitol has been estimated to be more than 500,000 Ton per year, having an increasing market (10). The industrial production of sorbitol is traditionally performed by catalytic hydrogenation of D-glucose syrup at a concentration of about 50% (w/v). In Brazil, sorbitol is produced in a sole industrial plant and a large part of its commercial demand is dependent on importation.

Gluconic acid and its salts are only prepared by the oxidation of glucose (or raw materials that contain glucose). The methods applied can be chemical, electrolytic, catalytic or biotechnological. Nowadays, the latter way is preferred, using *Aspergillus niger* or *Gluconobacter suboxydans* as micro-organisms. Industrially, gluconic acid and/or its salts are principally used for the removal of zinc coating from metallic objects, the removal of paint and varnish from surfaces, and in the food and pharmaceutical industries as stabiliser (5).

In the presence of both glucose and fructose the ethanologenic bacterium *Zymomonas mobilis* produces gluconic acid and sorbitol. According to Zachariou and Scopes (11), two enzymes are involved in this bioconversion: glucose-

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fructose oxidoreductase (GFOR) (EC 1.1.99) and glucono-δ-lactonase (GL) (EC 3.1.1.17). GFOR catalyses the reduction of fructose to sorbitol and the oxidation of glucose to glucono-δ-lactone. In the sequence, glucono-δ-lactone is hydrolysed by glucono-δ-lactonase to form gluconic acid. The authors observed that GFOR is preferentially induced by glucose and that the enzyme activity in *Z. mobilis* extracts was higher when increasing substrate concentration was used for cell growth. The localisation of GFOR in the periplasm of *Z. mobilis* was later observed by Loos *et al.* (6) and confirmed by Aldrich *et al.* (2). Afterwards, Loos *et al.* (7) described the osmoprotective role of sorbitol for *Z. mobilis*, suggesting a physiological function of GFOR.

Some processes for producing sorbitol and gluconic acid have been proposed in which, instead of purified enzymes, previously grown, concentrated, and permeabilised *Z. mobilis* cells were used (3,8). The reason for the permeabilisation of cells was to release essential soluble co-factors needed for the conversion of both glucose and gluconate to ethanol and other catabolic products.

In a previous work, we proposed to use fresh cells of *Z. mobilis* to avoid operational difficulties and reduce costs in large scale production (9). High yields were obtained when the total initial substrate concentration was increased to 600 g/L, due to the sequential inhibition of the bacterial metabolism by substrates and products, leading to preferential utilisation of substrates via the GFOR/GL system. Since this method requires cell cultivation and enzymes formation for each bioconversion run, the cost for this phase must be compensated for by the ethanol produced. In this context, the aim of this work was to study the formation of GFOR/GL and ethanol by *Z. mobilis*, with different glucose concentrations, in batch and fed batch modes. The effect of fermentative ethanol on enzymes activity is discussed. Furthermore, the production of gluconic acid and sorbitol with *Z. mobilis* cells grown under different conditions is evaluated.

MATERIALS AND METHODS

Micro-organism and Growth Conditions

Zymomonas mobilis ATCC 29191, used in all experiments, was maintained in liquid medium at 4ºC. The medium used for maintenance, inocula production, and fermentation experiments had the following composition (g/L): glucose, 20 (maintenance), 100 (inoculum), 42 to 230 (batch experiments); $(NH_4)_2SO_4$, 1.0; $MgSO_4.7H_2O$, 0.5; KH_2PO_4, 1.0; yeast extract, 5.0. Concentrated glucose solutions (500 g/L) were prepared and sterilised separately and added to the medium before inoculation. In fed-batch experiments, initial glucose concentration was 150 g/L, and the feed medium had the same nutrient formulation but a sugar concentration of 500 g/L. Sterilisation of nutrients and glucose solutions was done at 121ºC for 20 min.

Fermentation runs were carried out in a Biostat ED bioreactor (B. Braun Biotech, Germany). The temperature was kept at 30ºC, the pH was controlled at 5.5 with 5M NaOH, and the impeller speed was 400 rpm. Initial volumes in batch and in fed-batch runs were, respectively, 15 and 12 L. In fed batch runs, 3 L of feed medium, at a flow rate of 0.46 L/h, were added to the medium when the cell concentration reached ca. 3-4 g/L. Inocula for all runs, corresponding to 10% of the total volume, were grown under reciprocal agitation for 12 h at 30ºC.

Bioconversion of Glucose and Fructose to Gluconic Acid and Sorbitol

Bioconversion of glucose and fructose to sorbitol and gluconic acid was performed in a 500 mL bioreactor, containing 300 mL of a solution with 300 g/L of each substrate and 30 g/L of fresh or permeabilised cells of *Z. mobilis*, under magnetic stirring. The pH was automatically controlled at 6.4 by adding 10M NaOH and the temperature was 39ºC. Cell permeabilisation was done by treating concentrated *Z. mobilis* suspensions with cetyltrimethylammonium bromide (CTAB) as described by Rehr *et al*. (8).

Analytical Methods

Cell concentration was determined by measuring the optical density of cell suspensions at 560 nm. Turbidimetric measurements gave a linear relationship with dry cell mass for each case. In samples from fermentation runs, glucose was assayed enzymatically by using a glucose oxidase / peroxidase test-kit (CELM, Brazil; product number 3863). Ethanol was analysed by gas chromatography (HP model 5890, USA) with a FID detector. GFOR/GL activity was estimated by incubating 2 g/L of *Z. mobilis* cells in a solution containing 0.8 M each of glucose and fructose, in a 1 M phosphate/citrate buffer (pH 6.4), at 39ºC, for 20 min. (4). Alternatively, GFOR/GL activity was measured in cell extracts after disruption in a Retsch (Germany) mill. One unit of enzymatic activity was defined by the production of one gram of gluconic acid per hour under the assay conditions. Gluconic acid determination was done by enzymatic method (Boehringer-Mannheim, Germany; product number E0428191). Samples from bioconversion experiments containing glucose, fructose, and sorbitol were analysed by liquid chromatography (Merck-Hitachi, Germany) with a RI detector, using an Eurokat-Pb column (Knauer, Germany).

RESULTS AND DISCUSSION

Initially 6 batch runs, with initial glucose concentration (S_0) from 42 to 230 g/L, were carried out. The general results are presented in Table 1.

From the values calculated for the cell yield ($Y_{X/S}$) and the maximal specific growth rate ($\mu_{X,m}$) of each run, it is clear that sugar concentrations up to 105 g/L have no inhibitory effect on

cell growth, while S_0 = 153 g/L led to decreasing $Y_{X/S}$ and $\mu_{X,m}$. In the extreme case (S_0 = 230 g/L), the cell yield was less than 40% of that measured at non inhibitory conditions.

With S0 from 42 to 209 g/L, similar ethanol yields, between 0.45 and 0.50 g/g, were calculated (Table 1). Since *Z. mobilis* is an obligate anaerobe, it depends on the formation of ethanol to accomplish its energetic metabolism, and, therefore, would normally convert glucose to ethanol with similar yields. On the other hand, the use of an initial glucose concentration of 230 g/L resulted in lower ethanol yield (0.33 g/g).

With respect to the specific GFOR/GL activity in *Z. mobilis* cells, increasing values were measured with S_0 up to 153 g/L. With higher S_0 however, the positive effect of increasing glucose concentrations on GFOR activity, reported by Zachariou and Scopes (11), was no longer observed. In fact, with S_0 = 209 g/L, the specific GFOR/GL activity was dramatically reduced (Table 1). Taking into account both the specific activity and the cell mass, the total GFOR/GL activity (68.7 U/L) achieved with S_0 = 153 g/L was significantly higher than in any other condition.

Fig. 1 shows the time course for GFOR/GL specific activity in *Z. mobilis* cells, with S_0 of 42, 105, 153, and 209 g/L. As seen, the best activities were measured for the two runs with higher S_0. In these cases, increasing specific activities were observed in the first 8 hours, approximately, followed by decreasing values of activity for both sugar concentrations. With S_0 = 153 g/L, activity reached its minimum when the substrate was completely depleted and afterwards increased to a value close to the maximum previously measured. With S_0 = 209 g/L, however, the low activity remained constant even after the total consumption of glucose.

To elucidate such behaviour, considering that the positive effect of increasing glucose concentrations on GFOR induction reported in the literature (11) was observed with free enzyme preparations, cells harvested at different times in runs with S_0 = 153 and 209 g/L were disrupted and the free GFOR/GL activity was determined. As seen in Fig. 2, until 6 to 8 h of fermentation, similar specific activities were found by both methods. In the sequence, however, free GFOR/GL analysis has shown quite higher activities when compared with the results obtained with non disrupted cells. Since GFOR/GL are periplasmic enzymes, their catalytic action in fresh cells depends on the transport of

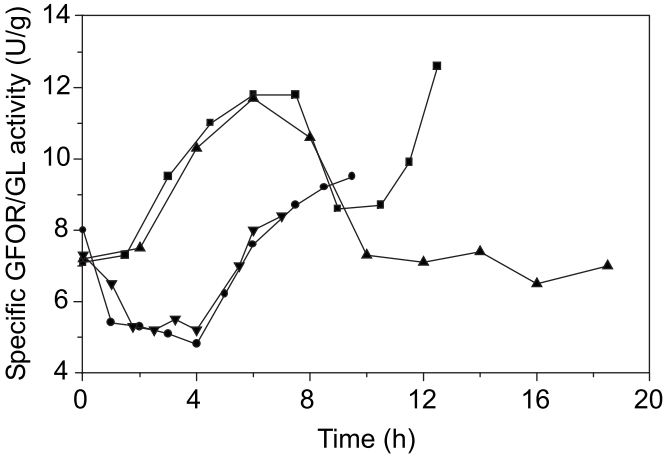

Figure 1. Variation of GFOR/GL specific activity with time in fresh *Zymomonas mobilis* cells grown, in batch mode, in different initial glucose concentrations. ▼ S_0 = 42 g/L; ● S_0 = 105 g/L; ■ S_0 = 153 g/L; ▲ S_0 = 209 g/L.

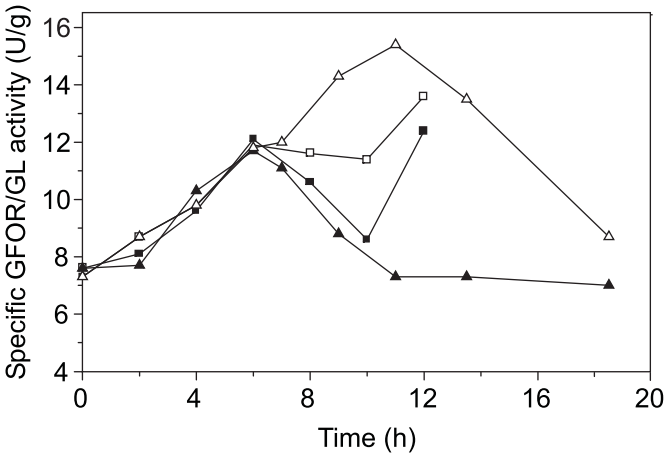

Figure 2. Variation of GFOR/GL specific activity with time in cells of *Zymomonas mobilis* grown, in batch mode, in 153 g glucose / L (■ fresh cells; □ enzyme extract) and 209 g glucose / L (▲ fresh cells; △ enzyme extract).

Table 1. Batch fermentation of glucose by *Zymomonas mobilis* ATCC 29191 with different initial glucose concentrations (S_0).

S_0 (g/L)	Process time (h)	Cell yield (g/g)	Ethanol yield (g/g)	Max. specific growth rate (h^{-1})	Specific GFOR/GL activity (U/g)	Total GFOR/GL ctivity (U/L)
42	6.0	0.043	0.48	0.49	8.4	17.7
89	6.5	0.042	0.49	0.48	9.7	36.9
105	8.5	0.041	0.49	0.48	9.9	39.3
153	10.4	0.035	0.50	0.43	12.6	62.2
209	16.0	0.023	0.45	0.27	7.0	36.3
230	42.0	0.017	0.33	0.17	3.6	15.1

substrates through the cell wall. As such, our results were probably due to some change in the cell wall that partially obstructed the flux of glucose and fructose to the periplasm during the enzymatic assay.

Among the factors that could modify the cells and, consequently, interfere on the substrate/enzyme interaction, ethanol, due to its high concentration in the medium at such conditions, was an obvious first choice for evaluation. For that purpose, an experiment under low pressure (100 mm Hg), to extract part of the formed ethanol from the bioreactor, was carried out. Since the attainment of a large product concentration depends, as already shown in this work, on the use of inhibitory S_0, that test was done in fed batch mode and compared with a run at identical conditions but under normal pressure. The general results of these experiments are summarised in Table 2. To help the discussion, data of the batch run with S_0 = 230 g/L are included in Table 2.

By comparing the results of runs in batch and fed batch modes at 760 mm Hg, one can immediately conclude that fed batch is a suitable way to perform this fermentation, when large amounts of glucose are needed, since it results in improving cell and ethanol yields in a shorter time. The most interesting result, however, was the apparent GFOR/GL specific activity achieved in the run under low pressure, that was almost the double of that measured in the fed batch fermentation under atmospheric pressure (Table 2 and Fig. 3). Considering that the final ethanol concentration was less than 65 g/L in the low pressure fermentation, whereas in the normal pressure run ethanol concentration reached 114 g/L, it is clear that ethanol hindered the action of GFOR/GL in fresh *Z. mobilis* cells grown on high glucose concentrations. Furthermore, under low pressure the cell yield was approximately 30% higher. Total GFOR/GL activity was 68.7 U/L, significantly larger than that calculated for the room pressure fed-batch experiment (29.4 U/L).

Cells of *Z. mobilis*, cultivated under different conditions, were evaluated for the bioproduction of gluconic acid and sorbitol. The general results are shown in Table 3. For the calculation of yields and specific productivities, the dilution of medium, due to the addition of NaOH solution to control pH, was considered. Similar good results were obtained in runs carried out with fresh cells grown on 150 g/L of glucose, in batch mode, or under low pressure in fed batch mode. On the other hand, due to the low specific GFOR/GL activity, the bioconversion experiment with cells from fed-batch cultivation at normal atmospheric pressure presented significantly poorer results. Although such results were expected, this last experiment was done to observe whether the osmotic pressure of the bioconversion medium would be high enough to disrupt the cells, and therefore, facilitate the access of substrates to enzymes.

Furthermore, low-activity cells were permeabilised with CTAB and used in a bioconversion experiment. By this procedure, larger pores were opened in the cell wall to allow an easier access of substrates to GFOR/GL, but the enzymes still remained in cell periplasm (8). As shown in Table 3, with

Table 2. Batch and fed-batch fermentation of glucose by *Zymomonas mobilis* ATCC 29191 under different pressures (S_0).

S_0 (g/L)	Pressure (mm Hg)	Process time (h)	Cell yield (g/g)	Ethanol yield (g/g)	Max. specific growth rate (h^{-1})	Specific GFOR/GL activity (U/g)	Total GFOR/GL Activity (U/L)
230[1]	760	42.0	0.017	0.33	0.17	3.6	15.1
227[2,3]	760	16.0	0.024	0.50	0.44	5.2	29.4
223[2,3]	100	15.0	0.030	-	0.45	9.8	68.7

[1]Batch mode; [2]Fed-batch mode; [3]Equivalent glucose concentration (glucose mass/final volume).

Table 3. Bioconversion of glucose and fructose to gluconic acid and sorbitol, respectively, by *Zymomonas mobilis* cells grown under different conditions (initial glucose + fructose = 600 g/L).

	Growth condition			
	Batch (S_0 = 150 g/L)	Fed-batch at 100 mmHg	Fed-batch at 760 mmHg	Fed-batch at 760 mmHg (CTAB permeabilised cells)
Process time (h)	6.0	6.0	14.0	6.5
Gluconic acid (g/L)	290	290	240	290
Sorbitol (g/L)	288	290	233	290
Gluconic acid yield [1] (g/g)	1.07	1.04	0.89	1.04
Sorbitol yield [2] (g/g)	0.98	0.93	0.85	0.94
Specific gluconic acid productivity (g/g.h)	1.78	1.67	0.62	1.52
Specific sorbitol productivity (g/g.h)	1.64	1.64	0.61	1.43

[1]Maximum theoretical yield = 1.089 g gluconic acid / g glucose; [2]Maximum theoretical yield = 1.011 g sorbitol / g fructose.

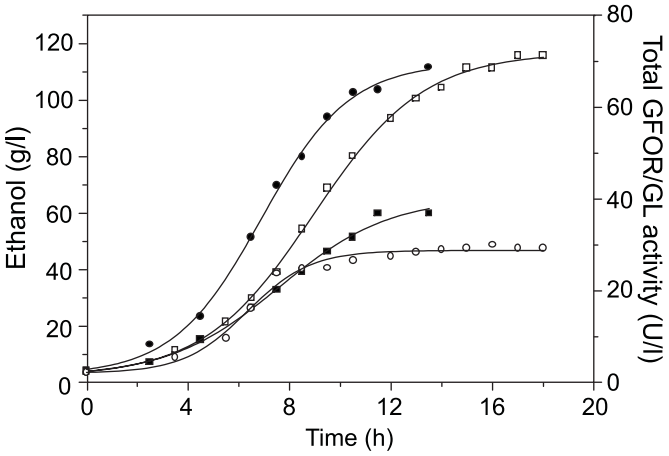

Figure 3. Time course of *Zymomonas mobilis* cultivation at 760 mmHg (□ ethanol; ○ GFOR/GL total activity) and at 100 mmHg (■ ethanol; ● GFOR/GL total activity).

permeabilised *Z. mobilis* high values for both final concentration of products, yields, and specific productivities were achieved, confirming the hypothesis proposed with respect to changes in the cell wall when an excessive substrate concentration is used for cell cultivation.

Although our results have confirmed the findings of Zachariou and Scopes (11), i.e. periplasmic glucose-fructose oxidoreductase activity is improved when *Zymomonas mobilis* is grown on high glucose concentrations, the catalytic potential of enzymes present in fresh cells is reduced due to the exposition to high levels of ethanol. As such, assuming that fresh cells would be employed for the production of sorbitol and gluconic acid, the *Z. mobilis* growth step must be carefully optimised.

RESUMO

Atividade de glicose-frutose oxidorredutase em células íntegras e permeabilizadas de *Zymomonas mobilis* cultivadas em diferentes concentrações de glicose

Sorbitol e ácido glucônico são produzidos por células previamente cultivadas da bactéria produtora de etanol *Zymomonas mobilis* pela ação das enzimas periplasmáticas glicose-frutose oxidorredutase (GFOR) e glucono-δ-lactonase (GL). A atividade de GFOR/GL em células a serem empregadas nesta bioconversão depende das condições de crescimento. Em cultivo em regime descontínuo, com concentrações iniciais de glicose (S_0) entre 42 e 230 g/L, as maiores atividades específica e total de GFOR/GL foram obtidas com S_0 = 153 g/L (12,6 U/g de células e 62 U/L), enquanto maiores S_0 levaram a atividades decrescentes em células não tratadas. Com S_0 = 209 g/L, a atividade específica final foi de 7 U/g, mas, após a ruptura das células, atividade superior a 15 U/g foi medida. Uma vez que em descontínuo observou-se inibição por substrato com S_0 a partir de 153 g/L, ensaios em regime descontínuo alimentado, com glicose equivalente a 230 g/L em descontínuo, foram realizados. Embora a inibição pelo substrato tenha sido superada, a atividade permaneceu baixa (5,2 U/g). Um novo ensaio em descontínuo alimentado, conduzido sob baixa pressão para remover o etanol do meio, resultou em atividade específica de 9,8 U/g e total de 68,7 U/L. Estes resultados indicam que as baixas atividades em células de *Z. mobilis* cultivadas com maiores S_0 se deveram a mudanças na parede celular, provocadas por concentrações elevadas de etanol, que dificultaram o transporte de substrato para as enzimas intracelulares durante a bioconversão. Esta conclusão foi confirmada em ensaios de bioconversão com células provenientes de cultivos em diferentes condições.

Palavras-chave: *Zymomonas mobilis*, glicose-frutose oxidoreductase, glicose.

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