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BIOCATALYSIS

Production of organic acids by periplasmic enzymes present in free and immobilized cells of *Zymomonas mobilis*

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Abstract In this work the periplasmic enzymatic complex glucose-fructose oxidoreductase (GFOR)/glucono-δ-lactonase (GL) of permeabilized free or immobilized cells of *Zymomonas mobilis* was evaluated for the bioconversion of mixtures of fructose and different aldoses into organic acids. For all tested pairs of substrates with permeabilized free-cells, the best enzymatic activities were obtained in reactions with pH around 6.4 and temperatures ranging from 39 to 45 °C. Decreasing enzyme/substrate affinities were observed when fructose was in the mixture with glucose, maltose, galactose, and lactose, in this order. In bioconversion runs with 0.7 mol l^{-1} of fructose and with aldose, with permeabilized free-cells of *Z. mobilis*, maximal concentrations of the respective aldonic acids of 0.64, 0.57, 0.51, and 0.51 mol l^{-1} were achieved, with conversion yields of 95, 88, 78, and 78 %, respectively. Due to the important applications of lactobionic acid, the formation of this substance by the enzymatic GFOR/GL complex in Ca-alginate-immobilized cells was assessed. The highest GFOR/GL activities were found at pH 7.0–8.0 and temperatures of 47–50 °C. However, when a 24 h bioconversion run was carried out, it was observed that a combination of pH 6.4 and temperature of 47 °C led to the best results. In this case, despite the fact that Ca-alginate acts as a barrier for the diffusion of substrates and products, maximal lactobionic acid concentration, conversion yields and specific productivity similar to those obtained with permeabilized free-cells were achieved.

Keywords *Zymomonas mobilis* · Glucose-fructose oxidoreductase/glucono-δ-lactonase · Organic acids · Immobilized cells

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Introduction

In the last two decades the ethanol-producing bacterium *Zymomonas mobilis* has been the subject of considerable research efforts due to its ability to produce sorbitol and gluconic acid from a mixture of fructose and glucose, in reactions catalyzed by its own periplasmic enzymatic complex of glucose-fructose oxidoreductase (GFOR; EC 1.1.1.99), and glucono-δ-lactonase (GL; EC 3.1.1.17) [3, 4, 20, 24, 31]. Research has been particularly focused on the biotechnological production of sorbitol, because this polyol has several important applications in the pharmaceutical and food industries [11, 23, 28]. However, since sorbitol and gluconic acid are formed in equimolar amounts when mediated by the GFOR/GL complex, the industrial application of this enzymatic system has been hindered by the fact that the commercial demand for sorbitol is significantly higher than that for gluconic acid and its salts.

In order to avert this problem, some technical alternatives have been tried. For instance, it has been reported that purified GFOR from *Z. mobilis* oxidizes different aldoses other than glucose, to their respective lactones, which are then hydrolyzed to form linear aldonic acids by the action of GL [21, 31]. One of these products is lactobionic acid,

which is obtained from the oxidation of lactose; it has a very high commercial value as a component of the U.W. (University of Wisconsin) solution, used to preserve human organs to be transplanted [25, 26], as an active principle for many cosmetics [8, 9, 27, 30], and also as part of drug delivery systems [12, 29]. Therefore, it would be extremely interesting if the synthesis of a group of organic acids could be coupled with the production of sorbitol, equilibrating the commercial demand for these products.

Immobilization techniques have been evaluated in some studies on the bioconversion of fructose/glucose to sorbitol/gluconic acid by GFOR/GL present in *Z. mobilis* cells. Immobilization supports such as calcium alginate [3, 5, 14], k-carrageenan [10, 20], and different polymers [6] are described in the literature. Notwithstanding, as for other biotechnological processes with immobilized enzymes, many aspects of functionality and reaction kinetics remain to be improved or optimized, such as the preservation of enzymatic activity, stability with varying pH and temperatures, design of special reactors, and the mode of operation of the process [16, 32].

The aims of this work were to study the action of the enzymatic GFOR/GL complex contained in free permeabilized cells of *Z. mobilis* ATCC 29191 on mixtures of fructose and aldoses (maltose, galactose, and lactose), and measure the formation of maltobionic, galactonic, and lactobionic acids, compared with the usual substrates of these reactions (fructose and glucose). Reactions were carried out under different pH, temperatures, and substrate concentrations. Furthermore, permeabilized *Z. mobilis* cells immobilized in Ca–alginate matrices were studied for the specific bioconversion of fructose and lactose to sorbitol and lactobionic acid, respectively, under the same conditions as for free cells.

Materials and methods

Microorganism

Zymomonas mobilis strain ATCC 29191 was used in this work. For immediate use, cultures were kept in liquid medium suspension, at 4 °C, according to procedures previously published [15]. Cultures were monthly replicated in order to keep cell viability.

Medium and cultivation conditions

Liquid medium [15] used for maintenance, inoculum growth, cell growth, and enzyme production had the following composition (in g l^{-1}): glucose, 20 (maintenance), 100 (inoculum), 150 (biomass and enzyme production); $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5; KH_2PO_4, 1.0; Prodex Lac® yeast extract (Prodesa S.A., Brazil), 7.5. For inocula preparation, 5 g l^{-1} of $CaCO_3$ was added to the medium to avoid an excessive pH drop during the experiments, with initial pH set to 6.2. Concentrated glucose solutions and $CaCO_3$ were separately sterilized and added to the medium before inoculation. Sterilization of nutrients and glucose solutions was accomplished by autoclaving at 1 atm for 15 min. Inocula were prepared in 500 ml anaerobic bottles, with CO_2 release filters, filled with 450 ml of medium and kept under orbital agitation of 200 rpm (Certomat U, Sartorius Stedim Biotech, Germany), at 30 °C, for approximately 10 h. Batch cultivations of *Z. mobilis* cells were carried out in a 5.5 l stainless-steel bioreactor, equipped with two flat-blade impellers, designed and built in our laboratory. The temperature was kept at 30 °C, the impeller speed was 450 rpm, and the pH was maintained at 5.5 with 5 M NaOH using a Consort model R735 (Consort, Belgium) pH controller. Cell mass was harvested from the medium by centrifugation at 6,000*g*, at 4 °C, for 20 min, using a Sigma 4K-15 centrifuge (Sartorius Stedim Biotech, Germany) and the cells were re-suspended in distilled water to a concentration of 50 g l^{-1}.

Cell permeabilization

Zymomonas mobilis cells were permeabilized by mixing equal volumes of concentrated biomass and 0.2 % (mass fraction) cetyl trimethylammonium bromide (CTAB) solution, this suspension being gently stirred for 10 min, as described by Rehr et al. [20]. According to these authors, this procedure avoids the fermentative metabolism of *Z. mobilis*, but the activity of GFOR/GL is unaffected. Cells were cross-linked with glutaraldehyde 0.5 % (volume fraction) under magnetic stirring during 10 min at room temperature [10]. Cells were centrifuged and re-suspended into distilled water to achieve the final concentration of 50 g l^{-1}.

Immobilization method

The permeabilized and cross-linked cells were immobilized in Ca-alginate beads by entrapment as described in a previous work [14]. The Ca-alginate beads were kept at 4 °C in distilled water and were subsequently used for the determination of the enzymatic activity and bioconversion assays.

Enzymatic tests

The following conditions were defined as the standard procedure for the determination of GFOR/GL enzymatic activity: substrate concentration, 0.7 mol l^{-1}; free-permeabilized cell concentration, 4.0 g l^{-1}; immobilized-permeabilized cell concentration, 10.0 g l^{-1}, temperature, 39 °C; pH 6.4. The catalytic activity present in free-permeabilized cells of

Z. mobilis, on fructose/aldoses (glucose, maltose, galactose, and lactose) was evaluated on different pH (5.2–7.2), and temperatures (37–54 °C). The thermal stability of the enzymatic complex in free-permeabilized *Z. mobilis* was assessed after exposing it to temperatures of 39, 43, and 45 °C for up to 12 h, at pH 6.4. To estimate the kinetic parameters of the Michaelis–Menten model, K_m and V_m, the GFOR/GL activity in free-permeabilized cells was determined, at the standard conditions, in solutions containing equimolar concentrations of fructose and glucose (0.1–2.5 mol l^{-1}), maltose (0.1–1.5 mol l^{-1}), galactose (0.1–1.9 mol l^{-1}), or lactose (0.1–1.3 mol l^{-1}). Since the solubility of lactose at 40 °C is approximately 0.60 mol l^{-1}, as observed by Machado et al. [13], in the present work this solution was prepared with boiling water, in which the solubility of this sugar is about 1.10 mol l^{-1} [2]. Thus, among the group of experiments in this discussion, tests with saturated solutions of lactose were included. For permeabilized Ca-alginate immobilized cells of *Z. mobilis*, GFOR/GL activity was compared at pH varying from 5.2 to 9.7 and temperatures between 34 and 59 °C, in reaction media containing fructose/lactose concentrations in the range 0.1–1.3 mol l^{-1}. The stability of the immobilized system was evaluated in tests that consisted of four successive 3 h bioconversion runs, using the same Ca-alginate beads, at pH values of 6.4, 7.0, 7.5, and 7.9, at 39 °C, and temperatures of 39, 43, 47, and 50 °C, at pH 6.4. In these tests, the concentrations of lactose/fructose and cells in the liquid phase were 0.7 mol l^{-1} and 20.0 g l^{-1}, respectively. After every 3 h run, calcium alginate beads were treated with 0.3 mol l^{-1} $CaCl_2$ to assure their hardness.

Bioconversion experiments

The bioconversion assays were performed in a 600 ml glass reactor containing 240 ml of reaction medium. The mixture was kept under magnetic agitation and the temperature was maintained at the desired values by placing it into a water bath. The pH was controlled by automatic addition of NaOH solutions using a pH controller (Consort R735, Belgium): 10 mol l^{-1} for tests with fructose/glucose and 7 mol l^{-1} for mixtures of fructose and the other aldoses. Unless otherwise stated, bioconversion tests were carried out at pH 6.4 and 39 °C, and the following initial concentrations, in relation to the volume of the liquid phase, were employed: substrates, 0.7 mol l^{-1}; free-permeabilized cells, 25.0 g l^{-1}; immobilized-permeabilized cells, 20.0 g l^{-1}. For the particular case of the production of sorbitol and lactobionic acid by free-permeabilized *Z. mobilis* cells, bioconversion tests were also performed with fructose/lactose initial concentrations of 1.0 and 1.2 mol l^{-1}. Furthermore, the production of sorbitol and lactobionic acid were compared with the standard conditions in experiments performed with cell concentrations varying from 12.5 to 37.5 g l^{-1} and temperatures from 33 to 45 °C. The permeabilized immobilized cells were only tested over the pair fructose/lactose with the combination of pH 6.4 and 7.5, and temperatures of 39 and 47 °C. All the results presented in the graphs and tables represent the mean of experimental duplicates.

Analytical methods

Cell concentration was determined by measuring the optical density of cell suspensions at 560 nm. Turbidimetric measurements produced a linear relationship with dry cell mass for each case. The assays for determining GFOR/GL activity in *Z. mobilis* cells were done in 100 ml of reaction medium containing variable equimolar concentrations of fructose/aldoses, and either 4.0 g l^{-1} of free-permeabilized cells or 10.0 g l^{-1} of immobilized-permeabilized cells. The systems were kept in a water bath, at the desired temperature, under magnetic agitation, for 30–60 min, with pH being controlled by the automatic addition of 1 mol l^{-1} of standardized NaOH solution using the same pH controller described above. One unit of GFOR/GL was defined as the amount of enzymatic complex responsible for the production of 1 mmol of organic acid per hour under the assay conditions. GFOR/GL activities in this work are presented as units per gram of dry cells (U g^{-1}) as defined in a previous work [14, 15]. The concentration of organic acids in relation to the volume of liquid phase, in both activity and bioconversion tests, was stoichiometrically inferred from the volume of NaOH used to control the pH [14, 19, 22], or HPLC analysis [18]. As shown by Pedruzzi et al. [19], when permeabilized *Z. mobilis* cells are used in this process, the responses of both methods are practically identical. Once sorbitol and organic acids are formed in an equimolar basis, and assuming that no other product is formed in this process, the molar concentration of sorbitol may be considered as the same as the acids. The pH within the alginate beads was estimated as described in a previous work [14].

Parameters of evaluation

The conversion of aldoses to the respective organic acids was calculated by dividing the number of moles of product formed in the reaction by the number of moles of substrate in the initial solution of each experiment, taking into account the dilution caused by the alkali addition for pH control. The maximal specific organic acid formation rate (q_{max}, mmol acid g^{-1} cell h^{-1}), which depends on the activity of GFOR/GL, was determined during the first hours of the bioconversion process, when the curve representing the variation of product concentration with time has a linear

profile. The specific productivity, with the same units as q_{max}, corresponds to an average rate, which is determined at the end or in the course of the process, taking into account the dilution caused by the volume of alkali used to control the pH.

Results and discussion

Enzymatic assays with permeabilized free-cells of *Z. mobilis*: effects of pH, temperature and concentration of fructose/aldoses

The variation of the enzymatic activity of GFOR/GL, contained in the permeabilized free-cells of *Z. mobilis* measured at different pH and temperatures for mixtures of fructose and aldoses are shown in Fig. 1a, b.

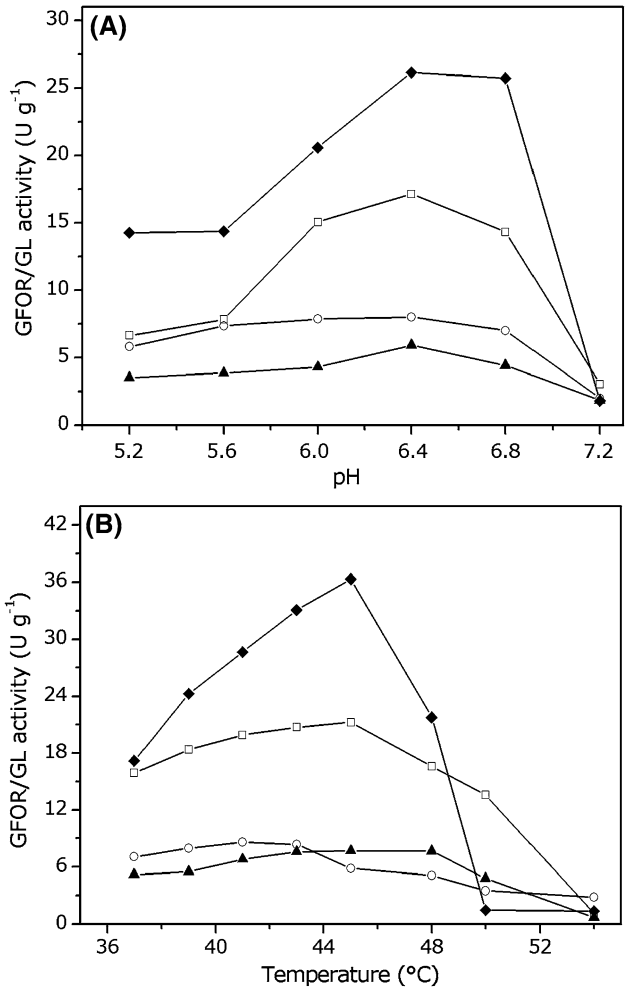

Fig. 1 Effects of pH at 39 °C (**a**) and temperatures at pH 6.4 (**b**) on the activity of glucose-fructose oxidoreductase/glucono-δ-lactonase in permeabilized free-cells of *Zymomonas mobilis* ATCC 29191. Fructose/aldose concentration in the liquid phase was 0.7 mol l^{-1}, and cell concentration was 4.0 g l^{-1}. *Filled diamond* glucose; *open square* maltose; *open circle* galactose; *filled triangle* lactose

The optimal pH for the reaction was around 6.4 for GFOR on fructose/glucose, with some variation for the different pairs of substrates. As expected, the highest activities were achieved with fructose/glucose, as a result of the preferential enzyme-substrate affinity, with decreasing responses to the mixtures of fructose with maltose, galactose, and lactose, respectively (Fig. 1a).

As shown in Fig. 1b, concerning the tested temperatures, GFOR/GL activity for the mixture fructose and glucose or maltose showed values increasing as temperatures rose to 45 °C. For lactose, the maximum enzyme activity was measured at temperatures as high as 48 °C. These temperatures are higher than those reported as the optimal for purified GFOR by Zachariou and Scopes [31], within the range 39–42 °C, for the fructose/glucose mixture. For fructose/galactose, GFOR/GL activity was enhanced for temperatures from 39 to 43 °C, with a peak at 41 °C, but decreased at 45 °C. High reaction temperatures cannot be immediately assumed as ideal for bioconversions because enzymes can be inactivated in long-term operations. Therefore, the effect of the temperature on the activity of GFOR/GL in mixtures fructose/glucose and fructose/lactose were evaluated after pre-exposing free-permeabilized cells of *Z. mobilis* to 39, 43, and 45 °C for 12 h. When GFOR/GL complex activity was measured on its natural substrates (fructose and glucose), high enzyme thermal stability at 39 and 43 °C was observed, with remaining activities close to 100 % of the initial, whereas at 45 °C, 91 % of the activity was maintained. For GFOR/GL determined on mixtures of fructose/lactose after pre-incubation at 39, 43 and 45 °C for 12 h, the activities were reduced to 92, 86 and 82 % of the original, respectively. Such difference in thermal stability could be due to the higher affinity of glucose for GFOR in comparison to lactose. When a small part of the enzyme is affected by temperatures up to 43 °C, the enzyme/substrate relationship for the pair fructose/glucose would still be high enough to avoid significant decreases in activity that could be measured. However, for fructose/lactose as substrates, any small decrease in the enzyme/substrate relationship would be detected.

The effect of the concentration of fructose/aldoses on the catalytic action of GFOR/GL was tested at pH 6.4 and 39 °C (Fig. 2). As expected, due to the high affinity between GFOR and glucose, the highest activity was obtained for glucose/fructose as substrate for the reaction, followed by fructose with maltose, lactose, and galactose. The maximal activities for each pair of substrates were achieved with concentrations as high as 2.1, 0.90, 1.2, and 1.1 mol l^{-1}, respectively, as can be seen in Fig. 2.

The results obtained for the equimolar substrate concentration reactions were used to calculate the kinetic parameters of the Michaelis–Menten equation, K_m and V_m, using the Lineweaver–Burk approach. Data obtained for fructose/lactose could not be fitted into the model due to the low

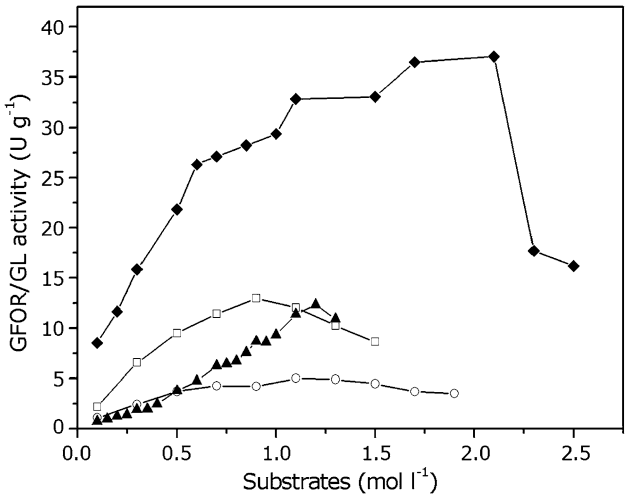

Fig. 2 Effect of equimolar fructose/aldose concentrations on the activity of glucose-fructose oxidoreductase/glucono-δ-lactonase in permeabilized free-cells of *Zymomonas mobilis* ATCC 29191. Cell concentration in the liquid phase, 4.0 g l^{-1}; pH, 6.4; temperature, 39 °C. *Filled diamond* glucose; *open square* maltose; *open circle* galactose; *filled triangle* lactose

affinity between GFOR/GL and lactose. As can be seen in Fig. 2, for the fructose/lactose pair, the variation of enzyme activity with substrate concentration, up to the maximal value, had a sigmoid profile, whereas for the other substrates, the curves presented hyperbolic shapes that needed to be linearized in double-reciprocal plots. For the other substrates combinations, the values found for K_m (in mol l^{-1}) and V_m (in units per gram of cell; U g^{-1}), respectively, were: fructose/glucose, 0.68 and 52; fructose/maltose, 2.0 and 47; fructose/galactose, 0.60 and 7.6. It was confirmed that glucose is a preferential substrate for GFOR, with the lowest K_m and highest V_m values.

Bioconversions with permeabilized free-cells of *Z. mobilis* in media containing fructose and different aldoses

In these experiments, the bioconversion process mediated by permeabilized free-cells of *Z. mobilis* was compared in mixtures containing 0.70 mol l^{-1} of fructose and either glucose, maltose, galactose or lactose at the standard conditions: substrate concentration, 0.70 mol l^{-1}, cell concentration, 25 g l^{-1}, pH 6.4 and 39 °C. Results are presented in Table 1, showing the influence of the different affinities between GFOR and each aldose used as substrate on the parameters evaluated.

Despite the fact that the bioconversion of fructose/lactose rate was lower than those observed for the other pairs of substrates (Table 1), an impressive 78 % conversion of lactose into lactobionic acid was achieved at 24 h of reaction. Comparatively, Satory et al. [21], using free GFOR, obtained a conversion of 90 % in a 90 h process.

Bioproduction of lactobionic acid by permeabilized free-cells of *Z. mobilis*: effects of lactose/fructose concentrations and temperature

The influence of the concentration of substrates on the formation of lactobionic acid was evaluated in long-term tests with equimolar concentrations of fructose/lactose of 0.70, 1.0, and 1.20 mol l^{-1}, at pH 6.4 and 39 °C. As shown in Table 2, increasing values for products concentration and specific productivities were found at 24 h of process, as the initial substrate concentration increased from 0.70 to 1.2 mol l^{-1}. The maximal specific rates of lactobionic acid formation (q_{max}), determined at the beginning of each run, were practically identical for initial fructose/lactose concentrations of 0.70 or 1.0 mol l^{-1}, whereas for the saturated concentration (1.2 mol l^{-1}), smaller q_{max} and conversion rates were obtained. Since a higher conversion was achieved with 0.70 mol l^{-1} of substrates, which is also the optimal condition for rheological characteristics due to lower viscosities, this condition was chosen for further experiments.

To test the effect of cell concentration on the reaction parameters, different amounts of permeabilized free-cells (12.5, 20.0, and 37.5 g l^{-1}) were compared to the standard condition of 25.0 g l^{-1} for the production of sorbitol/

Table 1 *Zymomonas mobilis* ATCC 29191 permeabilized free-cells bioconversion of aldoses (glucose, maltose, galactose, or lactose), in medium containing equimolar concentrations of fructose

Organic acid	Final concentration[a] (mol l^{-1})	Conversion (%)	Maximal specific organic acid formation rate (mol g^{-1} h^{-1}) × 10^{-3}	Specific productivity (mol g^{-1} h^{-1}) × 10^{-3}
Gluconic[b]	0.64	95	17.4	1.1
Maltobionic[c]	0.57	88	7.0	0.95
Galactonic[c]	0.51	78	5.1	0.85
Lactobionic[c]	0.51	78	3.9	0.85

Initial fructose/aldose concentration in the reaction, 0.7 mol l^{-1}; initial cell concentration, 25.0 g l^{-1}; pH, 6.4; temperature, 39 °C

[a] Concentration expressed in relation to the initial volume of the liquid phase plus the volume of alkali used for pH control; [b] Calculations made at 5 h of process; [c] Calculations made at 24 h of process

Table 2 *Zymomonas mobilis* ATCC 29191 permeabilized free-cells bioconversion as a function of initial lactose concentrations, in media containing equimolar concentrations of fructose

Initial lactose/fructose concentration[a] (mol l^{-1})	Lactobionic acid concentration[b] (mol l^{-1})	Conversion (%)	Maximal specific organic acid formation rate (mol g^{-1} h^{-1}) × 10^{-3}	Specific productivity (mol g^{-1} h^{-1}) × 10^{-3}
0.7	0.55	86	4.4	1.0
1.0	0.69	80	4.5	1.3
1.2	0.75	72	3.5	1.4

Initial cell concentration in the liquid phase, 25.0 g l^{-1}; pH, 6.4; temperature, 39 °C; process time, 24 h

[a] Concentration expressed in relation to the initial volume of the liquid phase; [b] Concentration expressed in relation to the initial volume of the liquid phase plus the volume of alkali used for pH control

Table 3 *Zymomonas mobilis* ATCC 29191 permeabilized free-cells bioconversion of lactose, in medium containing equimolar concentrations of fructose as a function of initial cell concentration

Initial cell concentration[a] (g l^{-1})	Lactobionic acid concentration[b] (mol l^{-1})	Conversion (%)	Maximal specific organic acid formation rate (mol g^{-1} h^{-1}) × 10^{-3}	Specific productivity (mol g^{-1} h^{-1}) × 10^{-3}
12.5	0.42	64	3.7	1.5
20.0	0.46	70	3.1	1.0
25.0	0.55	85	4.2	0.98
37.5	0.56	86	3.0	0.67

Initial fructose/lactose concentration in the liquid phase, 0.7 mol l^{-1}; pH, 6.4; temperature, 39 °C, process time, 24 h

[a] Concentration expressed in relation to the initial volume of the liquid phase; [b] Concentration expressed in relation to the initial volume of the liquid phase plus the volume of alkali used for pH control

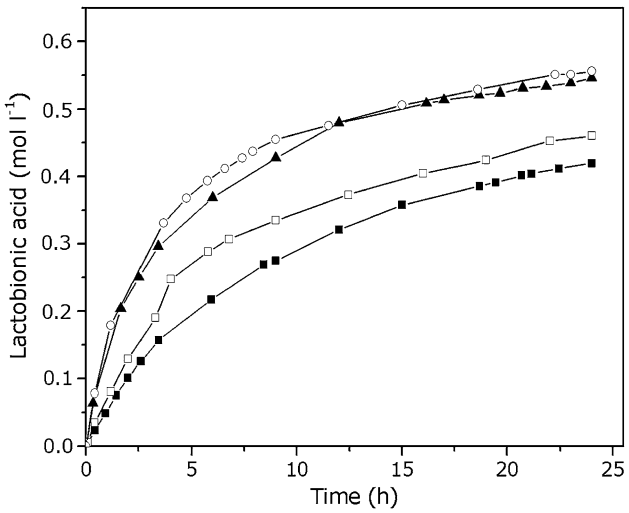

Fig. 3 Time course of lactobionic acid concentration in the bioconversion of lactose, in medium containing equimolar concentration of fructose, by permeabilized free-cells of *Zymomonas mobilis* ATCC 29191 at different concentrations in the liquid phase. Initial fructose/lactose concentration in the liquid phase, 0.7 mol l^{-1}; pH, 6.4; temperature, 39 °C. *Filled square* 12.5 g l^{-1}; *open square* 20.0 g l^{-1}; *filled triangle* 25.0 g l^{-1}; *open circle* 37.5 g l^{-1}

lactobionic acid on a 0.70 mol l^{-1} solution of fructose/lactose. Results are presented in Table 3, while the kinetic profiles for lactobionic production are shown in Fig. 3

Increasing lactobionic acid concentrations were found after 24 h of reaction, as the cell concentration was raised to 25.0 g l^{-1}. The use of 37.5 g l^{-1} of cells did not produce any increment in the final product concentration resulting in a lower specific production rate, suggesting an excess of enzyme in the reaction medium. Therefore, further experiments were carried out using the cell concentration of 25.0 g l^{-1}.

The influence of temperature on the bioproduction of lactobionic acid was assessed and the results are shown in Table 4. Similarly to findings of other authors for the catalytic action of GFOR/GL on fructose/glucose [3, 31], the best results were obtained at 39 °C for any aspect considered. To explain the superior performance of the process at 39 °C, both the interaction of enzyme activity with temperature, and enzyme activity with thermal stability along the reaction must be considered. As such, this result cannot be compared with that depicted in Fig. 1b for fructose/lactose as substrates, in which an approximately 10 % higher GFOR/GL activity was measured at 45 °C in comparison to 39 °C, but the time dependence of enzyme thermal stability was not significant.

Enzymatic assays with permeabilized immobilized-cells of *Z. mobilis*: effects of fructose/lactose concentration, pH and temperature

The activity of the GFOR/GL system of immobilized cells as a function of pH and temperature is depicted in Fig. 4a,

Table 4 *Zymomonas mobilis* ATCC 29191 permeabilized free-cells bioconversion of lactose, in medium containing equimolar concentrations of fructose as function of temperature

Temperature (°C)	Lactobionic acid concentration[a] (mol l^{-1})	Conversion (%)	Maximal specific organic acid formation rate (mol g^{-1} h^{-1}) × 10^{-3}	Specific productivity (mol g^{-1} h^{-1}) × 10^{-3}
33	0.43	65	2.4	0.77
37	0.49	74	2.7	0.87
39	0.55	85	4.4	0.98
42	0.53	82	4.1	0.96
45	0.53	82	3.0	0.95

Initial fructose/lactose concentration in the reaction, 0.7 mol l^{-1}; initial cell concentration, 25.0 g l^{-1}; pH, 6.4; process time, 24 h

[a] Concentration expressed in relation to the initial volume of the liquid phase plus the volume of alkali used for pH control

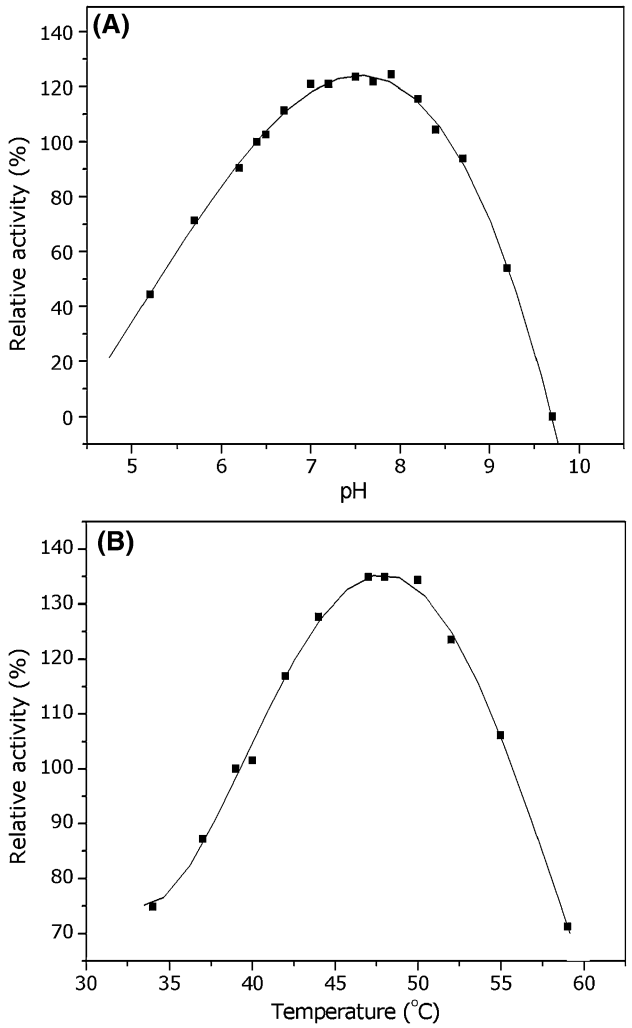

Fig. 4 Effect of pH at 39 °C (**a**) and temperatures a pH 6.4 (**b**) on the activity of glucose-fructose oxidoreductase/glucono-δ-lactonase in permeabilized immobilized-cells of *Zymomonas mobilis* ATCC 29191. Fructose/lactose concentration in the liquid phase, 0.7 mol l^{-1}; cell concentration in the liquid phase, 10.0 g l^{-1}. The enzymatic activity measured at the standard conditions (pH 6.4 and 39 °C) corresponds to 100 %

b, respectively, considering pH 6.4 and 39 °C as the standard 100 %.

In the first set of experiments, the catalytic action of the GFOR/GL system present in Ca-alginate immobilized cells of *Z. mobilis* was compared as a function of substrate concentration, which varied from 0.10 to 1.3 mol l^{-1}, with increasing activities being observed up to 0.90 mol l^{-1}. With 0.90–1.2 mol l^{-1}, similar results were achieved (ca. of 2.0 units of GFOR/GL per gram of cell), whereas with 1.3 mol l^{-1}, an activity of about 1.5 U g^{-1} was measured. There was a remarkable reduction of enzymatic activity for the immobilized system; approximately six times lower than that found for free cells with the same substrate concentration. According to Zanin and Moraes [32] and Mateo et al. [16], the use of immobilized enzymatic systems can hinder the diffusion of substrates and products, decreasing reaction rates, effects that are dependent on the immobilization technique, type of support, and the characteristics of substrates. In the case of the enzymatic system in the present work, the control of important parameters such as pH, temperature, substrate and product concentration in the micro-environment inside the alginate beads, could be especially difficult. Therefore, GFOR/GL would be exposed to sub-optimal conditions for its catalytic action, as it has been reported for the bioconversion of fructose/glucose to sorbitol/gluconic acid by Ca-alginate immobilized *Z. mobilis* [14].

The highest activities for the immobilized system at 39 °C were measured at pH between 7.0 and 8.0, 24 % higher than the standard condition of pH 6.4. The accumulation of lactobionic acid inside the beads would result in an inadequate pH for the optimal enzymatic activity. Therefore, higher external pH would provide for a balanced internal pH, more adequate for GFOR/GL system activity.

Concerning the temperature, activities were enhanced up to 35 % for 47–50 °C, as compared against the standard of 39 °C. These experiments defined the optimal ranges for pH and temperature for GFOR/GL reaction performed over shorter times of 30–60 min. However, enzymatic systems

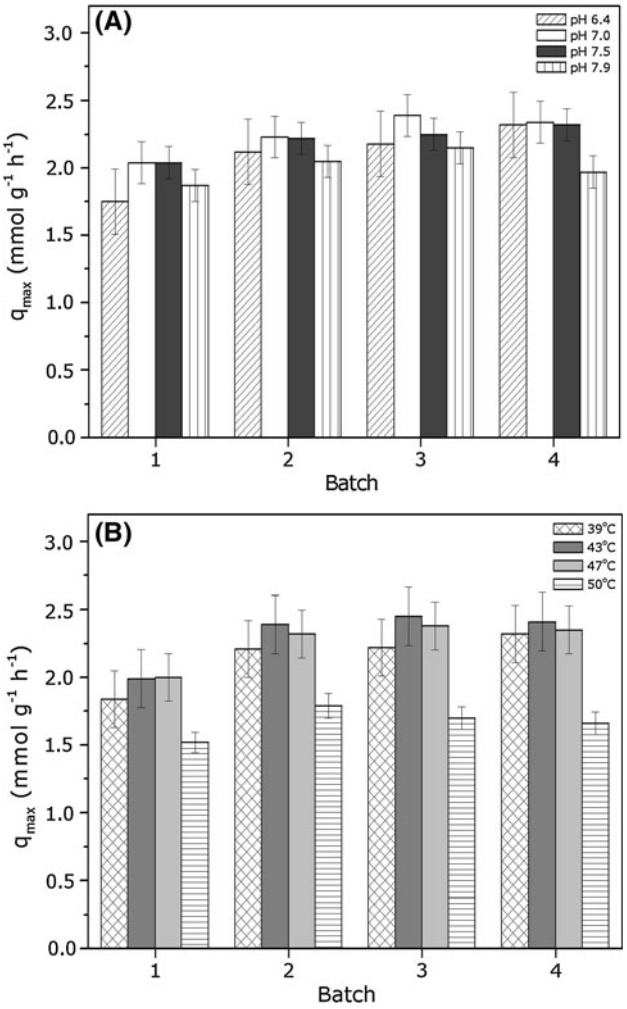

Fig. 5 Maximal specific rate of lactobionic acid formation (q_{max}) in successive 3 h bioconversion batches with permeabilized immobilized-cells of *Zymomonas mobilis* ATCC 29191 at different pH at 39 °C (**a**), and temperatures at pH 6.4 (**b**). Initial fructose/lactose concentration in the liquid phase of each batch, 0.7 mol l^{-1}; cell concentration in the liquid phase, 20.0 g l^{-1}

are usually used in bioconversion reactions for several hours, thus it would be important to study the effects of these two parameters on the bioconversion of fructose/lactose to sorbitol/lactobionic acid by the immobilized cells. Results of q_{max} as a function of pH and temperature for four successive batches of 3 h of reaction are shown in Fig. 5a, b. As mentioned before, the inactivation of GFOR/GL system is associated to the catalytic action of the enzymes themselves [7, 17]. Thus, the test of stability as performed in this work is more significant than a simple exposition of enzymes to inactivating conditions.

As can be seen in Fig. 5a, b, for both pH and temperature, q_{max} presented relatively lower values in the first batch, probably due to the fact that the beads had been recently prepared and still had an excessive rigidity that hindered the diffusion of substrate, as previously reported by Bertasso et al. [1] and Malvessi et al. [14]. From the second batch of experiments, small variations in q_{max} were observed, except for the assay at pH 7.9, which presented a lower specific rate in the fourth cycle (Fig. 5a). This result contrasts with those for the tests of activity (Fig. 4a) and could be attributed to the instability of GFOR/GL complex due to the exposure of enzymes to a high pH for a longer time. With respect to batches at different temperatures, from the second to the fourth cycle, similar q_{max} were measured for each particular temperature, indicating that GFOR/GL in immobilized-permeabilized cells of *Z. mobilis* has a good thermal stability at 39, 43, and 47 °C (Fig. 5b). However, at 50 °C, a lower q_{max} was calculated compared to other temperatures. These results substantially differ from those for activity-conditions tests (Fig. 4b) and demonstrate that the time of reaction strongly affects this system.

Bioproduction of lactobionic acid by permeabilized immobilized-cells of *Z. mobilis*: combined effects of pH and temperature

The bioconversion of fructose/lactose (0.7 mol l^{-1}) to sorbitol/lactobionic acid was evaluated combining two values of pH (6.4 and 7.5) and temperatures (39 and 47 °C), which were chosen among the best observed in the tests of stability illustrated in Fig. 5. From Table 5, maximal q_{max} values, measured up to 3–4 h of run, were obtained in experiments at 47 °C. However, such high specific rates did not lead to important differences in terms of conversion and specific productivity, in comparison to the further bioconversion runs. On the contrary, at pH 7.5 and 47 °C, which were individually the optimal values for pH and temperature in both activity and stability assays, the smallest conversion and productivity were found.

The results suggest that the decreasing rate of enzymatic reaction along the process is due to the reduction of substrate concentration. In the beginning of the process, when high concentrations of substrate are present, high q_{max} is achieved; lactobionic acid quickly accumulates inside the beads, and the diffusivity resistance delays its neutralization by the alkali used to control pH. Therefore, a higher temperature would be favorable to the process, increasing diffusivity of substrates and products within the Ca-alginate beads. As the concentration of substrates decreased, reducing lactobionic acid rate formation, the equilibrium of product concentration inside and outside the beads would be more easily reached despite the diffusion barrier. At the end of reaction at pH 7.5 and 47 °C, mass transfer would have been improved by the higher temperature resulting in an excessive increment of internal pH, hindering the catalytic action of GFOR/GL. This hypothesis is confirmed since the internal pH of Ca-alginate beads after 24 h of each batch

Table 5 *Zymomonas mobilis* ATCC 29191 permeabilized immobilized-cells bioconversion of lactose, in medium containing equimolar concentrations of fructose as a function of temperature and pH

Temperature (°C)/pH	Lactobionic acid concentration[a] (mol l^{-1})	Conversion (%)	Maximal specific organic acid formation rate (mol g^{-1} h^{-1}) × 10^{-3}	Specific productivity (mol g^{-1} h^{-1}) × 10^{-3}	Final internal pH
39 °C/pH 6.4	0.48	73	1.6	1.0	6.1
39 °C/pH 7.5	0.47	72	1.7	0.97	6.6
47 °C/pH 6.4	0.49	75	2.1	1.0	6.4
47 °C/pH 7.5	0.45	68	2.0	0.92	6.8

Initial fructose/lactose concentration in the reaction, 0.70 mol l^{-1}; cell concentration, 20.0 g l^{-1}; process time, 24 h

[a] Concentration expressed in relation to the initial volume of the liquid phase plus the volume of alkali used for pH control

(Table 5) shows that the highest final lactobionic acid concentration and conversion were attained when the pH inside the beads was around 6.4, optimal for the action of GFOR/GL on fructose/lactose with permeabilized free-cells, as was shown in Fig. 1. These results confirm findings of a previous work of this group [14], which indicated that decreasing pH, while keeping the temperature constant, is the best approach for producing gluconic acid and sorbitol with Ca-alginate immobilized *Z. mobilis* cells. In the present work, good conversion yields and productivities could be achieved by controlling the temperature at 47 °C and with a pH gradient from 7.5 to 6.4 along the bioconversion process.

Conclusions

The results of this work have demonstrated the possibility of obtaining high concentrations of different aldonic acids by the enzymatic complex GFOR/GL present in permeabilized cells, both free and Ca-alginate immobilized, of *Z. mobilis.* Although GFOR/GL presented higher affinities for maltose and galactose than for lactose, applications for the products obtained from the first two aldoses are still to be developed. On the other hand, lactobionic acid, formed through the action of GFOR/GL on lactose, has important applications and a significant commercial value. Thus, designing a process using the GFOR/GL system capable of producing balanced amounts of sorbitol and lactobionic acid becomes an important technological alternative.

We have confirmed that Ca-alginate immobilized *Z. mobilis* cells can be used for repeated batches and long-term reactions for the production of high concentrations of sorbitol and lactobionic acid, and established the operational conditions of pH and temperature to be carefully controlled.

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