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Study of the deactivation of β-galactosidase entrapped in alginate-carrageenan gels

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Abstract

The stability of β-galactosidase entrapped in Ca-alginate–K-κ-carrageenan gels under operation conditions was studied. The thermal deactivation of the immobilised enzyme and the biocatalyst protein loss due to gel swelling were taken into account in the mass balance of the enzymatic reaction rate expression.

Time-temperature effect was the most important factor in the biocatalyst deactivation reaction. However, results showed that the enzyme entrapped in gels was partially lost by gel swelling, which was a source of error in predicting results in continuous processes. The enzyme loss determined in this work showed a non-linear behaviour and it depended on mixing conditions of the reactor.

Values of protein loss were used in the modelling of a fixed-bed reactor with similar flow conditions to reduce the error in predicting the operation conditions to maintain a constant conversion.

For reaction conditions similar to those analysed in this work, the β-galactosidase was well entrapped in alginate-carrageenan matrices.

Keywords: Biocatalyst deactivation; Enzyme entrapment; Alginate-carrageenan matrices; Protein loss; Packed-bed reactor model

1. Introduction

Enzymes are usually used in an environment quite different from its natural habitat. Most enzymatic reactions are performed in aqueous media, which favours inactivation. Water acts as a reactant in inactivation reactions and also as a lubricant in conformational changes associated with protein unfolding [1]. One of the most used strategies to improve the stability of enzymes is the immobilisation in solid carriers. This technique besides those advantages also is convenient for a better control of the operation, flexibility in reactor design, and a facilitated product recovery without catalyst contamination [2–4].

Entrapment is one of the simplest methods of immobilisation and consists of the inclusion of enzymes or cells within polymeric matrices. Despite of the problems associated with entrapment within polymers, the method has been extensively used for a wide variety of processes [5,6]. The immobilisation by entrapment has been accepted as an appropriate technique to fix the β-galactosidase that catalyses the enzymatic hydrolysis of lactose. Both, the substrate (lactose) and the products (glucose and galactose), are compounds of low molecular size with high diffusion rates on several supporting matrices [7,8].

There is a variety of polymers available for enzyme entrapment [9]. Alginate, a naturally occurring polysaccharide that forms gels by ionotropic gelation, is the most popular of all supports [10–12]. Banerjee et al. [13] used beads of calcium-alginate gels as supporting matrix to immobilise β-galactosidase, but calcium concentrations gradually affected the activity of the enzyme.

Mammarella [14] studied the activity of soluble β-galactosidase in presence of mono and divalent ions and found that some monovalent cations, in particular K^+, had a positive effect on the enzyme activity. Moreover,

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κ-carrageenan, another naturally occurring polysaccharide which forms gel with K^+ ions, increased the enzyme activity. Unfortunately, carrageenan gels are very weak and break easily, reducing the application of the polymer as supporting matrix for enzyme immobilisation [15,16]. However, the mixture of gelled hydrocolloids acts synergistically, the K-κ-carrageenan favours the enzymatic reaction, while, the Ca-alginate provides the necessary mechanical resistance [17].

The β-galactosidase was immobilised in a Ca-alginate–K-κ-carrageenan gel, exhibiting better activity and mechanical stability [17]. However, leaching of the enzyme from the hydrogel network occurs when the enzyme molecular size is smaller than porous diameter. Furthermore, the gel structure obtained with the hydrocolloids can be modified during the enzymatic reaction by the action of the surrounding medium [18], producing swelling after weakening the supporting gel [19,20]. Therefore, the efficiency of the enzyme entrapment can be different due to the modification of the carrier structure [9].

The biocatalyst deactivation depends on different factors and it is commonly studied for reactor designs to maximise the operational conditions in order to ensure the stability of immobilised enzymes [21]. Thus, the immobilisation method can be selected according to the behaviour of the working system. Deactivation models may lead to better approaches.

The enzymatic deactivation has been traditionally considered as a consequence of the protein denaturalisation by the reaction conditions (solvent, pH, temperature, reaction medium ionic force, etc.) or the reaction system with inhibition due to permanent blockade of enzyme active sites by the present inhibitors [21,22]. The inactivation by chemical reagents can often be avoided rather easily by keeping them out of the reaction medium. Temperature, however, produces opposed effects on enzyme activity and stability and therefore, it is a key variable in any biocatalytic process.

The catalytic durability of the biocatalyst during continuous operation is called operational stability and it is commonly estimated numerically by its apparent half-life, which is the elapsed time at which the catalytic activity is reduced to half value. This parameter is very important for the economic feasibility of the bioprocess concerned and it requires a more exhaustive analysis.

The objective of this work was to develop a new approach to determine the operational stability of β-galactosidase entrapped in alginate-carrageenan matrices by estimating the principal factors that reduce half life.

2. Theory

In continuous reactors, the apparent half-life of immobilised enzyme depends on the intraparticle diffusional limitation, the reactor working conditions (constant conversion or constant feedrate), the initial conversion, and the bioreactor system design (plug-flow reactor or continuous-flow stirred-tank reactor). Therefore, a mathematical model that takes into account the biocatalyst inactivation during operation will improve the methods to optimise or to control the process.

Most of the immobilised enzymes are used in buffer medium without inhibitors modifying the enzyme by irreversible reactions. Therefore, only the thermal deactivation is the principal factor in the reduction of the activity. In this case, a simple one-stage first-order kinetics has been used for considering the transition of a fully active native enzyme (E) to a fully inactivated species (E_d) in a single step, due to the thermal deactivation of enzymes [23]:

$$E \xrightarrow{k_d} E_d \tag{1}$$

where k_d is the deactivation rate constant. k_d is temperature dependent and follows the Arrhenius equation.

This mechanism leads to an exponential model, where the residual enzyme activity "a" at time t can be determined from the following equation:

$$a = \frac{E|_t}{E|_{t=0}} = e^{-k_d t} \tag{2}$$

where $E|_{t=0}$ and $E|_t$ are the quantities of active enzyme at initial time and at any other time, respectively, in mg of protein. A usual defined value is biocatalyst half-life. This value is the time at which activity is reduced to the half of the initial value.

However, biocatalyst half-life in a packed-bed reactor is different from the value for a continuous-flow stirred-tank reactor, operating at the same conditions. The biocatalyst enzyme loss can explain this difference. The experimental enzyme loss can be easily obtained in a batch reactor. Then, the results can be extended to a continuous reactor assuming multiple batch conditions.

Using a multiple batch system of ∞-stages, an equation for a continuous reactor can be obtained. The mass transfer effects on the immobilized enzyme were considered through the mass balance for each reactor. The final quantity of active enzyme in the ith-stage of a batch system, $E_i|_{t_i}$, is the initial quantity of active enzyme, $E_i|_{t_{i-1}}$, affected by the actual thermal deactivation and it can be expressed by:

$$E_i|_{t_i} = E_i|_{t_{i-1}} e^{-k_d t_i}, \quad i = 1, 2, \ldots, n \tag{3}$$

where t is the time period of the ith-stage.

The $E_i|_{t_{i-1}}$ can be calculated by the mass balance in the $(i-1)$ stage:

$$E_i|_{t_{i-1}} = \left(1 - \frac{E_{i-1}^{liq}|_{t_{i-1}}}{E_{i-1}|_{t_{i-1}}}\right) E_{i-1}|_{t_{i-1}} = \eta_{i-1} E_{i-1}|_{t_{i-1}} \tag{4}$$

where η is the effectiveness factor of the enzyme retention in the biocatalyst and E^{liq} is the quantity of enzyme remained in the liquid.

Replacing $E_i|_{t_{i-1}}$ of Eq. (3) with Eq. (4), a general expression for enzyme thermal deactivation and enzyme loss, can

be obtained [24]:

$$E_i|_{t_{i-1}} = E_1|_{t=0} \prod_{i=1}^{i-1} \left(1 - \frac{E_{i-1}^{liq}|_{t_{i-1}}}{E_{i-1}|_{t_{i-1}}}\right) e^{-k_d \sum_{i=1}^{i-1} t_i} e^{-k_d t_i}$$

$$= E_1|_{t=0} \prod_{i=1}^{i} \eta_{i-1} e^{-k_d \sum_{i=1}^{i} t_i} \quad (5)$$

In this case, the residual enzyme activity a'_i at any time can be determined from the following equation:

$$a'_i = \frac{E_i|_{t_i}}{E_1|_{t=0}} = \prod_{i=1}^{i} \eta_{i-1} e^{-k_d \sum_{i=1}^{i} t_i} \quad (6)$$

When the thermal deactivation of the immobilised enzyme and biocatalyst enzyme loss rates are slow, compared to the enzymatic reaction rate, the total reaction coupled with enzyme deactivation and enzyme loss can be expressed in terms of residual activity:

$$v'_i = v\, a'_i \quad (7)$$

where v'_i is the reaction rate with thermal deactivation and enzyme loss in the *i*th-stage and v is the initial reaction rate.

For the lactose hydrolysis to glucose and galactose by β-galactosidase, with reaction kinetics defined as Michaelis–Menten type with competitive inhibition by product, the residual reaction rates for an *n*-stages system can be finally expressed by:

$$v'_{n+1} = \frac{V'_{max}[S]}{K'_m(1 + ([Ga]/k'_p)) + [S]} \prod_{i=1}^{n} \eta_i e^{-k_d \sum_{i=1}^{n} t_i} \quad (8)$$

where V'_{max} is the intrinsic maximum reaction rate; K'_m, the intrinsic Michaelis–Menten constant; k'_p, the intrinsic inhibition constant by product; [S], the substrate concentration (lactose) in the biocatalyst; and [Ga], the product concentration (galactose), which can be assumed equal to $[S] - [S]_0$. The $E_{i-1}^{liq}|_{t_{i-1}}/E_{i-1}|_{t_{i-1}}$ ratio is assumed to be equal to the protein extraction ratio.

This reaction kinetic rate can be used to obtain the k_d value to predict substrate concentrations in a packed-bed reactor or introduced in the mass balance of a stirred-tank reactor.

3. Experimental

3.1. Microbial lactase

The β-galactosidase enzyme from *Kluyveromyces fragilis* (Lactozym 3000 L, Novo Nordisk A/S, Denmark) with an initial activity of 3000 LAU/ml was used in this study. One microbial lactase unit (LAU) is defined as the quantity of enzyme that will liberate 1 μmol of glucose per min at 37 °C and pH 6.5, using lactose 4.7% (w/w) as substrate. During the whole study, enzyme extract was stored in a refrigerator at 4 °C, and activity did not change.

3.2. Hydrocolloids

Commercial hydrocolloids were used for enzyme entrapment. Sodium alginates, Keltone low viscosity (LV) and Keltone high viscosity HV, were from Kelko (Chicago, USA) and Gelacid C-3 κ-carrageenan was from Biotec S.A. (Buenos Aires, Argentina).

3.3. Lactose solution

Lactose (reagent grade, Mallinckrodt, St. Louis, USA) was dissolved in phosphate buffer of pH 6.85 preparing lactose solutions 2.5–10.0% (w/v), which were used as substrate. The solution also contained potassium sorbate 0.1% (w/v) to prevent microbial growth in the reactor for long-term operation study.

3.4. Enzyme immobilisation

A solution contained 1.0% (w/v) low viscosity sodium alginate, 1.4% (w/v) high viscosity sodium alginate, 0.3% (w/v) κ-carrageenan and 12.0% (v/v) β-galactosidase was prepared into a stirred glass container at room temperature. The solution composition was previously determined to ensure the highest enzyme load and K^+ incorporation with the lowest viscosity of the solution [17].

The biocatalyst beads obtained by forcing the mixture dropwise through a needle at a rate of 30 drops/min [14,17], into a solution of calcium chloride 2.0% (w/v) (reagent grade, Mallinckrodt, St. Louis, USA) and potassium chloride 4.0% (w/v) (reagent grade, Mallinckrodt, St. Louis, USA). The droplet forms gel spheres of approximately 2.4 ± 0.2 mm diameter, entrapping the enzyme in a three-dimensional lattice of ionically cross-linked alginate and carrageenan. Final treatment and storage was carried out as explained in a previous work [14,17].

3.5. Determination of protein

Protein was quantified using a Proti-2 kit for total protein determination (Wiener Lab, Rosario, Santa Fe, Argentina) followed by absorbance measurement at 495 nm in a spectrophotometer (Spectronic Genesys 5, Milton Roy, Rochester, NY, USA) [25]. Hydrolysed casein (protein 13.6% (w/w), previously determined by Kjeldhal method) was used as standard. Measurements were performed in duplicate.

3.6. Estimation of entrapped protein in biocatalyst beads

The quantity of entrapped enzyme per gram of support was calculated by subtracting the protein quantity remained

in the calcium chloride 2.0% (w/v) and potassium chloride 4.0% (w/v) solution after the immobilisation step from the initial protein quantity presented in the hydrocolloid solution before gelling.

3.7. Determination of sugar

Glucose concentration was quantified using a kit for enzymatic glucose determination (Wiener Lab, Rosario, Santa Fe, Argentina) followed by absorbance measurement at 505 nm in a spectrophotometer (Spectronic Genesys 5, Milton Roy, Rochester, NY, USA), [26]. Measurements were performed in duplicate.

Concentrations of galactose and lactose were obtained from glucose concentration by assuming that 1 mol of glucose and 1 mol of galactose were formed from 1 mol of hydrolysed lactose.

3.8. Reactors

A glass vessel (4.50 cm × 3.86 cm; length × diameter), with agitation and with a jacket for water recirculation at 37 °C, was used as stirred batch reactor to study the biocatalyst activity behaviour during operation conditions.

A glass column (34.0 cm × 1.47 cm; length × diameter), with a jacket for water recirculation at 37 °C, was used with 39.94 g of biocatalyst (36.06 cm^3 of initial bead volume) as the isothermal packed-bed reactor. Packed-bed reactors are often used for reaction system with product inhibition. In this case, the efficiency is larger since the inhibition effect decreases due to the low difference between substrate and product concentrations in the whole reactor. Reactors were maintained at 37 °C, the optimum temperature of the immobilised enzyme.

For the isothermal packed-bed reactor, the differential mass balance for the substrate can be expressed by:

$$D_z \frac{\partial^2[S]^b}{\partial z^2} + D_r\left(\frac{\partial^2[S]^b}{\partial r^2} + \frac{1}{r}\frac{\partial[S]^b}{\partial r}\right) - u_z\frac{\partial[S]^b}{\partial z} = -\frac{V'_{max}[S]e^{-k_d t}}{K'_m(1+([Ga]/k'_p)) + [S]} \quad (9)$$

where D_z and D_r are axial and radial dispersion coefficients, respectively; z and r are axial and radial coordinates, respectively; $[S]^b$ is the lactose concentration in the liquid stream; and u_z is the superficial fluid velocity in the reactor. Usually, the influence of axial dispersion is more important than the radial one; and for packed-bed reactors with large length/diameter ratios, radial dispersion can be rejected. In this case, Eq. (9) can be reduced to:

$$D_z\frac{\partial^2[S]^b}{\partial z^2} - u_z\frac{\partial[S]^b}{\partial z} = -\frac{V'_{max}[S]e^{-k_d t}}{[K'_m(1+([Ga]/k'_p)) + [S]]} \quad (10)$$

with boundary conditions:

$$z = 0^+, \quad ([S]^b - [S]^b) = \frac{D_z}{u_z}\frac{d[S]^b}{dz} \quad (11)$$

$$z = Z, \quad \frac{d[S]^b}{dz} = 0 \quad (12)$$

where $[S]^b_0$ substrate concentration at initial time in the liquid stream.

To solve the equation system (10)–(12), the substrate concentration inside catalyst spherical particle, [S], should be known; therefore, a mass balance considering diffusion and chemical reaction, in steady state, was considered:

$$\frac{1}{r^2}\frac{d}{dr}\left(r^2 D_S\frac{d[S]}{dr}\right) = R_S \quad (13)$$

where D_S is the diffusion coefficient of the substrate (lactose), and the boundary conditions are:

$$r = 0, \quad \frac{d[S]}{dr} = 0 \quad (14)$$

$$r = R, \quad \frac{d[S]}{dr} = \frac{k_l}{D_S}([S]^b - [S]) \quad (15)$$

where k_l is the external mass transfer coefficient and R is the bed radius (cm).

The mathematical model proposed for the substrate concentration profile in one direction (z) takes into account mass transport equations according to the operating conditions such as: the hydrodynamic conditions, axial dispersion flow; the external and internal mass transfer resistance and the kinetic reaction rate. Thus, the reactor model involves a second-order partial differential equation, which can be solved by the method of orthogonal collocation with n internal points [14,27].

3.9. Biocatalyst activity

Biocatalyst samples of 1.00 ± 0.01 g were tested in a stirred batch reactor using aliquots of 10.0 ± 0.1 ml of the lactose solutions at 37 °C for periods of 30 min. The activity assays were carried out until the biocatalyst activity was decreased to the 50% of the initial activity. The biocatalyst activity was determined by measuring the glucose concentration at different times. The activity determined immediately after the immobilisation process was considered as the initial activity of the biocatalyst.

During the whole study, the prepared biocatalyst was stored at 4 °C in a solution of potassium sorbate 0.1% (w/v) and it lost very low activity.

3.10. Estimation of loss protein due to swelling effect

The quantity of loss enzyme per gram of support was estimated by adding both, the determined protein quantity in the solution of each reaction batch and the one determined in

each storage solution. Gel swelling effect was determined in a previous work [14], using an empirical correlation:

$$\Delta R = 2.323 \times 10^{-4} t \frac{(1-\varphi)^3}{(1+\varphi)^2} \quad (16)$$

where

$$\varphi = \frac{8.483 \times 10^{-2}}{1 + Re_p} \quad (17)$$

where Re_p is the Reynolds number based on the particle.

3.11. Data validation

For k_d validation obtained in batch assays, experimental values of the lactose conbiocatalyst in the continuous fixed-bed reactor were compared with values obtained by Eq. (10), for different reaction conditions. Those theoretical values were predicted using a program written in Matlab® language for the same reaction experimental conditions using a fixed-bed reactor model with axial dispersion, operating in steady state with a kinetics reaction of Michaelis–Menten with product inhibition for the immobilised enzyme. The program considered gel swelling and biocatalyst deactivation effects. Kinetic constants were determined in a previous work [14], being $K'_m = 0.075$M; $k'_p = 0.037$M; and $k'_2 = 0.00017\,\text{mol}\,\text{l}^{-1}\text{mg}^{-1}\,\text{min}^{-1}$. The V'_{max} value was obtained by relating k'_2 value to the initial protein retention; thus, V'_{max} value was $0.139\,\text{mol}\,\text{l}^{-1}\,\text{min}^{-1}$.

Moreover, an experimental value of $4.35 \times 10^{-6}\,\text{cm}^2/\text{s}$ was obtained for D_S, while k_l and D_z were estimated using empirical correlations available in literature:

$$k_l = \frac{1.09}{\varepsilon}\left(\frac{D_S}{2R}\right)^{2/3} u_z^{1/3},$$

$$1.6 \times 10^{-5} < Re_p < 55 \text{ and } 165 < Sc < 70600 \quad (18)$$

and

$$D_z = \frac{2u_s D_S}{\varepsilon}, \quad 0.1 < Re_p < 50 \quad (19)$$

where ε is the void fraction of packed bed reactor.

4. Results and discussion

The biocatalysts obtained by entrapping β-galactosidase in alginate-carrageenan matrices shown an initial activity of 213 μmol glucose min^{-1} g^{-1} of beads, and an initial retention of 20.5 mg protein per gram of beads.

The accumulated protein loss from biocatalyst in reaction conditions, including the corresponding washing and storage periods, is shown in Table 1, together with the amount of enzyme in the biocatalyst which is related to the residual relative activity defined by Eq. (2). After 18 assays in a well-agitated system, a protein loss of 10% was determined. In the first 9 assays, only 2.7% was lost. The loss protein rate increased in the last assays. This result shows that, in this case, the β-galactosidase enzyme is well entrapping into alginate-carrageenan matrices.

Table 1
Protein loss from biocatalyst and residual relative activity in reaction conditions

Assay no.	Accumulated protein loss (mg/g of biocatalyst)	Residual relative activity
1	0.00 ± 0.025	1.00 ± 0.01
2	0.00 ± 0.025	0.99 ± 0.01
3	0.05 ± 0.025	0.98 ± 0.01
4	0.10 ± 0.025	0.97 ± 0.01
5	0.15 ± 0.025	0.96 ± 0.01
6	0.25 ± 0.025	0.95 ± 0.01
7	0.35 ± 0.025	0.95 ± 0.01
8	0.45 ± 0.025	0.94 ± 0.01
9	0.55 ± 0.025	0.93 ± 0.01
10	0.70 ± 0.025	0.92 ± 0.01
11	0.85 ± 0.025	0.91 ± 0.01
12	1.00 ± 0.025	0.90 ± 0.01
13	1.15 ± 0.025	0.89 ± 0.01
14	1.30 ± 0.025	0.88 ± 0.01
15	1.50 ± 0.025	0.87 ± 0.01
16	1.70 ± 0.025	0.86 ± 0.01
17	1.85 ± 0.025	0.85 ± 0.01
18	2.05 ± 0.025	0.82 ± 0.01

The comparison between quantities of initial and residual active enzyme after each stage showed the influence of enzyme loss and thermal deactivation (Fig. 1). As it can be observed, the protein loss is not the main factor in the biocatalyst deactivation.

A linear regression of the $\ln[E_i|_{t_i}/(E_1|_{t_0}\prod_{i=1}^{i}\eta_{i-1})]$and $\ln[E_i|_{t_i}/E_1|_{t_0}]$ experimental values against operation time were used to obtain k_d values (Fig. 2). In this case, k_d values were 0.0180 and $0.0220\,\text{h}^{-1}$, with a good adjustment ($r^2 = 0.9939$ and 0.9803, respectively). This result indicates that time-temperature effect was the most important factor of reaction conditions in the biocatalyst deactivation but it

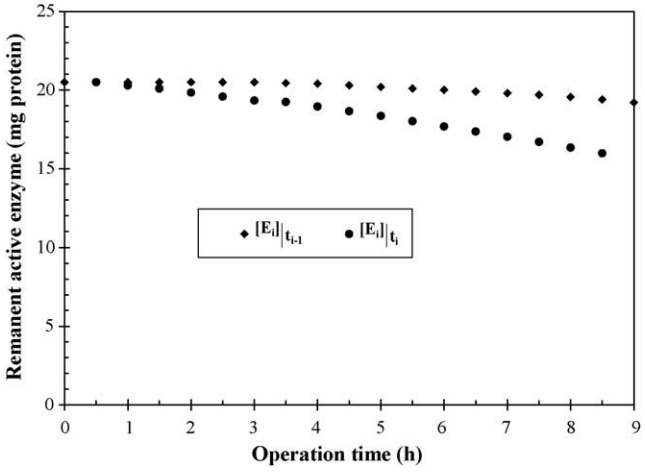

Fig. 1. Available and residual activated enzyme quantity after each experimental stage.

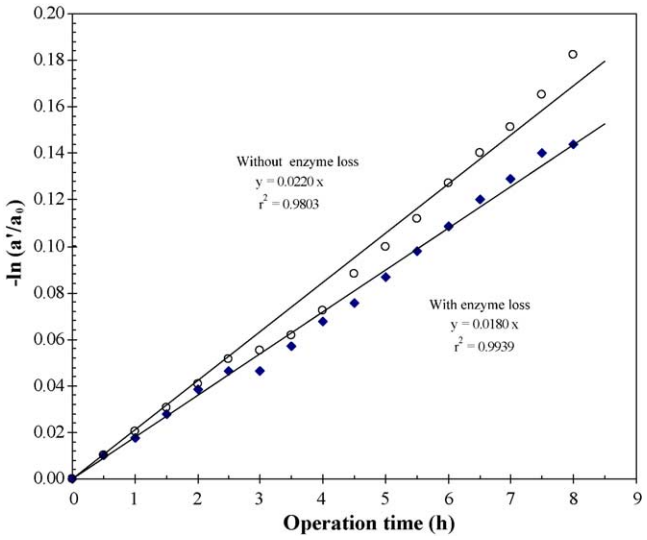

Fig. 2. Linear regression of experimental values to obtain k_d values.

could be better determined after considering the enzyme loss of biocatalyst.

The biocatalyst behaviour in the fixed-bed reactor operating in steady-state, was study for different operational conditions. Theoretical and experimental conversion values obtained for the same reaction conditions are shown in Fig. 3 using the deactivation constant with and without the effect of protein loss. As it is observed, for the analysed flow-rate range, the theoretical model using a deactivation constant with the effect of protein loss, predicted the experimental behaviour with smaller error, which was less than 2.5%.

Likewise, due to that the gel swelling is non-linear and depends on the agitation and the operation time of the reactor, the biocatalyst protein loss is dependent on flow condition.

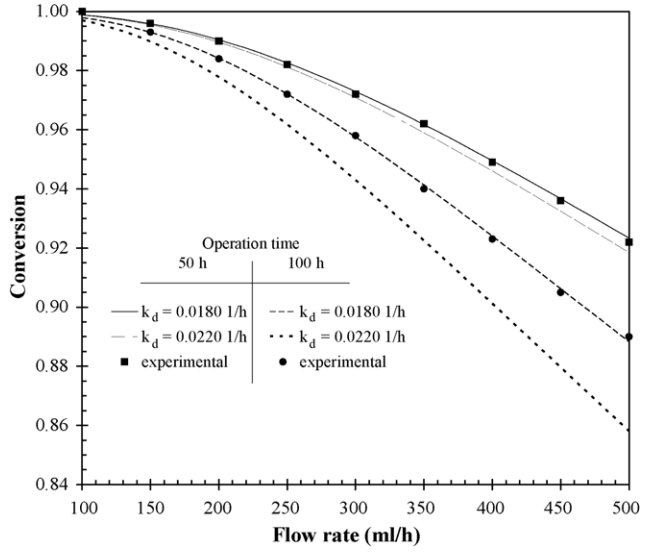

Fig. 3. Calculated conversion values and experimental data obtained by feeding the fixed-bed reactor with 5.0% (w/v) lactose solution.

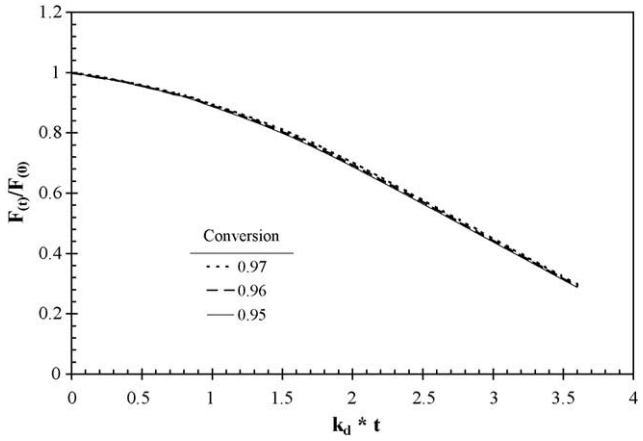

Fig. 4. Reactor feed flow variation for maintaining a constant conversion value for a 5.0% (w/v) lactose solution.

Nevertheless, in the operation time for a half-life of biocatalyst in a packed-bed reactor, this factor is very small.

Thus, the reactor operation conditions for maintaining constant conversion, predicted using numerical solutions for the reactor model with the deactivation constant considering the effect of protein loss, is observed in Fig. 4 for a substrate concentration equal to 5.0% (w/v). For a conversion of 97%, the initial flow rate ($F_{(0)}$) was approximately 350 ml h^{-1}, while for a conversion of 95%, the initial flow rate was approximately 450 ml h^{-1}. The reactor productivity prediction after 200 h of operation, with a deactivation constant that includes protein loss, reduced the error in 9.9% for a 95% conversion and in 8.1% for a 97% conversion, because the higher flow rate used to obtain the lower conversion value, increases the enzyme loss.

5. Conclusions

The β-galactosidase entrapment in alginate-carrageenan matrices shows good efficiency as immobilisation method for the reaction conditions analysed.

Time-temperature effect was the most important factor of reaction conditions in the biocatalyst deactivation. Thermal deactivation constant was obtained after considering the enzyme loss of biocatalyst in a batch reactor and extended to continuous reactors.

Errors to predict lactose conversions in a continuous reactor were considerably reduced using a deactivation constant with the effect of protein loss.

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