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Production of lactobionic acid and sorbitol from lactose/fructose substrate using *GFOR*/*GL* enzymes from *Zymomonas mobilis* cells: A kinetic study

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ABSTRACT

In this work, we have investigated the kinetics of the biotechnological production of lactobionic acid (*LBA*) and sorbitol by the catalytic action of glucose–fructose oxidoreductase (*GFOR*) and glucono-δ-lactonase (*GL*) enzymes. The cells of bacterium *Zymomonas mobilis* ATCC 29191 containing this enzymatic complex were submitted to permeabilization and reticulation procedures. The effect of the concentration of substrates on the rate of product formation using a mobilized cell system was investigated. The application of higher fructose concentration seems to not affect the initial rate of formation of the bionic acid. Under conditions of low initial concentration of lactose, the experimental kinetic data of the bi-substrate reaction were modelled by assuming a rate equation of the classical ping-pong mechanism. The found kinetic parameters displayed a low affinity of the *GFOR* enzyme for both substrates. The enzymatic system did not exhibit normal *Michaelis–Menten* kinetics in response to a change of concentration of lactose, when fructose was held constant, presenting a sigmoid relationship between initial velocity and substrate concentration. A rate equation based on *Hill* kinetics was used to describe the kinetic behaviour of this enzyme-substituted reaction at higher lactose concentrations. The results from batch experiments using immobilized cells within Ca-alginate beads revealed that there is no pronounced occurrence of mass transfer limitations on *LBA* production for beads with 1.2 mm in average diameter. This discussion aids for defining the best operating conditions to maximize the productivity for *LBA* and sorbitol in this bioconversion and also for reducing the complexity of downstream separation processes.

1. Introduction

Sorbitol (S) is a non-toxic and slightly hygroscopic compound with a mildly sweet flavour, being employed in large scale as texturizer, humectant, stabilizer and conditioner in several industries: pharmaceutical, cosmetic, confectionery products, etc. [1,2]. The world market demand for sorbitol is constantly growing; Bizzari et al. [3] have reported that there has been a growth rate around 1–2% per year since 1997. Lactobionic acid (*LBA*) is a polyhydroxyl bionic acid (α-hydroxy acids) with important uses as active ingredient: (i) in skincare products for hydration and anti-aging treatments, (ii) in solutions for the storage of organs for transplantation due to its strong chelating and antioxidant properties, (iii) in surfactant products containing detergent or cleaner formulations. Thus, *LBA* and derivatives can be recognized as high-value products [4].

Glucose–fructose oxidoreductase (*GFOR*) is an exclusive enzyme from Gram negative bacterium *Zymomonas mobilis* [5]. Using *Z. mobilis* in a fermentative process to produce ethanol from sucrose – or, alternatively, from a mixture of glucose and fructose – [6–8], *GFOR* is reported as the enzyme responsible for the sorbitol formation [5,9]. This enzyme uses as native substrates glucose and fructose to produce glucono-δ-lactone and sorbitol, at an equimolar ratio, through the hydrogen carrier NADP tightly bound to the enzyme. At high concentration of substrates, sorbitol is accumulated in the cells as a natural response in order to overcome osmotic stress in high-sugar media [10]. Applying the extraction and purification procedures of *GFOR*, glucono-δ-lactonase (*GL*) enzyme was also identified [5], which accelerates the hydrolysis of the glucono-δ-lactone to produce the gluconic acid.

GFOR is a tetrameric enzyme, determined by gel electrophoresis [5], and takes place in the periplasmic space of the bacterial cells [11,12]. The tetrameric structure consists in two domains as a classical dinucleotide-binding, determined by X-ray crystallography [13]. The N-terminal arm is responsible to the stabilization of the tetrameric configuration of the *GFOR* [13,14]. Each enzyme subunit contains the hydrogen carrier NADP that is not released

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during catalysis [13]. The structure of the enzyme is one of the most important factors for understanding and modelling the kinetics in bi-substrate systems [15].

In the context of valorisation of ordinary products, the capability of this endo-enzyme from *Z. mobilis* in allowing the hydrogenation of glucose coupled to fructose reduction has aroused interest from many research groups [16–20]; not only by the concomitant production of value-added products, but also by some attractive features such as weak inhibition and high specific activity [21]. Considering the required substrate system consisting of donor/acceptor of electrons, aldose sugars as alternative to glucose as well as other reducing sugars instead of fructose have been investigated to attempt the *GFOR* activity, and some sugars have shown some activity [5]. In particular, for the biochemical production of bionic acids and sorbitol, Satory et al. [22] have shown that the oxidizing action of *GFOR* includes a considerable number of donor substrates, for example monosaccharides, as xylose (to xylonic acid; 42% yield), galactose (to galactonic acid; 35% yield); or disaccharides, as maltose (to maltobionic acid; 30% yield), lactose (to lactobionic acid; 9% yield). In fact, literature exploring the use of *GFOR* with other substrates is lacking, and there is only a few works discussing the replacement of glucose [22,23]. Carra et al. [24] have investigated the optimized conditions of pH (range from 5.2 to 7.2) and temperature (from 37 to 54 °C) to promote the higher enzymatic activity using substrate pairs of 0.7 M fructose and different aldoses (maltose, galactose and lactose) besides glucose. Among the aldose sugars, lactose has been recognized as an attractive raw material for synthesis of food products and pharmaceutical derivatives. The use of this sugar is extremely interesting, since it can be obtained from the whey, a by-product of the manufacture of cheese and, many times, considered as a source of pollution.

In large scale, the perspective for the biotechnological industrial production of sorbitol and gluconic acid has been discussed in Silveira and Jonas [2], where they have pointed some crucial aspects: the simplicity of the reactive process and the use of the enzyme as well as the downstream processes for final products. With regard to the enzyme complex, experiments with *GFOR* in permeabilized cells of *Z. mobilis* for the production of sorbitol and gluconic acid [16–17,25,26] provide results similar to those obtained with purified enzymes. The use of *GFOR* enzyme has been mentioned as a low-competitiveness option to produce *LBA* [27,28], mainly because the low affinity for fructose and the high *Michaelis* constant of lactose. The final mixture obtained from this process has been also considered as one disadvantage, but Pedruzzi et al. [29] have shown that the recovery of the products (*LBA*, sorbitol) and substrates (fructose, lactose) can be achieved by adsorption techniques using a proper adsorbent. Also, the process of lactose oxidation integrated with separative systems, including the SMB and pseudo-SMB technologies, was analyzed by Borges da Silva et al. [30] as one alternative to allow enzymatic reactions to occur at highest substrate concentrations and to improve its productivity for the formation of lactobionic acid and sorbitol. Here, our goal fits in improving the biotechnological process and simplifying the downstream processes to produce sorbitol and lactobionic acid by enzymatic catalysis from glucose–fructose oxidoreductase and glucono-α-lactonase enzymes.

Gollhofer et al. [31] have commented on the recognition of the biotechnical potential of this enzyme, and the efforts for optimizing the enzymatic production of sorbitol and gluconic acid, as shown in elsewhere [17,32–34]. When studying the production of low sugar fruit juices by using this oxidoreductase, Aziz et al. [35] have remarked that it is surprising that so far only few concrete applications involving the enzyme, especially in food processing, have been described.

The objective of this work is to present kinetic studies on lactobionic acid and sorbitol production using the enzymatic glucose–fructose oxidoreductase/glucono-δ-lactonase system of *Z. mobilis*, for the achievement of improvements upon this enzymatic route. In this production, the effect of the concentration of substrates on the activity of *GFOR*/*GL* present in mobilized cell system was investigated. The action of the enzymes glucose–fructose oxidoreductase and glucono-δ-lactonase present in calcium alginate-immobilized *Z. mobilis* cells was also evaluated in this process of lactose oxidation.

1.1. Kinetic reaction pathway of GFOR

The catalytic cycle of *GFOR* enzyme (in purified form) on glucose and fructose was found as a classical ping-pong system [5], confirmed through the double-reciprocal kinetic plots. The overall reaction can be described as two half-reactions [21]:

$$\text{E-NADP}^+ + \text{G} \underset{k\_1}{\overset{k1}{\rightleftarrows}} \text{E-NADP}^+ \cdot \text{G} \underset{k\_2}{\overset{k2}{\rightleftarrows}} \text{E-NADPH} \cdot \text{GL} \underset{k\_3}{\overset{k3}{\rightleftarrows}} \text{E-NADPH} + \text{GL} \quad (1)$$

$$\text{E-NADPH} + \text{F} \underset{k\_4}{\overset{k4}{\rightleftarrows}} \text{E-NADPH} \cdot \text{F} \underset{k\_5}{\overset{k5}{\rightleftarrows}} \text{E-NADP}^+ \cdot \text{S} \underset{k\_6}{\overset{k6}{\rightleftarrows}} \text{E-NADP}^+ + \text{S} \quad (2)$$

where G, GL, F and S represent glucose, glucono-δ-lactone, fructose and sorbitol, respectively; E-NADP$^+$ and E-NADPH are enzyme-bound coenzymes at different redox states, forming intermediate complexes during the reaction. The parameters k are kinetic rate constants of the elementary reactions. In this double replacement mechanism, the enzyme works in such way that one substrate can bind at a time, with a single active site for all substrates [21,36]. There is one molecule tightly bound NADP(H) per subunit of *GFOR*. Hardman and co-workers [21,36] have extensively investigated the kinetic behaviour of the enzyme under specific operation conditions and the main rate constants have been characterized (using native substrates at low concentration). The overall rate-determining step in the direction of the glucose oxidation has been ascribed to the lactone dissociation. The reverse reaction, i.e., the hydrogen transfer from sorbitol to enzyme-bound NADP$^+$, has been considered negligible. From the estimated values for maximum rate with lactose/fructose and glucose/fructose, Satory et al. [22] have suggested a common rate limiting step in the oxidation of both substrates. So, taking all into consideration, the following approach on the kinetic reaction pathway of *GFOR* for the lactose/fructose substrate pair can be described as

$$\text{E-NADP}^+ + \text{L} \underset{k\_a}{\overset{k_a}{\rightleftarrows}} \text{E-NADPH} \cdot \text{Lct} \xrightarrow{k_b} \text{E-NADPH} + \text{Lct} \quad (3)$$

$$\text{E-NADPH} + \text{F} \underset{k\_c}{\overset{k_c}{\rightleftarrows}} \text{E-NADP} \cdot \text{S} \xrightarrow{k_d} \text{E-NADP}^+ + \text{S} \quad (4)$$

where L and Lct represents lactose and lactono-δ-lactone, respectively (scheme shown in Fig. 1). Assuming a pseudo-steady state for all enzyme substrate complexes, and using the overall balance for the enzyme, the reaction rate (v) can be expressed as:

$$\frac{[C_{E_o}]}{v} = \frac{1}{k_d} + \frac{1}{k_b} + \frac{(k_a + k_b)}{k_a k_b [C_L]} + \frac{(k_{\_c} + k_d)}{k_c k_d [C_F]} \quad (5)$$

or in the general form:

$$v = \frac{v_{\max}}{1 + (K_L/[C_L]) + (K_F/[C_F])} \quad (6)$$

with

$$K_F = \frac{k_{\_c} + k_d}{k_d + k_b} \cdot \frac{k_b}{k_c} \quad (7)$$

$$K_L = \frac{k_a + k_b}{k_d + k_b} \cdot \frac{k_d}{k_a} \quad (8)$$

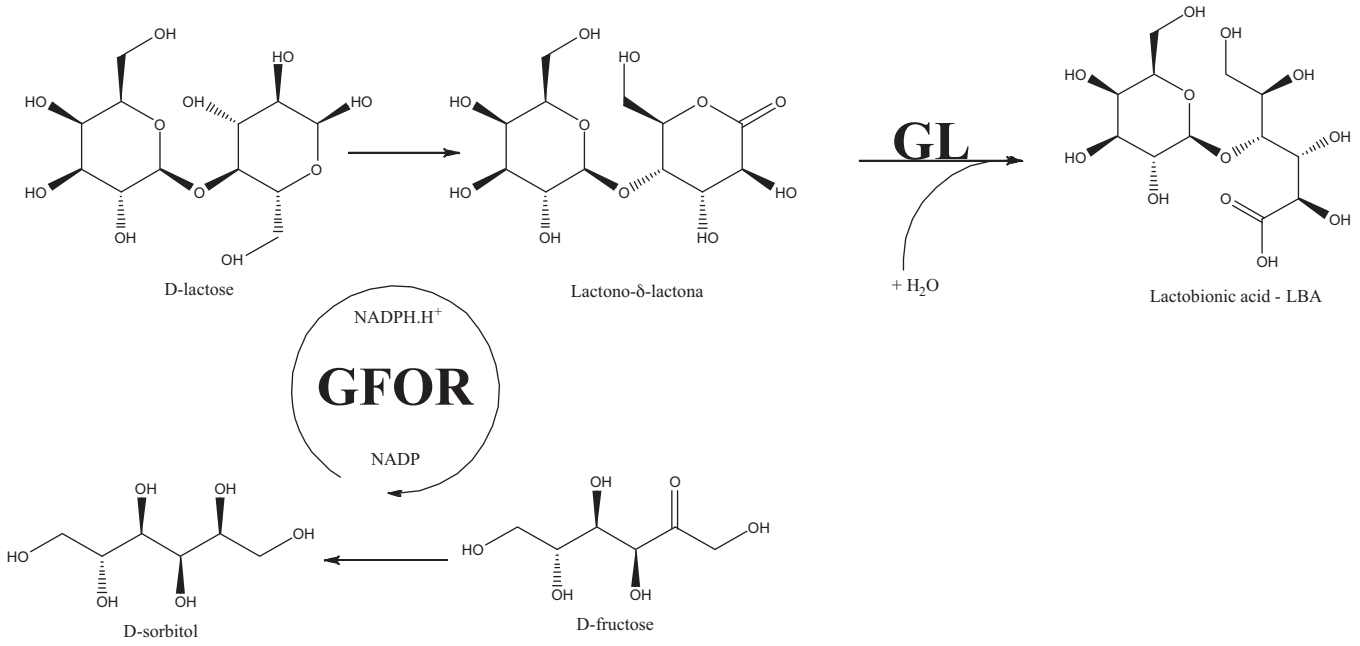

Fig. 1. Reaction scheme for the production of *LBA* and sorbitol with successive oxidation-reduction cycles of NADP in GFOR; lactone hydrolysis by GL.

$$v_{\max} = \frac{k_d k_b}{k_d + k_b}[C_{E_o}] \quad (9)$$

where C_i is the concentration of species i ($i = L, F$ and E_o). The parameters of the reaction rate given in Eq. (6) can be determined from Lineweaver–Burk method and/or mass balance of the species in the reactor. At the end, the kinetic pathway of *GFOR* can be used to describe the consumption of both substrates and formation of sorbitol and lactono-δ-lactone.

2. Materials and methods

2.1. Chemicals

Sorbitol (>98%), lactobionic acid (≥97%), sulphuric acid (97.5%; for HPLC purposes), KOH (>86%), and Na-alginate (bioreagent identified as alginic acid sodium salt from brown algae (product no. 71238, Fluka) and employed to immobilize the bacteria cells) were purchased from Sigma–Aldrich (Steinheim, Germany). The sugars used in kinetic assays, D-lactose 97% and D-fructose 99%, were purchased from Alfa Aesar (Karlsruhe, Germany). All others chemicals used were p.a. grade.

2.2. Microorganism and culture conditions

Bacterial strains *Z. mobilis* ATCC 29191 were kindly donated by Professor M.M. Silveira, University of Caxias do Sul, Brazil. The bacteria cells were grown in a 2 L fermentor SGI/SET2 (SGI, France) under conditions of controlled pH (pH 5.5; using a 5 M KOH solution) and temperature (30 °C) as well as stirring speed (350 rpm). The culture medium was composed of glucose (150 g L^{-1}), used as the carbon source, yeast extract (5 g L^{-1}) and some micronutrients [37]. The mass cell production was obtained through several batch fermentations in a semi-continuous mode (operational volume of 1.5 L). The cell concentration for each fermented volume was about 2.3 g L^{-1} (dry mass). In our methodology, a volume of 1.1 L of the growth medium was centrifuged (Digtor 20 centrifuge, Ortoalresa, Span) and, afterwards, the whole cells were re-suspended at 25 g L^{-1}. During this procedure, the bioreactor was recharged with a fresh culture medium of same removed volume to be fermented with the remained cells from the previous stage.

The whole cells were washed with deionized water three times before the permeabilization procedure and, also, between the permeabilization and crosslinking treatments. Cells were permeabilized with CTAB (0.2%, w/v), when the same amount of solution of cells and CTAB were mixed under slow magnetic stirring during 10 min at 4 °C. Cells were crosslinked with glutaraldehyde (0.5%, v/v), when the same amount of solution of cells and glutaraldehyde were mixed under slow magnetic stirring during 15 min at ambient temperature [37]. The reason for the cross-linking of the cells is to prevent losses of enzyme from the periplasmic space of the permeabilized cells [38,39]. Finally, these whole cells were washed three times with deionized water and the resulting solution (144 g L^{-1}) was kept at 4 °C for further applications.

2.3. Cells immobilization in calcium-alginate

The permeabilized and crosslinked cells were immobilized in Ca-alginate beads by entrapment in a traditional process of dripping in $CaCl_2$ solution. Four grams of Na-alginate were dissolved in 150 mL deionized water, which was achieved by slow mechanic shaking overnight (100 rpm). Forty milliliters of concentrated cells (144 g L^{-1}) were added to the Na-alginate solution under shaking condition for 3 h. The volume was collected to a volumetric flask and brought up to 200 mL in volume with deionized water. The drops were formed by simply dripping aqueous Na-alginate solution with cells into 500 mL $CaCl_2 \cdot 2H_2O$ (40 g L^{-1}). Dripping was generated by a peristaltic pump (model 305, Watson-Marlow, USA) that provides reasonably smooth flows using a sterile silicone tube (3 mm, internal diameter) and a needle (40 mm long, 0.5 mm internal diameter) in vertical position over 1.2 m of distance to the surface of the $CaCl_2$ solution (kept under shaking condition at 350 rpm). All obtained beads were transferred to another reservoir containing 500 mL fresh $CaCl_2$ solution and then stayed at 4 °C overnight. Afterwards, the whole beads were washed and stored in deionized water at 4 °C to be used in enzymatic kinetic experiments. In order to evaluate mass transfer limitations on the reaction kinetic, beads were fractioned in four groups according its size using *Retsch* test sieves (mesh numbers: 10, 14 (*Endecotts* sieve), 18 and 20; according to ASTM E11:01). Those size fractions retained on 14 and 18 mesh sieves were used in further experiments (see Table 1).

2.4. Analytical methods

The cell concentration was determined by turbidity measurements (absorbance at 560 nm) in a 7800 UV/vis spectrophotometer (Jasco, Japan) and by gravimetrical analysis (dry mass). The quantification of lactose, fructose, *LBA* and sorbitol was carried out using the methodology described by Pedruzzi et al. [40]. An indirect method to determine the *LBA* production, through the amount of added KOH moles during the bioconversion, was also employed. The alkali solution (KOH) was added to the reactor in order to control the value of pH during the reaction.

Table 1
Different sizes of Ca-alginate gel beads containing permeabilized and crosslinked cells of *Z. mobilis*.

Diameter ($\varnothing_p$; mm)	Average diameter ($\varnothing_a$; mm)	Mass of beads fraction (g)	Total (%)
$\varnothing_p < 1.00$	–	1.76	0.84
$1.00 < \varnothing_p < 1.41$	1.20	37.4	17.8
$1.41 \leq \varnothing_p < 2.00$	1.70	151	71.9
$\varnothing_p < 2.00$	–	19.75	9.46

2.5. Bioconversion kinetics

Bioconversion experiments were performed in a closed bioreactor vessel of 150 mL nominal volume. The initial work volume was a solution of 100 mL containing lactose, fructose and biocatalyst cells. The sugar mass corresponding to the desired concentration of substrates was dissolved in 50 mL deionized water at 60 °C. After cooling to 25 °C, that volume was brought up to 90 mL with deionized water and then introduced into the bioreactor. The start-up of an experimental run was given when 5 mL of concentrated cell solution (144 g L^{-1}) and 5 mL of deionized water (used to wash the test-tube containing the cell solution) were added to the bioreactor. Hence, a total biocatalyst concentration of 7.2 g L^{-1} was obtained.

The operating conditions were: shaking frequency of 350 rpm (magnetic stirrer), reaction temperature at 39 °C and value of pH equal to 6.4. The pH control was made by the automatic addition of KOH using gravity flow of alkali and one solenoid valve, which was activated when the pH controller from fermentor SGI/SET2 measured any drop in pH set point because the acid production (by the glass electrode). The used concentration for the alkali solution was dependent on the concentration of the limiting substrate (lactose), ranging from 0.2 M to 5.0 M. The addition of alkali solution into the reactor was restricted to an upper limit of 15 mL. The added KOH volume was registered in a pipette, and converted in mol of produced acid by stoichiometric relation 1:1 (indirect method for *LBA* quantification). The control of pH was sensitive within from 0.1 up to maximum 0.2 pH units, which is satisfactorily narrow for the enzymatic reaction.

Samples from the reactor were collected during each experiment using a precision sampling syringe. In order to stop the reaction occurring in samples, these samples were promptly diluted, filtered in cellulose acetate membrane (0.45 μm, Whatman) and kept at 0 °C to subsequent analyses in HPLC. Two different dilution rates were applied depending on the initial concentration of substrates at the beginning of the reaction: 1:50 when starting with high concentration and 2:25 with low concentration in given experiment. The number of samples taken in each run was around sixteen with a higher frequency of sampling in the first hours (for a proper determination of the initial rate of reaction). The total volume was also measured at the end of each experiment run. The alkali additions and some small evaporation were taken into account for the determination of *LBA* concentration by the indirect method. The chromatograms determined by HPLC showed that the bioconversion reaction did not show any detectable by-product and therefore the substrates lactose and fructose can be only converted to *LBA* and sorbitol, respectively.

In the case of using immobilized cells, the series of reaction experiments were performed in a closed bioreactor vessel of 250 mL nominal volume. The initial work volume was 150 mL containing lactose, fructose and Ca-alginate beads with immobilized cells. The initial sugar content corresponding to the desired concentration of substrates was dissolved in 90 mL deionized water at 70 °C. After cooling it till 25 °C, the volume was brought up to 100 mL and introduced into the bioreactor. Subsequently, 50 mL of the prepared Ca-alginate beads (with 14.4 g L^{-1} immobilized cells) was added to the bioreactor starting the enzymatic reaction. All the other operating conditions were the same as described before.

Some experimental runs were carried out in duplicate (the number of trials was limited by the amount of cells produced). The duplicate experiments agreed fairly well. Also, all measurements in each category of experiments (mobilized and immobilized cell systems) were made in duplicate. The experimental points correspond to average values.

3. Results and discussion

Fig. 2a and b shows the experimental evolution of concentration of lactobionic acid during the course of enzymatic reactions of lactose oxidation and fructose reduction using permeabilized and reticulated cells of *Z. mobilis*, which contain the *GFOR* and *GL* enzymes. Through these figures, one can verify the effect of the concentration of each one of two substrates on the rate of formation of the bionic acid during the process. In these experimental runs, high concentrations of substrates were used, since it consists of a more favorable condition to the action of the *GFOR* enzyme [19,32]. As one can confirm from the shown experimental data, the measurements of consumption of the alkali used to pH control maintained a good kinetic agreement with the amount of produced product measured during the course of reaction by using the HPLC technique. Hence, both analytical procedures can be considered adequate. In Fig. 2a, the initial concentration of fructose was chosen as 350 mM and the initial concentration of lactose was varied from 400 mM to 700 mM. The higher lactose concentration in the reactive medium led to a significant increase of the rate of formation of the lactobionic acid. However, the maximum feasible initial concentration of this disaccharide to be used in the enzymatic reaction is constrained by its solubility at the reaction temperature (39 °C); in this case, it

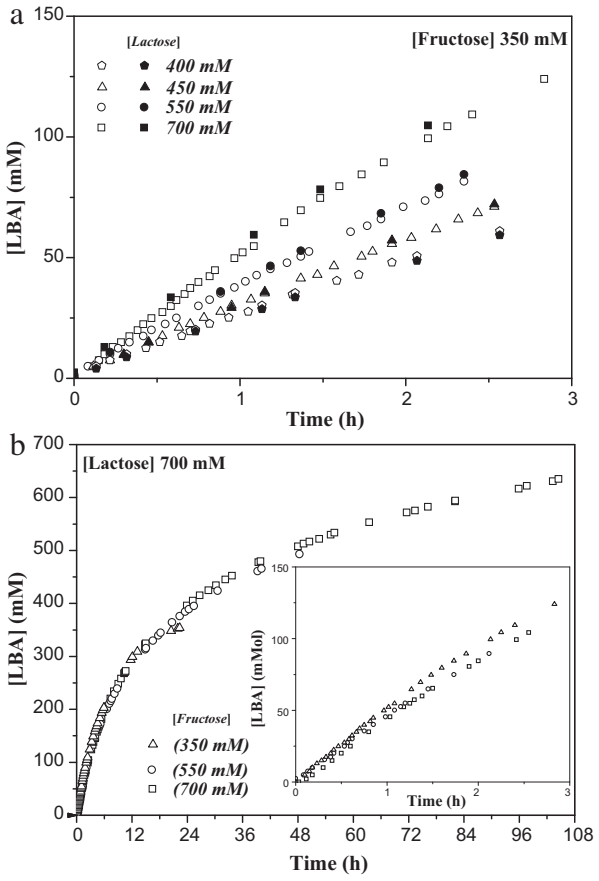

Fig. 2. Concentration–time profile of *LBA* by the action of the GFOR/GL (from cells of bacterium *Z. mobilis* ATCC 29191) on the lactose/fructose substrate pair. Initial substrate solution: (a) 350 mM fructose in the presence of different concentration of lactose; (b) 700 mM lactose in the presence of different concentration of fructose. Quantification of *LBA*: by the indirect method (open symbols), by using HPLC analysis through the chromatographic peaks (closed symbols). Reaction conditions: 39 °C, pH 6.4, 350 rpm, [*X*] = 7.19 g L^{-1} (cell concentration, in dry mass).

is around 30 g per 100 g solution at 39 °C [41]. For the condition of 700 mM lactose at the beginning of the reaction and different initial concentrations of fructose, one can see from Fig. 2b that when the monosaccharide concentration was increased from 350 to 700 mM, there was no remarkable influence on the initial rate of the enzymatic reaction (the activity of the enzyme does not seem to be affected). An overlapping of the experimental curves took place at the first hours of the bioconversion and, of course, the reaction was finished earlier for the case of lower fructose concentration available in the reactive medium.

Under the applied reaction conditions (pH 6.4 and 39 °C), when 700 mM lactose/350 mM fructose pair substrate was used, the maximum possible conversion yield was attained after 24 h of operation. At 700 mM equimolar mixture of sugars, the reaction was stopped before the total substrate consumption (concentration of *Z. mobilis* cells, on dry basis, was only 7.2 g L^{-1} that represents a low value when compared to 25–30 g L^{-1} presented in the literature [42]). This fact can be also related to the affinity of the enzyme for the substrates; it may even be low enough to discontinue the reaction under conditions of very low substrate concentrations. In the production of the *LBA*, results shown in Fig. 2a and b revealed that the application of higher fructose concentration did not affect decisively the initial rate of formation of the bionic acid. The rate was dependent on the concentration of lactose and, of course, the amount of biocatalyst. Therefore, these parameters can be optimized further for defining the best operating conditions

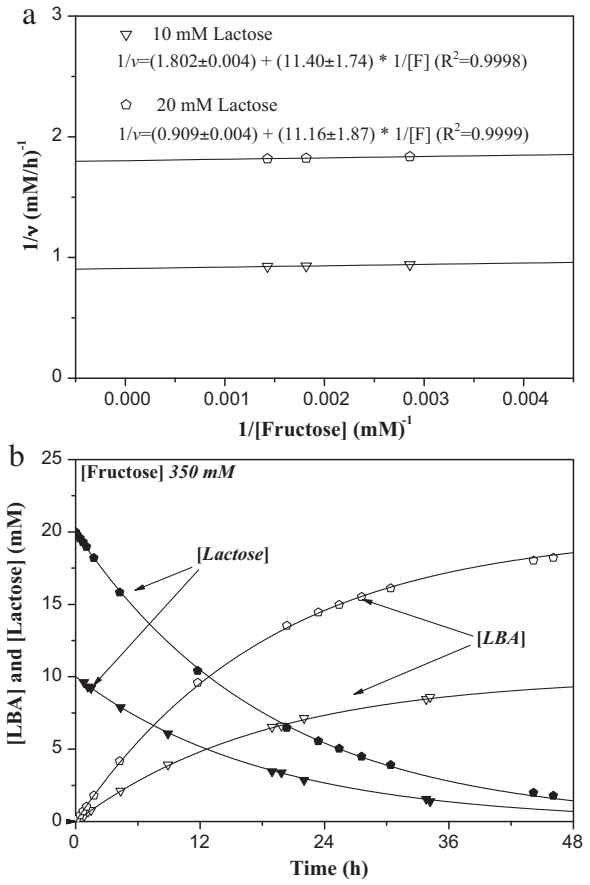

Fig. 3. (a) *Lineweaver–Burk* plots of initial reaction rates of GFOR/GL producing *LBA* and sorbitol, at low initial concentration of lactose (classical ping-pong model). (b) Comparison between experimental profiles and kinetic curves from the ping-pong equation; *LBA* (open symbol) and lactose (closed symbol). Quantification by HPLC analysis. Conditions: 39 °C, pH 6.4 (KOH 0.2–0.8 M), 350 rpm, [*X*] = 7.19 g L^{-1} (in dry mass).

to maximize the productivity for *LBA* and sorbitol in this bioconversion. Applying high lactose concentration, besides the higher enzymatic activity, the total consumption of fructose could benefit the downstream separation processes, since it reduces the complexity of the formed mixture (one equimolar mixture of products and lactose, in excess).

Considering Eq. (6) describing this case of two-substrate enzyme-substitute system, the ping-pong kinetic parameters can be obtained if lactose is held at constant concentration and fructose concentration is varied, or vice versa. For instance, initial rate data from Fig. 2 can be plotted using the Lineweaver–Burk technique. Since only initial experimental points of the concentration curve are taken in determining kinetic parameters by the application of Lineweaver–Burk plots, the global mass balance in the batch reactor is also a valid tool to obtain these parameters using all the points of the experimental curves. An initial estimation of the kinetic parameters was determined by the Lineweaver–Burk technique. A nonlinear regression method was performed in such way the values of the parameters must minimize the sum of the residual values for the set of experimental data. Besides runs with high initial concentrations of lactose, some runs were also carried out at low initial concentrations of this substrate (hypothetically, at non-inhibitory substrate concentration), maintaining the same concentration of 7.2 g L^{-1} free cells of *Z. mobilis* (dry basis). Fig. 3a presents the double reciprocal plot from initial reaction rates of *GFOR*/*GL* producing the lactobionic acid and sorbitol, with fructose as variable substrate and under conditions of low initial concentration of lactose. In accordance to classical ping-pong mechanism, straight, and parallel, lines are verified in the range of studied concentration of fructose 350–700 mM (correlation $R^2 > 0.999$). Fig. 3b shows some experimental and predicted profiles of the oxidation of lactose to lactobionic acid by the studied enzymatic system at two different initial substrate conditions: 10 mM lactose and 20 mM lactose with 350 mM fructose. The parameters of the classical ping-pong model – Eq. (6) – at low concentration of lactose, are listed in Table 2. There was a good agreement between the experimental data and the kinetic behaviour predicted by such model. With respect to the *LBA* formation, the overall kinetic model should include both the formation rate and the hydrolysis rate of the formed lactone. However, we have observed that, under the current reaction conditions, the hydrolysis reaction of lactono-δ-lactone is sufficiently rapid. In all cases, the experimental kinetic profiles of *LBA* (measured by indirect and HPLC methods) were in close agreement to the experimental kinetic of sorbitol (results not shown) and, therefore, it was assumed that the formation rate of the acid could be expressed by Eq. (6). In fact, in the case of glucose as substrate, published results have shown that gluconolactone is also hydrolyzed very rapidly having a quite short half-life compared to the rate limiting step of its respective catalytic process [5,31]. Models describing the spontaneous hydrolysis of gluconolactone, and other aldono-δ-lactones, have shown clearly how fast this reaction is [43].

Table 2
Kinetic parameters of models accounting for the double displacement mechanism to predict the kinetic of lactose oxidation by GFOR at low and high initial concentration of lactose.

Parameters	Values—at low $[C_L]_o$ [a]	Values—at high $[C_L]_o$ [b]
υ_{max} (mM/h)	66.0 ± 16.2	276.0
K_L (mM)	(1.183 ± 0.289) × 10^3	1.86× 10^3
K_F (mM)	(0.739 ± 0.218)× 10^3	1.85× 10^2
n	–	1.6

[a] Classical ping-pong model – Eq. (6) – applied to the lactose oxidation by GFOR at low concentration of lactose (obtained from *Lineweaver–Burk* technique; 95% confidence interval).
[b] Ping-pong model with *Hill* coefficient applied to the lactose oxidation by GFOR at high concentration (global mass balance applying a procedure of best fitting; adjusted R^2 value of 0.9801).

The obtained value of the apparent *Michaelis–Menten* constant for lactose (i.e., 1.18 M) is in agreement to the published value (1.2 M) by Satory et al. [22]. The kinetic constant for fructose was not determined by these authors, but the found value of K_F in our work is in similar order of magnitude to those ones obtained when the enzymatic complex catalyzed the bioconversion of native substrates, i.e., $K_m \sim 0.4$–1.4 M [5,21,28]. The calculated values of K_L and K_F were quite high, which reveals one major inconvenient of this enzymatic pathway for the *LBA* production: the low affinity of the *GFOR* enzyme for both substrates (thus, it requires high levels of substrate concentration to promote higher productivities of the oxidation reaction).

In Fig. 4, the experimental values of the initial rate of lactobionic acid formation were plotted as a function of the initial lactose concentration at fixed initial fructose concentration of 350 mM. At low concentration, the visualization was not so clear, but we have shown that the kinetic behaviour followed the *Michaelean* model for bi-substrate reaction. Hence, from the parameters of the classical ping-pong model, the lines showed the graphical presentation of Eq. (6) for the current enzymatic system, under the mentioned conditions, applied to the entire range of concentration of lactose at three different conditions of fructose. It was found that the initial rate of reaction was underpredicted by the model, which is not able to predict the experimental kinetic behaviour at high concentrations. One can see that the experimental points followed a sigmoid trend with increasing concentrations of lactose. A sigmoid profile is typical of regulatory enzymes where allosteric and/or cooperative

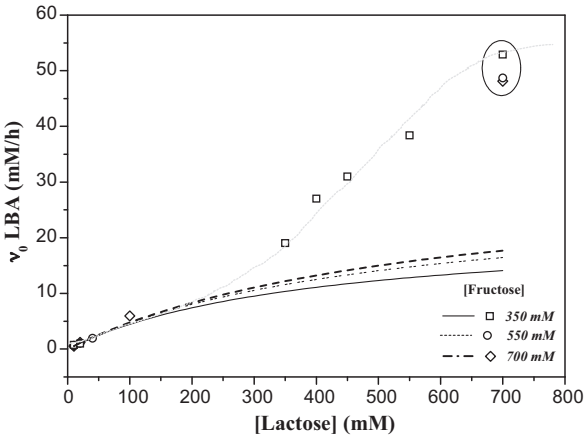

Fig. 4. Profile of the initial reaction rate as a function of the initial concentration of lactose at a given concentration of fructose: 350 mM (□ —), 550 mM (○ ···) and 700 mM (◊ – –); (symbols) experimental points; (black lines) classical ping-pong kinetic equation. The circle points out the effect of fructose concentration, at 700 mM lactose, on reaction rate of GFOR/GL producing *LBA* and sorbitol. Conditions: 39 °C, pH 6.4 (KOH 5 M), 350 rpm, [*X*] = 7.19 g L^{-1} (in dry mass).

effects are taking place [15]. At this point, it cannot be ascertained whether this peculiar behaviour was not caused by the low affinity of the enzyme for these substrates or due to any additional control mechanism for the regulation of enzyme activity when other aldoses, lactose in this case, are used as alternative to glucose. As described in literature, *GFOR* can be structurally changed by the addition of one substrate [5]. As can be expected, the sigmoid curve is interrupted by the limitation of solubility of the lactose at the reaction conditions. Moreover, the initial rate data from the experiments at 700 mM lactose decreased as the initial concentration of fructose increased (experimental points within the circle shown in Fig. 4). This decline in reaction rate at such concentration level could in principle be explained by the inhibition of the *GFOR* by the increased fructose concentration and therefore the competition for substrate binding sites. But we could not discard the possibility of deactivation of the enzyme [28] or problems linked to the increased viscosity of the medium solution, as pointed out by Zachariou and Scopes [5] for the case of glucose/fructose pair substrate at higher equimolar concentrations.

Some works have reported the activity loss of *GFOR* (purified) by conformational and chemical changes on the tetrameric structure of the enzyme during the translocation hydrogen step to produce the lactone [28,44]. This statement concurs with previous studies whereby it has been assumed that the inactivation of the catalytic cycle from *GFOR* in free cells of *Z. mobilis* involves an unusual mechanism, through the oxidation of cysteine residues [31]. In all of these investigations the researchers have employed purified *GFOR*, or a French pressure cells, to obtain the crude cell extracts. However, the cytoplasmic membrane makes up highly permeable after permeabilization procedure, and all small compounds (metallic ions and cofactors) can be transferred out from the cells by diffusion [16]. These authors have obtained a high production of sorbitol and gluconic acid performed by the *GFOR* enzyme in permeabilized cells of *Z. mobilis* bacteria without the interference of other parallel metabolic pathway. In addition, Jang et al. [38] have produced sorbitol and gluconic acid with *GFOR* enzymes in permeabilized and crosslinked cells of *Z. mobilis*. In this case, they have reported that the stability of the enzyme was apparent and without loss of activity of the enzyme for 30 days in a continuous process. In the present work, kinetic experimental runs were carried out with permeabilized and crosslinked cells and, for each experiment, fresh cells were used. Hence, the interference of inactivation processes on the presented kinetic profiles might not be pertinent.

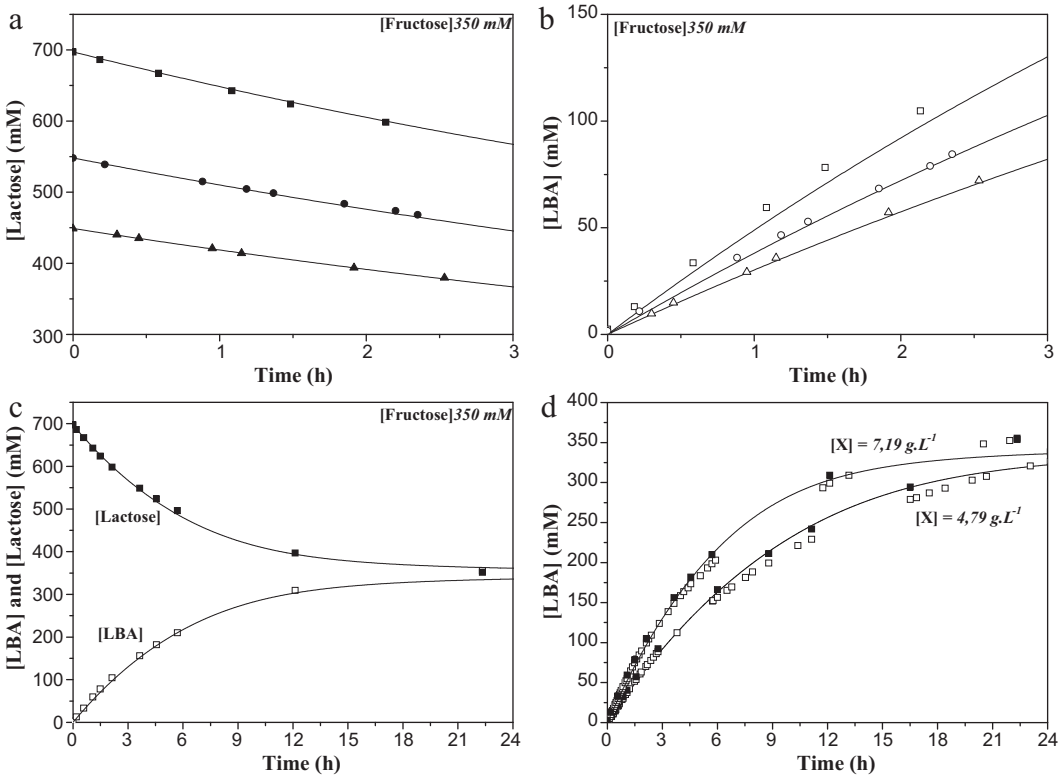

Fig. 5. Concentration–time profiles of (a) lactose consumption and respective; (b) *LBA* production by the action of GFOR/GL (from permeabilized free-cells of *Z. mobilis*); (c) complete kinetic for 700 mM lactose/350 mM fructose substrate pair; (d) concentration–time profiles of *LBA* production using different biocatalyst concentration. (symbols) Experimental points; (lines) ping-pong kinetic equation with Hill coefficient. Quantification of *LBA*(d): by the indirect method (open symbol), by using HPLC analysis through the chromatographic peaks (closed symbol). Conditions: 39 °C, pH 6.4, 350 rpm ([*X*] in dry mass).

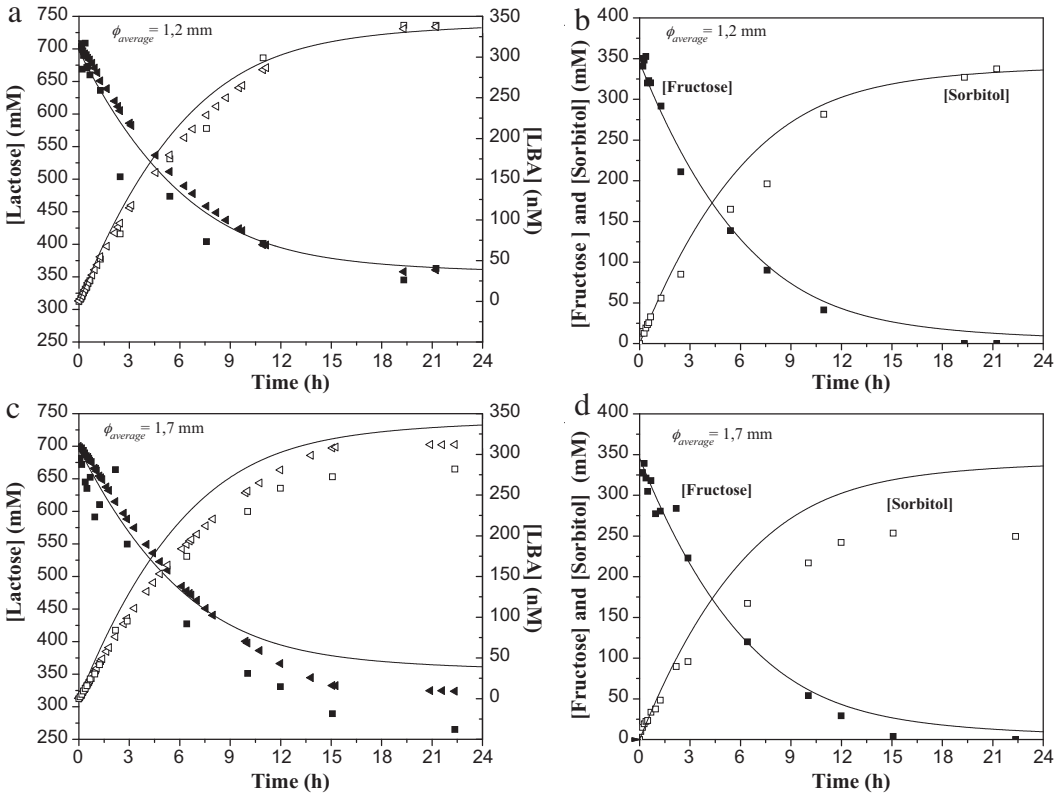

Fig. 6. Concentration–time profiles of products (open symbol) and substrates (closed symbol) by the enzymatic reaction with *Z. mobilis* cells immobilized within Ca-alginate beads. Average diameter ($\phi_{average}$): (a and b) 1.2 mm; (c and d) 1.7 mm; (symbols) experimental points, (lines) ping-pong kinetic equation with Hill coefficient. (◄ △, indirect method of quantification) Conditions: $[C_L]_o$ = 700 mM, $[C_F]_o$ = 350 mM, 39 °C, pH 6.4, 350 rpm, $[X]_{immobilized}$ = 14.2 g L^{-1} (in dry mass).

In order to describe proper behaviour of the kinetic of complex *GFOR*/*GL* to produce *LBA* and sorbitol at high substrate conditions, the ping-pong model with a *Hill* coefficient was assessed. As previously mentioned, the application of the ping-pong model for the case of lactose/fructose pair substrate is based on the *Michaelis–Menten* kinetics used to describe the steps of oxidation and reduction of native substrates (glucose/fructose pair) in each one of the active sites of the *GFOR* enzyme. One rate equation based on *Hill* kinetics could be derived from the idea that the binding effect of one substrate with the enzyme might be able to induce structural changes and variations in binding affinity on other active sites. Making an analogy from the ping-pong equation, the following rate equation can be assumed:

$$v = \frac{v_{max}}{1 + (K_L/[C_L]^n + (K_F/[C_F]^n))} \quad (10)$$

where n is the called *Hill* coefficient, which is linked to the number of active sites in the tetrameric form of *GFOR* ($n \leq 4$).

One major advantage of the proposed *Hill*-based rate equation is its ability to accurately describe the rate of product formation in the desired range of substrate, even if the substrate binding mechanism is unresolved. As stated by Hanekorn [45], in the field of computational systems biology, *Hill* equations take a place between purely mechanistic equations and purely empirical formulations. The results depicted in Fig. 5 showed that *Hill* equation – Eq. (10) – fitted the experimental data very well.

Fig. 5a–c shows experimental data regarding the initial kinetic behaviour of lactose consumption (a and c) and *LBA* production (b and c) by the action of *GFOR*/*GL* from the permeabilized, and crosslinked, free-cells of *Z. mobilis* (under the known reaction conditions and at high lactose concentration). Fig. 5d presents the kinetic profiles of *LBA* production when the bioconversion is performed for two different concentrations of biocatalyst: 4.79 and 7.19 g of dried cell per liter of solution into reactor. By solving the global mass balance of the enzymatic batch system, the suggested kinetic equation was fitted to the experimentally measured kinetic curves at fixed initial fructose concentration (350 mM). Therefore, the estimation of new parameters of Eq. (10) was found from a 'best fit' procedure in which the sum of residual between the experimental and calculated concentrations for the four compounds had to be minimized. These parameters are listed in Table 2. As may be noticed in Fig. 5, the concentration–time profiles calculated by the ping-pong kinetic equation with *Hill* coefficient could be used to evaluate sigmoid kinetic data for this bi-substrate reaction at higher initial concentration of lactose (however, at very low substrate conditions, *Hill* equation fails to predict proper behaviour). Being aware on the limitations of the application of such rate equation, since it incorporates key kinetic information, it can be useful as a tool in more complex computational models of process design and optimization, enabling a formulation to accurately describe the enzymatic kinetic behaviour for a desired concentration range of operation.

Concerning the found value of the *Hill* coefficient (=1.6), as it was previously described the configuration of *GFOR* enzyme corresponds to a tetrameric group with a N-terminal arm responsible for this structure [13]. Kingston et al. [13] have found an unexpected structural similarity between the *GFOR* and glucose-6-phosphate dehydrogenase (G6P) enzymes, where the possibility that both enzymes have been developed from a common ancestor has been discussed. G6P is the second enzyme of the *Entner–Doudoroff* glycolytic pathway [46]. For instance, the response of this enzyme to glucose-6-phosphate concentration becomes sigmoidal, with a *Hill* coefficient up to 2. At low ionic strength in the absence of phosphoenolpyruvate, the response to glucose 6-phosphate concentration

was shown to be the *Michaelis–Menten*, but at physiological ionic strength and pH, a *Hill* coefficient of 1.2–1.4 was found even in the absence of phosphoenolpyruvate. The K_m values for NAD and NADP were also ionic-strength-dependent, increasing rapidly as salt concentration increased [46].

3.1. Bioconversion with immobilized cells in calcium-alginate

The immobilization of enzymes, or whole cells, into polymeric matrix may offer several technological advantages. However, it becomes imperative that such polymers are smart carriers designed so as not to cause additional difficulties, as for instance, enabling the conditions of pH, of temperature, of substrate concentration, of availability to the additives and stabilizing agents, etc., within the matrix of immobilization are the same than those found in the bulk solution of the bioreactor. Moreover, the limitations of mass transfer of compounds between the interior and exterior of the particles should not add complexities to the process of enzymatic bioconversion. Taking into account these considerations, we investigated the potential for the lactose oxidation of the *Z. mobilis*, prepared as shown before, entrapped into calcium alginate.

Fig. 6 shows the experimental points for the rate of lactose oxidation (a and c) and fructose reduction (b and d) (and respective product formation) with *Z. mobilis* cells (CTAB permeabilized and treated with glutaraldehyde) immobilized on Ca-alginate beads. The curves (represented by lines) depicted in Fig. 6 correspond to the kinetic behaviour described by the ping-pong equation with *Hill* coefficient, taking into account the cell concentration mixed with the Ca-alginate solution and used in each experimental run of the bioconversion. A comparison between the rates from the experimental measurements and these profiles provided us a rapid impression of mass transfer limitations occurring during the process. There was no remarkable interference on the rate of product formation for the case of particles with average diameter of 1.2 mm. The value of the formation rate of bionic acid for this size range was 41.6 mM h^{-1}, while when the reaction was carried out using beads with 1.7 mm average diameter, this rate decreased to 33.6 mM h^{-1}. Bead size represents a critical parameter influencing mainly the productivity and the enzymatic activity of the system. The activity of enzymatic *GFOR*/*GL* system of *Z. mobilis* immobilized in alginate has been evaluated for the gluconic acid production by Malvessi et al. [34]. In this study, the average size of the beads was about 2 mm, and they have noticed temperature and pH levels differ significantly from the inner space of the beads to the external medium. Ryu et al. [47] have pointed out that beads with 1.00–1.15 mm in diameter may overcome mass transfer limitations and give improved values of kinetic constants. The diffusion coefficients of small carbohydrates in gel matrix as calcium alginate have been reported to be slightly lower than the corresponding diffusivity in water [48,49]; however, such mass transfer coefficient can vary depending on alginate concentration, amount of cells entrapped into matrix, concentration of compounds, among others [50]. According to our experimental results, no considerable mass transfer limitation was observed when permeabilized cells were immobilized with k-carrageenan [38].

From these data, it was evident that immobilized cells in Ca-alginate, converting 700/350 mM lactose/fructose substrates, represent one good operational alternative to produce *LBA* and sorbitol at equimolar ratio. However, additional studies should be performed to verify the mechanical stability of the Ca-alginate matrix containing the whole *Z. mobilis* cells during a longer operation period due to the breakage of smaller beads.

4. Conclusions

In this work, we have investigated the kinetics of lactobionic acid and sorbitol production, from lactose/fructose substrates, using the enzymatic glucose–fructose oxidoreductase/glucono-δ-lactonase system of *Z. mobilis* in two different forms: mobilized cell system and cells immobilized within calcium alginate beads. During the mobilized cells assessment, under the applied operational conditions, lactose concentration was critical parameter for the *GFOR*/*GL* complex of *Z. mobilis* in order to ensure maximum reaction rates. Higher initial concentrations of this substrate led to a larger action of the enzymatic complex. On the other hand, the use of higher initial fructose concentration for this bioconversion did not cause any relevant increase on the rate of product formation.

For conditions of low substrate concentrations, the kinetic behaviour of the lactose oxidation and fructose reduction by the *GFOR* can be modelled by using one classical ping-pong equation as occurring for the case of the action of this enzyme on native substrates (glucose and fructose). At the range of high substrate concentrations, a sigmoid relationship was obtained between the initial rate of reaction and the concentration of lactose on fixed initial concentration of fructose. This peculiar behaviour may be caused by the low affinity of the enzyme for the current substrates or due to an additional control mechanism for the regulation of enzyme activity when lactose is used as alternative to glucose. Since *GFOR* can be structurally changed by the addition of one substrate, this fact requires further investigation. The ping-pong model with *Hill* coefficient was able to describe the kinetic profile at high substrate concentrations.

In a stirred batch process, two different sizes of Ca-alginate beads entrapping permeabilized, and crosslinked, *Z. mobilis* cells were investigated for the production of *LBA* and sorbitol: 1.2 mm and 1.7 mm (average diameter). The higher rate of lactobionic acid formation was obtained with the smaller gel Ca-alginate bead, in which mass transfer resistance seems to be not significant. Further studies might point out whether there will be easier breakage of these smaller gel particles in long term processes and if it will lead to more pronounced loss of enzyme activity.

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