

Article

Catalyst Replacement Policy on Multienzymatic Systems: Theoretical Study in the One-Pot Sequential Batch Production of Lactofructose Syrup

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Abstract: One-pot systems are an interesting proposal to carry out multi-enzymatic reactions, though this strategy implies establishing an optimal balance between the activity and operability of the involved enzymes. This is crucial for enzymes with marked differences in their operational stability, such as one-pot production of lactofructose syrup from cheese whey permeate, which involves two enzymes— β -galactosidase (β -gal) and glucose isomerase (GI). The aim of this work was to study the behavior of one-pot sequential batch production of lactofructose syrup considering both enzymes immobilized individually, in order to evaluate and design a strategy of replacement of the catalysts according to their stabilities. To this end, the modelling and simulation of the process was carried out, considering simultaneously the kinetics of both reactions and the kinetics of inactivation of both enzymes. For the latter, it was also considered the modulating effect that sugars present in the medium may have on the stability of β -gal, which is the less stable enzyme. At the simulated reaction conditions of 40 °C, pH 7, and 0.46 (IU_{GI}/IU _{β -gal}), the results showed that considering the stability of β -gal under non-reactive conditions, meaning in absence of the effect of modulation, it is necessary to carry out four replacements of β -gal for each cycle of use of GI. On the other hand, when considering the modulation caused by the sugars on the β -gal stability, the productivity increases up to 23% in the case of the highest modulation factor studied ($\eta = 0.8$). This work shows the feasibility of conducting a one-pot operation with immobilized enzymes of quite different operational stability, and that a proper strategy of biocatalyst replacement increases the productivity of the process.

Keywords: multi-enzymatic reactions; one-pot; catalyst replacement policy; modulation factor; sequential batch; lactofructose syrup; β -galactosidase; glucose isomerase



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1. Introduction

The increasing use of enzymes as industrial catalysts [1–3], has evolved to the development of multienzyme processes [4–6], where two or more enzymes act coordinately to perform the conversion reaction [7]. An appealing proposal is to conduct the multi-step enzymatic reaction in a single vessel (one-pot) [8–11]. This strategy implies that the enzymes involved are able to act together without compromising their activity and operational stability, therefore reducing the intermediate product(s) inhibition, and avoiding unnecessary separation and purification steps [12–15]. In this way, fewer unit operations are required, solvent consumption reactor volume and operation time are reduced, yield is increased, and waste is considerably reduced [5]. However, the temperature and pH profiles of the enzymes can differ significantly, as well as their operational stability [5,14,16]. Therefore, compromised operation conditions should be established to optimally balance their activity and operational stability [14,17], which is a key determinant of reactor performance [18,19].

In that direction, mathematical modeling is a powerful tool for predicting the process output before its implementation, allowing to explore different reactor configurations and modes of operation to early on select the most promising options that will later be validated experimentally [7]. Mathematical modelling has been used in the case of enzyme reactors to identify optimal operation conditions, maximize substrate conversion into product, increase productivity, and maintain substrate conversion (and therefore product quality) during reactor operation within pre-established margins of variation [7,19].

Immobilization of enzymes is another powerful tool for its industrial implementation, usually producing a significant increase in enzyme stability and facilitating the reactor operation [4,20–22]. In the case of one-pot multienzyme systems, if each enzyme is immobilized separately, the resulting biocatalyst can be handled independently in a spinning basket reactor (Figure 1A) [5]. This configuration allows an easy control of the proportion of each biocatalyst required, their activity and operational stability; it also allows individual replacement of the spent immobilized enzyme according to its lifespan of use [14]. Since enzyme inactivation and its replacement is inevitable, modeling and simulation is a powerful tool for the effective use of the biocatalyst prior to replacement and the planning of reactor operation [23].

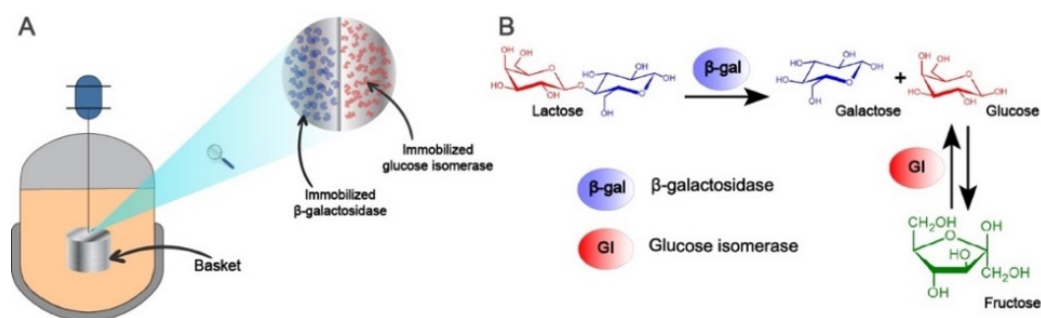


Figure 1. (A) Schematic representation of a spinning basket reactor with separate compartments for each immobilized biocatalyst. (B) Scheme of lactose hydrolysis with β -gal and glucose isomerization with GI.

A sound strategy for the upgrading of lactose is the production of lactofructose syrup (LFS) by the sequential hydrolysis of lactose with β -galactosidase (β -gal) and the isomerization of the resulting glucose by glucose isomerase (GI) (Figure 1B). The kinetics of both enzymes have been well characterized, having significant differences in thermal stabilization [24–33]. The resulting product is a mixture of sugars (fructose, glucose, galactose, and residual lactose) with a sweetening power similar to sucrose [8,19,34]. The production of this syrup has been conducted in a sequential operation in two separate reactors for hydrolysis and isomerization; however, its production in a one-pot strategy with co-immobilized β -gal and GI has been recently reported [8]. It is well documented that substrates and products of reactions, as well as some sugars, exert a modulation effect on the stability of both enzymes [35–39]. In the case of β -gal, galactose exerts a positive modulation effect (protection) while the lactose modulation effect is negative and glucose has no effect [40,41]; in the case of GI, both glucose and fructose exert a positive modulation effect [32,42].

Using modelling and simulation tools, the purpose of this work was to study the behavior of the one-pot sequential batch production of lactofructose syrup considering both enzymes immobilized individually, in order to evaluate and design a strategy of catalyst replacement according to their stabilities. β -gal from *Aspergillus oryzae* immobilized onto poly(methacrylate) beads and commercial immobilized GI from *Streptomyces murinus* (Sweetzyme) were selected for the purpose of this work, where different scenarios were considered in terms of the modulation effects exerted by lactose and galactose on β -gal, which is the less stable enzyme.

2. Results and Discussions

2.1. Selection of Operating Conditions

The performance of a one-pot sequential batch reactor for LFS production was simulated considering the thermal dependence of the kinetic and stability parameters of β -gal and GI at different GI/ β -gal activity ratio and temperatures (see materials and methods). The kinetics of LFS production per batch at three temperatures and three GI/ β -gal activity ratios are shown in the Figure 2. As expected, fructose production increases with a higher GI/ β -gal activity ratio approaching more rapidly to its equilibrium concentration. Similarly, increasing temperature favors fructose formation, given the thermophilic nature of GI. On the other hand, it can be observed that the rate of lactose hydrolysis decreases at the higher temperature as a result of β -gal inactivation.

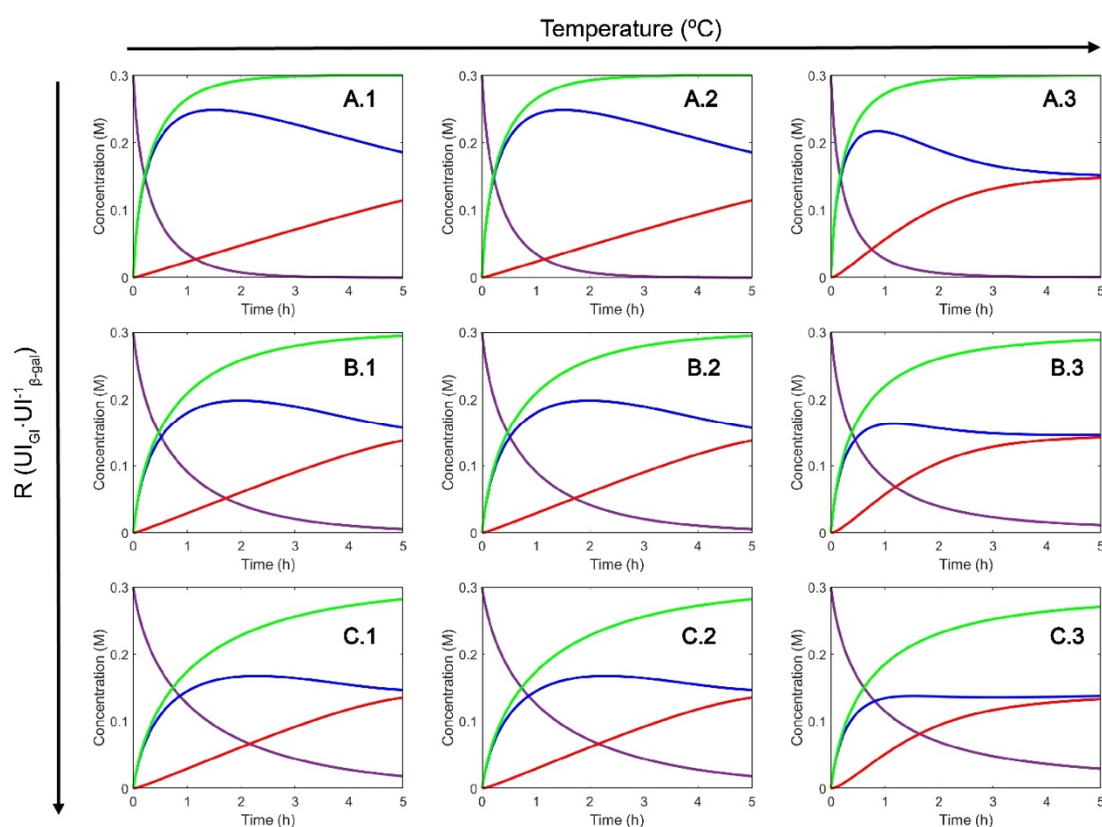


Figure 2. Substrates and products concentrations profiles during the one-pot production of lactofructose syrup in batch at different temperatures and GI/ β -gal activity ratio. (Temperatures: (1): 40 °C, (2): 45 °C and (3): 50 °C. GI/ β -gal activity ratio: (A.1–A.3): 0.1; (B.1–B.3): 0.3 and (C.1–C.3): 0.5. Purple: lactose; Green: galactose; Blue: glucose; Red: fructose).

These results reflect the importance of both enzyme ratio and temperature on reactor behavior, and the need to identify conditions that maximize the production of fructose syrup. To identify this condition, an evaluation of the effect of temperature and enzyme ratio on the productivity was carried out. The effect of GI/ β -gal activity ratio on the productivity of a batch of fructose syrup production for three different temperatures can be found in the Supplementary Materials. An optimum GI/ β -gal activity ratio for each temperature resulting in 0.46, 0.25, and 0.18 (IU_{GI}/IU_{β-gal}) corresponding to 40, 45, and 50 °C, respectively (Figure S4).

In order to select the temperature for the simulations, an analysis was carried out based on the specific productivity during one batch at different temperatures. Figure 3 shows the variation of the specific productivity related to β -gal in one batch conducted at the optimum GI/ β -gal activity ratio for each temperature. As it can be observed, specific productivities at 45 and 50 °C are significantly lower than at 40 °C, because the mass of β -gal

used is different at each temperature, being lower at 40 than at 50 °C. This shows the strong influence of the inactivation of β -gal on the productivity due to the thermal inactivation.

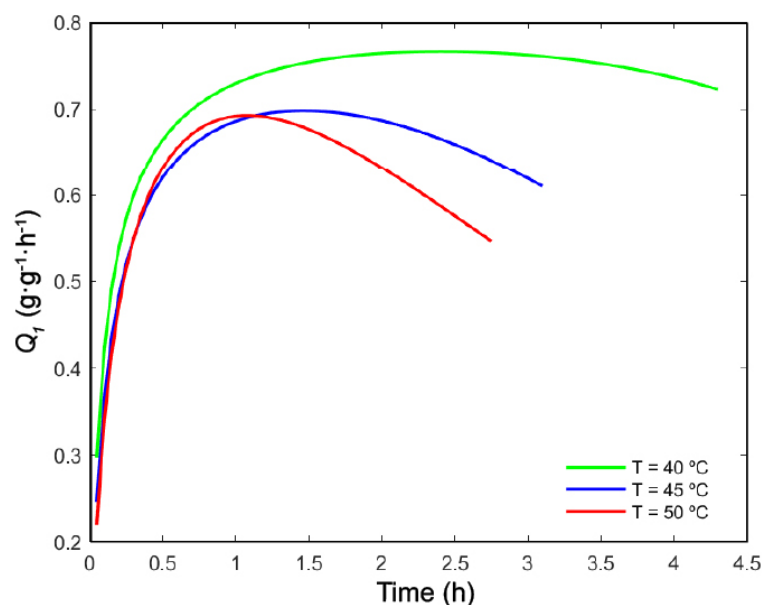


Figure 3. Specific productivity referred to β -galactosidase during one batch of lactofructose syrup production at different reaction temperatures and at the corresponding optimal GI/ β -gal activity ratio.

For the following simulations based on specific productivities, the temperature of 40 °C and an IG/ β -gal activity ratio of 0.46 (IUGI/IU β -gal) were selected, which are conditions that favor β -gal, the least stable enzyme in this case.

2.2. Effect of Modulation of β -gal Stability on the Production of Lactofructose Syrup in One-Pot Sequential Batch Operation

It has been shown that substrates and products of reaction (and other compounds like some polyols and sugars) may exert a modulation effect, usually protection, on enzyme stability [38,43]. In this case, it has been reported that galactose exerts a positive modulation (protection), but lactose modulation is negative [41]; therefore, stability under non-reactive conditions does not adequately predict the stability during reaction so that in many instances thermal inactivation of enzymes is overestimated [8].

Given the above, the eventual modulation effect that the sugars present in the reaction medium may have on the stability of β -gal was included, β -gal being the less stable enzyme. Under non-reactive conditions, the first order inactivation rate constant of *A. oryzae* β -gal was determined, obtaining a value of 0.0098 h⁻¹ at 40 °C (see Supplementary Materials), which is higher than the inactivation rate obtained under reactive conditions [8]; thus reflecting the protective effect of the sugars. In addition, previous works have shown that the use of modulation factors is a good approximation to simulate the stability of enzymes under reactive conditions [38,44]. Therefore, it was decided to evaluate the effect of different magnitudes of the global (positive) modulation factor (η).

Simulation of the one-pot production of LFS in sequential batch operation considering the operational stability of both enzymes with different global modulation factors (η) was carried out. The simulation considers one cycle of use of GI, since it is the most stable enzyme, and the results are shown in Figure 4.

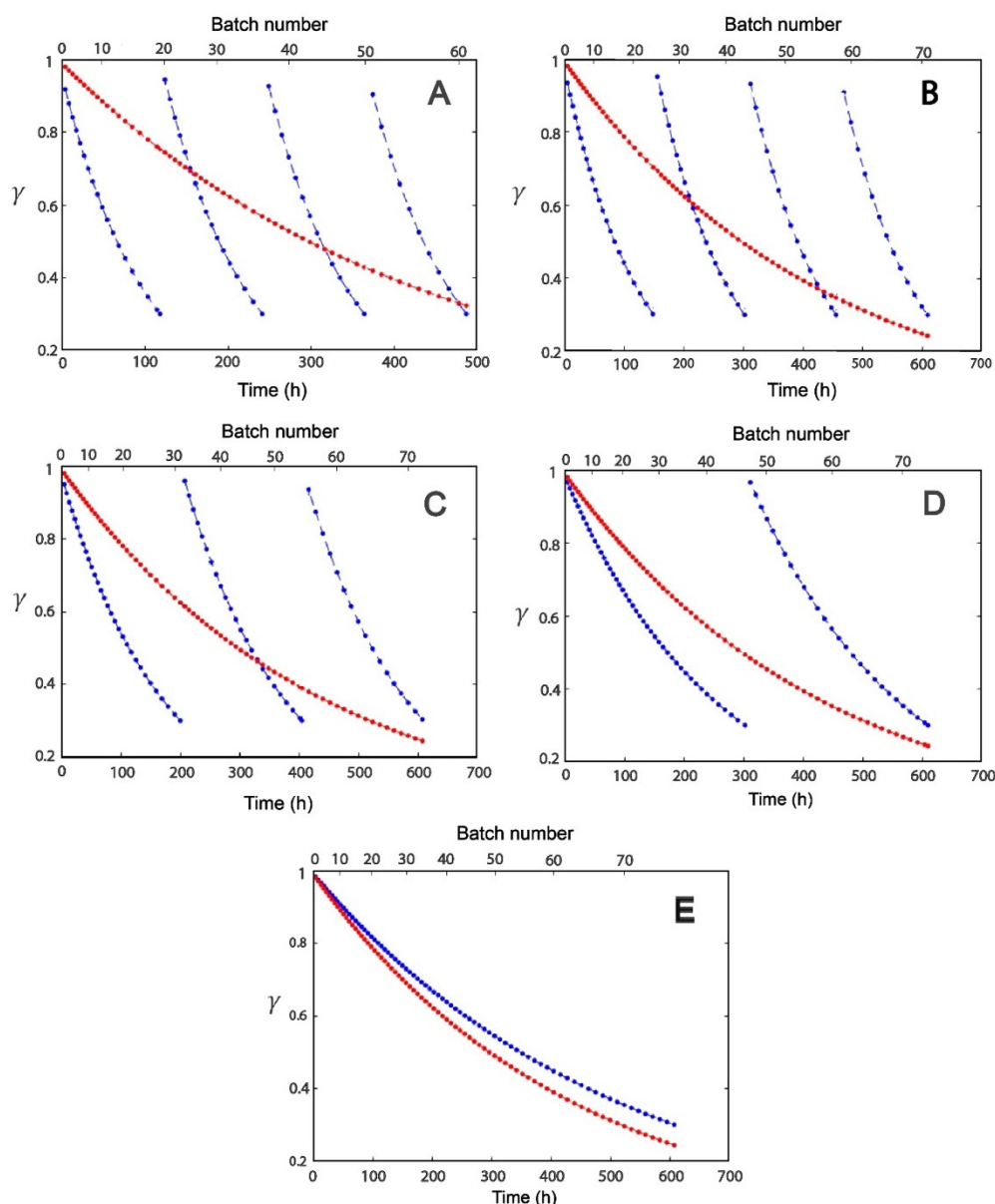


Figure 4. Operational stability of β -gal and GI in terms of reaction time and number of sequential batches during the one-pot production of lactofructose syrup in sequential batch operation. Simulations were performed considering 40 °C, pH 7, 10% (*w/w*) of initial lactose concentration and GI/ β -gal activity ratio of 0.46 ($IU_{GI}/IU_{\beta\text{-gal}}$). (A) $\eta = 0$; (B) $\eta = 0.2$; (C) $\eta = 0.4$; (D) $\eta = 0.6$; (E) $\eta = 0.8$. Operational stability of β -gal: blue lines; stability of GI: red lines.

As expected, the simulation in Figure 4 shows that the number of cycles of use required by β -gal with respect to one cycle of GI decreases as a higher η is considered. In the absence of modulation ($\eta = 0$; Figure 4A), it is observed that during one cycle of use of GI, the β -gal enzyme has to be replaced four times. Instead, when a $\eta = 0.8$ is considered, one cycle of use of GI will match one cycle of β -gal use, so the replacement of both enzymes would in this case be synchronic.

Working with combi-crosslinked enzyme aggregates (combi-CLEAs) of β -gal and GI for the production of lactofructose syrup from lactose in one-pot batch operation, Araya, et al. [8] reported that the productivity of fructose remained constant for five batches with an accumulated time of 50 h. This result is similar to the one obtained in this work, considering $\eta = 0.4$ (Figure 4D). Indeed, the decrease in productivity from the first to the ninth sequential batch in our simulation was 18.34%, corresponding to an accumulated time during the nine batches of 48.6 (h).

The effect of operational stability and the global modulation factor on the productivity of each batch was also evaluated and its evolution is shown in the Figure 5. Productivity decreases with the number of batches at all the evaluated conditions, as enzymes (especially β -gal) are progressively inactivated, so that the time required for each batch increases. It is important to analyze the productivity after each cycle of β -gal use; in this case, even though the productivity increases after β -gal replenishment, values obtained in the initial batches are no longer reached since GI is already inactivated to a certain extent. This situation worsens as the time of use of GI increases. After one cycle of use of GI, the reduction in productivity was of 63.8 and 71.7%, the former corresponding to an absence of modulation, and the latter to a modulation factor of $\eta = 0.8$. The results show that it is feasible to use two catalysts with different stability in a one-pot process, when both are immobilized independently, which allows to replace the more unstable catalyst at the right time, without losing the residual activity of the second enzyme, thereby enabling a more cost-effective use of the catalysts.

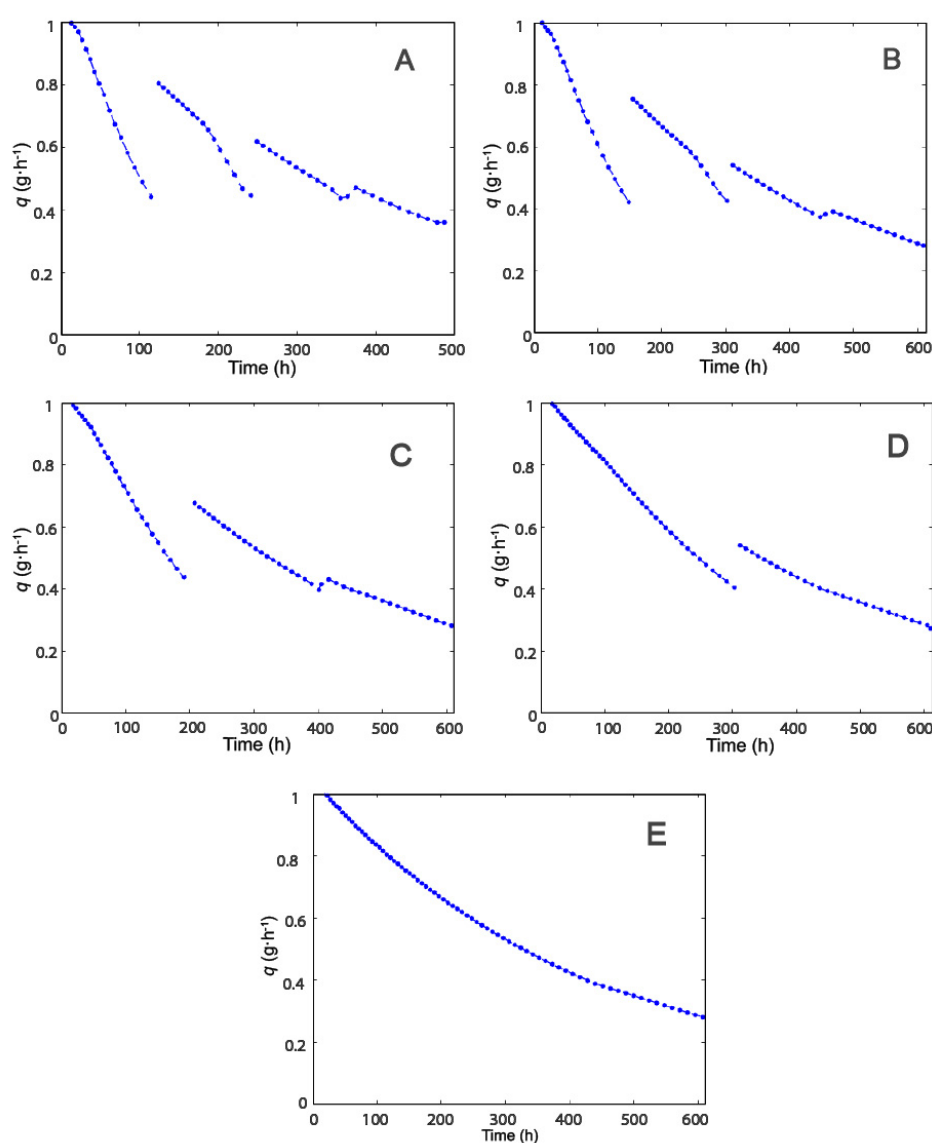


Figure 5. Evolution in productivity in sequential batch operation of lactofructose syrup production in one-pot considering one cycle of use of GI and one or several cycles of use of β -gal. Simulations were performed considering 40 °C, pH 7, 10% (*w/w*) of initial lactose concentration and GI/ β -gal activity ratio of 0.46 ($IU_{GI}/IU_{\beta\text{-gal}}$). (A) $\eta = 0$; (B) $\eta = 0.2$; (C) $\eta = 0.4$; (D) $\eta = 0.6$; (E) $\eta = 0.8$.

Although other alternatives to compensate the enzyme inactivation have been discussed in the literature, such as the addition of fresh enzyme, the increase of the reactor temperature [45,46] and the increase of the residence time in continuous reactor [19], none of them are compatible with the presented case of study. Moreover, the proposed catalyst replacement policy can be applied to other multi-enzyme systems where the enzymes involved might have quite different stabilities [17].

In order to assess the impact of the inactivation and the replacement of β -galactosidase on the production of LFS, the accumulated productivity was determined (see Equation (27)). The results show a decrease in accumulated productivity with the operation time (Figure 6). When inactivated β -gal is replaced by fresh enzyme, a slight increase in the accumulated productivity is observed because of the decrease of the times required in the initial batches. However, since GI is partially inactivated, the reaction times required in the initial batches are no longer sufficient to maintain the initial productivity. This effect is clearly noticeable after the first β -gal replacement and becomes less significant in the following. As a result, the number of batches that can be performed depends on the time of β -gal use (see Table S5). For all η values studied, the number of batches per β -gal use decreases. This is a clear effect of the batch duration time, which is increasing because the GI has not been replaced in the reactor and therefore has less and less activity. Therefore, the productivity values keep declining.

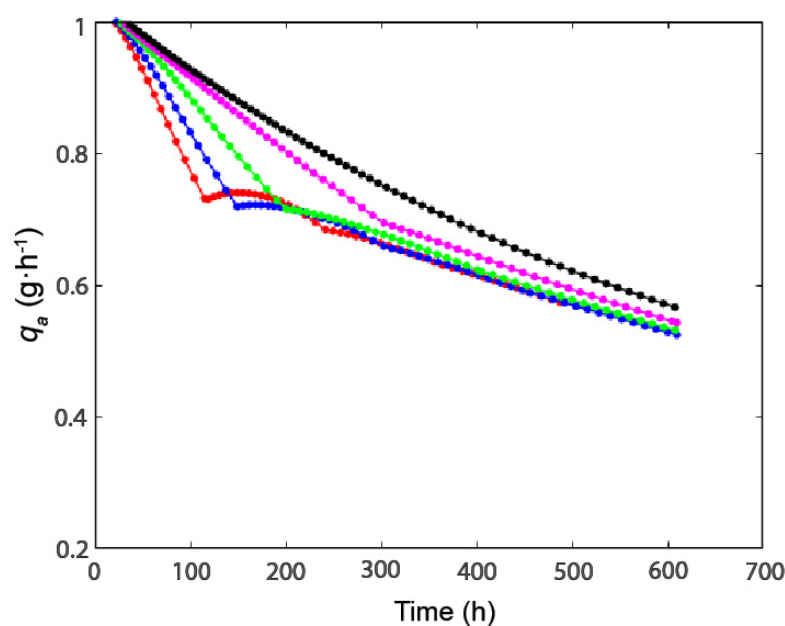


Figure 6. Variation in the accumulated productivity in the one-pot production of lactofructose syrup in sequential batch operation considering one cycle of use of GI and one or more cycles of β -gal use. Simulations were performed considering 40 °C, pH 7, 10% (*w/w*) of initial lactose concentration and GI/ β -gal activity ratio of 0.46 ($IU_{GI}/IU_{\beta\text{-gal}}$). Red line: $\eta = 0$; blue line: $\eta = 0.2$; green line: $\eta = 0.4$; purple line: $\eta = 0.6$; black line: $\eta = 0.8$.

3. Materials and Methods

3.1. Materials

This study was conducted using the commercial immobilized GI from *Streptomyces murinus* under the trade name Sweetzyme[®]IT, kindly donated by Novozymes (Bagsvaerd, Denmark), and the commercial soluble β -gal from *Aspergillus oryzae*, under the trade name Enzeco[®] Fungal Lactase Concentrate, kindly provided by Enzyme Development Corporation (New York, NY, USA). In the case of β -gal, the enzyme was immobilized in a methacrylate heterofunctional support (Relizyme Resindion, Milano, Italy) [47,48] (more details in Supplementary Materials). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.2. Modeling

The operation of a one-pot sequential batch reactor was simulated with β -gal and GI immobilized separately for the production of LFS from lactose. For process modeling purpose, a perfectly stirred and isothermal basket type reactor was used throughout the operation, and internal diffusional restrictions of the catalysts were not considered. For each batch of LFS production, the evolution of substrate and products concentrations was obtained, determining the specific productivity and the residual activity of both immobilized enzymes. As previously reported [8], the composition of LFS on a dry basis was: 6% *w/w* lactose, 47% *w/w* galactose, 26% *w/w* glucose, and 21% *w/w* fructose). Both immobilized enzymes were recovered at the end of each batch and used in the next one without intermediate addition of fresh catalyst. The cycle of use of each immobilized enzyme was defined as the number of batches that could be carried out until its activity dropped to 30% of its initial activity, which was the criterion adopted for its replacement. The specific productivity of the whole cycle of use for each immobilized enzyme was also determined. The scheme of the reactions involved is shown in Figure 1B.

3.2.1. Enzyme Kinetics

Rate equations and kinetic parameters were taken from the literature [28–30,49]. Hydrolysis of lactose by β -gal is competitively inhibited by galactose, so the rate equation corresponds to Equation (1).

$$v_1 = \frac{k_{cat1} \cdot e_1 \cdot Lac}{K_{m1} \cdot \left(1 + \frac{Lac}{K_I}\right) + Lac} \quad (1)$$

Temperature dependence of the kinetic parameters (k_{cat1} , K_{M1} and K_I) are represented by Arrhenius type equations:

$$k_{cat1} = k_{cat01} \cdot \exp(-E_a/RT), \quad (2)$$

$$K_{M1} = K_{M0} \cdot \exp(-\Delta H^\circ/RT), \quad (3)$$

$$K_I = K_{I0} \cdot \exp(-\Delta H^\circ_i/RT). \quad (4)$$

Isomerization of glucose into fructose by GI is represented by reversible kinetics according to the steady-state hypothesis of Briggs-Haldane. At favorable conditions for GI, activity and operational stability, the equilibrium constant of the reaction is close to 1 so the glucose–fructose conversion at equilibrium is close to 50% [18]. The rate equation for glucose isomerization can be expressed in terms of the difference in glucose concentration and glucose concentration at equilibrium, as represented by Equation (5):

$$v_2 = \frac{V_{M2} \cdot (Glu - Glu_e)}{K_{M2} + (Glu - Glu_e)} \quad (5)$$

where:

$$Glu_0 = Glu + Fru = Glu_e + Fru_e = (1 + K_e) \cdot Glu_e = (1 + K_e^{-1}) \cdot Fru_e, \quad (6)$$

$$V_{M2} = \left[1 + \frac{1}{K}\right] \frac{K_{mr} \cdot k_{cat2} \cdot e_2}{K_{mr} - K_{mf}}, \quad (7)$$

$$K_{M2} = \frac{K_{mr} \cdot K_{mf}}{K_{mr} - K_{mf}} \left[1 + \left(1 + \left(\frac{1}{K_{mf}} + \frac{K_e}{K_{mr}}\right) \frac{Glu_0}{1 + K_e}\right)\right]. \quad (8)$$

As in the case of lactose hydrolysis with β -gal, kinetic parameters of the isomerization reaction with GI (k_{cat2} , K_{mf} , K_{mr} , and K_e) are represented by Arrhenius type equations:

$$k_{cat2} = k_{cat02} \exp\left(\frac{E_{aVm f}}{R \cdot T}\right), \quad (9)$$

$$K_{mf} = K_{mf0} \exp\left(\frac{E_{a, Kmf}}{R \cdot T}\right), \quad (10)$$

$$K_{mr} = K_{mr0} \exp\left(\frac{E_{a, Kmr}}{R \cdot T}\right), \quad (11)$$

$$K_e = K_{e0} \exp\left(-\frac{E_{aK}}{R \cdot T}\right). \quad (12)$$

In the case of GI, the affinity constant (K_{M2}) depends on the initial concentration of glucose Glu_0 ; therefore, the energy of activation and the frequency factor in K_M vary with Glu_0 .

Dehkordi, et al. [49] observed that at constant temperature K_{M2} varied linearly with Glu_0 , as expected, and reported a general equation for K_{M2} which is explicit in Glu_0 and T :

$$K_{M2} = 10.37 \cdot 10^{10} \exp^{-\frac{7534}{T}} Glu_0 + 5.48 \cdot 10^{32} \exp^{-\frac{22,818}{T}}. \quad (13)$$

Values of the kinetic parameters of β -gal and GI immobilized used in the simulation of the production of lactofructose syrup are presented in Table 1. In this work, pH 7 was considered for the one-pot production of LFS, which is a compromise between the pH optima of β -gal and GI. In fact, the activity of GI decreases significantly below pH 7, while β -gal is fairly active at pH 7 even though its optimum pH is lower [8].

Table 1. Values of the kinetic parameters of β -gal and immobilized GI used in the simulation of the production of lactofructose syrup.

Enzyme	Parameter	Value	Unit	Reference
β -gal	k_{cat01}	6.74	mol/(h·IU)	[29,30]
	E_a	7.02	kcal/mol	
	K_{M0}	1.01×10^7	M	
	ΔH°	7.21	kcal/mol	
	K_{I0}	1.32×10^2	M	
	ΔH°_I	5.52	kcal/mol	
GI	k_{cat02}	1.46×10^{15}	mol/(h·IU)	[49]
	$E_{aVm f}$	29.5	kcal/mol	
	K_{mf0}	1.35×10^{21}	M	
	$E_{a, Kmf}$	32.9	kcal/mol	
	K_{mr0}	5.27×10^{13}	M	
	$E_{a, Kmr}$	21.2	kcal/mol	
	K_{e0}	4.10×10^4	-	
	E_{aK}	7.03	kcal/mol	

3.2.2. Enzyme Inactivation

Kinetics of enzyme inactivation for each enzyme was performed at pH 7 and temperatures of 40, 50, and 60 °C under non-reactive conditions. The results obtained (see Supplementary Materials) were fitting to a one-step first-order kinetics of inactivation. However, stability under operation conditions may vary depending on the presence of modulators of enzyme stability, such as substrates and products of reactions [40,41,43,50].

The equation for one-step first-order kinetics of inactivation under modulation can be expressed as:

$$-\frac{de}{dt} = k_D \cdot (1 - \eta) \cdot e. \quad (14)$$

Temperature dependence of the inactivation parameters is described by an Arrhenius type equation:

$$k_D = k_{D0} \cdot \exp\left(\frac{E_{aK_D}}{R \cdot T}\right). \quad (15)$$

The experimental data of inactivation of each of the immobilized enzymes under non-reactive conditions were adjusted by linear regression, using the function `fminsearch` of MATLAB[®], to obtain the values of the parameters of the Arrhenius equation, as shown in Table 2, and then used for the calculation of the inactivation constants (k_D) of each immobilized enzyme at different temperatures, using Equation (15).

Table 2. Parameters of the Arrhenius equation for the determination of the inactivation rate (k_D) of β -galactosidase and glucose isomerase.

Parameters	β -gal	GI
E_{aK_D} (Kcal/mol)	73.46	34.83
k_{D0} (h^{-1})	1.64×10^{49}	5.98

3.2.3. Mathematical Model for the One-Pot Production of Lactofructose Syrup in Sequential Batch Operation

The proposed model considers that the liquid phase in the reactor is completely mixed so that lactose concentration is the same at any point inside the reactor. The model was built up from a material balance of substrates and products considering enzyme inactivation throughout reactor operation. Since in this case there are two reactions in series, the product of the first reaction (glucose) becomes the substrate of the second. The following equations describe the system:

$$\frac{dLac}{dt} = -v_1 \cdot \gamma_1, \quad (16)$$

$$\frac{dGal}{dt} = v_1 \cdot \gamma_1, \quad (17)$$

$$\frac{dGlu}{dt} = v_1 \cdot \gamma_1 - v_2 \cdot \gamma_2, \quad (18)$$

$$\frac{dFru}{dt} = v_2 \cdot \gamma_2, \quad (19)$$

$$\frac{d\gamma_1}{dt} = -k_{D1} \cdot (1 - \eta) \cdot \gamma_1, \quad (20)$$

$$\frac{d\gamma_2}{dt} = -k_{D2} \cdot \gamma_2. \quad (21)$$

The operation curve of the one-pot sequential batch reactor (substrate conversion versus time) is obtained by the simultaneous solving of Equations (16)–(21). To do so, the proposed model was implemented in MATLAB[®], version R2019a and the equations were solved using `ode23s` solver.

Simulation of each batch during sequential batch operation was performed until reaching 90% conversion of lactose into glucose and 45% conversion of glucose into fructose. The GI/ β -gal activity ratio is:

$$R = \frac{e_2}{e_1}. \quad (22)$$

The enzyme concentration of each enzyme, can be determined from the mass balance as:

$$e_1 = \frac{M_{cat} \cdot a_1}{V_r \cdot \left(1 + \frac{R \cdot a_1}{a_2}\right)}, \quad (23)$$

$$e_2 = \left(\frac{M_{cat}}{V_r} - \frac{e_1}{a_1}\right) \cdot a_2. \quad (24)$$

3.2.4. Reactor Operation Conditions

The number of batches that can be performed within one cycle of use of an immobilized enzyme will depend on the process operating conditions, since the two enzymes have different kinetic properties and a great difference in operational stability. To enable the selective replacement of each immobilized enzyme, simulation was carried out considering a compartmentalized basket enzyme reactor, where the immobilized enzymes were contained in separate compartments in order to be manipulated independently (Figure 1).

The simulation of reactor operation was carried out at different temperatures, and kinetic and stability parameters were expressed as explicit functions of temperature using the above Arrhenius type equations. Each batch was performed at 0.3 M lactose (10% *w/w*) and pH 7, until reaching 90% lactose conversion and a minimum of 45% glucose isomerization (90% of the equilibrium conversion). Since the operating criterion adopted for sequential batch operation was a constant conversion of both reactions at the end of each batch, and there was no addition of fresh immobilized enzymes, the time for each batch progressively increases as the enzymes were inactivated.

3.3. Metrics Used in Reactor Operations

The following parameters were used for describing reactor operation:

Lactose conversion: the mass fraction of lactose hydrolyzed in the reactor:

$$x_{Lac} = \frac{Lac_0 - Lac}{Lac_0} \quad (25)$$

Productivity: the mass of fructose produced per unit reaction time, calculated at 90% lactose conversion

$$q = \frac{V_r \cdot Fru \cdot MW_F}{t} \quad (26)$$

Accumulated productivity: the total mass of fructose produced in the *m* batches that make up the cycle of use of the immobilized enzymes, divided by the accumulated time of the *m* batches, calculated at 90% lactose conversion:

$$q_a = \frac{V_r \cdot MW_F \sum_1^m Fru_i}{\sum_1^m t_i} \quad (27)$$

Accumulated specific productivity: the total mass of fructose produced in the *m* batches that make up the cycle of use of the immobilized enzyme, divided by the accumulated time of the *m* batches and the mass of immobilized enzyme (β -gal or GI) calculated at 90% lactose conversion:

$$Q_j = \frac{V_r \cdot MW_F \sum_1^m Fru_i}{(\sum_1^m t_i) \cdot M_j} \quad (28)$$

4. Conclusions

This work presents the design of a strategy of catalysts replacement in a multienzyme one-pot system operating in sequential batch operation, with each enzyme immobilized separately according to their respective stabilities. This strategy is useful when the stabilities of the enzymes differ significantly, since each enzyme can be individually replaced once the criterion for replacement has been reached, thus enabling a better use of each enzyme activity. However, this is not feasible when the enzymes are co-immobilized, where the less stable enzyme will determine the criterion for biocatalyst replacement, while the more stable enzyme may still be quite active.

Having a mathematical model to simulate the one-pot reactor operation allows establishing the strategies for the replacement of each immobilized enzyme considering their individual stabilities, therefore establishing a proper operational schedule for increasing productivity and thus boosting the profitability of the process.

This work shows that it is feasible and convenient to carry out the one-pot sequential batch operation with the enzymes immobilized separately when the stabilities of the enzymes are significantly different. We envision that this strategy could be applied to any multienzyme system operating in a single pot for an effective use and replacement of the biocatalyst with the concomitant benefits in the economics of the process.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/catal11101167/s1>: detailed description of the material and method regarding enzymes activity assays and immobilization; Figure S1: Immobilization kinetics of β -gal; Figure S2: Effect of temperature on the stability of β -gal; Tables S1 and S2: Inactivation parameters of β -gal; Figure S3: Effect of temperature on the stability of GI; Tables S3 and S4: Inactivation parameters of GI; Figure S4: Effect of the GI/ β -gal activity ratio (R) on the productivity (q) at different reaction temperatures; Table S5: Summary of the results the simulation of the one-pot production of lactofructose syrup.

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Nomenclature

β -gal:	β -galactosidase
GI:	glucose isomerase
a_1 :	specific activity of β -gal (IU/g)
a_2 :	specific activity of GI (IU/g)
e_1 :	enzyme concentration of β -gal (IU/L)
e_2 :	enzyme concentration of GI (IU/L)
E_a :	activation energy (Kcal/mol)
Fru:	fructose concentration (M)
Fru _e :	equilibrium concentration of fructose (M)
Gal:	galactose concentration (M)
Glu:	glucose concentration (M)
Glu ₀ :	initial concentration of glucose (M)
Glu _e :	equilibrium concentration of glucose (M)
ΔH° :	standard enthalpy change of dissociation of β -gal-lactose into β -gal and lactose (Kcal/mol)
ΔH°_1 :	standard enthalpy change of dissociation of β -gal-galactose into β -gal and galactose (Kcal/mol)
k_{cat1} :	catalytic rate constant of β -gal (mol/(h·IU))
k_{D1} :	thermal decay constant of β -gal (h ⁻¹)
k_{D2} :	thermal decay constant of glucose isomerase (h ⁻¹)
k_D :	thermal decay constant under operational conditions (h ⁻¹)
K_e :	equilibrium constant of GI
K_i :	inhibition constant of β -gal by galactose (M)
K_{m1} :	Michaelis-Menten constant for β -gal (M)
K_{m2} :	apparent Michaelis-Menten constant of GI (M)
K_{mf} :	Michaelis-Menten constant for fructose (mol)
K_{mg} :	Michaelis-Menten constant for glucose (mol)

Lac:	lactose concentration (M)
Lac ₀ :	initial concentration of lactose (M)
q:	productivity (g/h)
q _a :	accumulated productivity of fructose (g/h)
Q _j :	accumulated specific productivity of j catalyst (j =1: β-gal, j =2: GI) (g·g ⁻¹ ·h ⁻¹)
R:	universal gas constant (J/(mol·K))
T:	temperature (K or °C)
t:	time (h)
η:	modulation factor
m ₁ :	batch numbers in a use cycle of β-gal (–)
m ₂ :	batch numbers in a use cycle of GI (–)
M ₁ :	catalyst mass of β-gal (g)
M ₂ :	catalyst mass of GI (g)
v ₁ :	reaction rate of β-gal (mol/(g·h))
v ₂ :	reaction rate of GI (mol/(g·h))
V _r :	reaction volume (mL)
V _{m2} :	maximum apparent reaction rate of glucose isomerization (mol/(g·h))
V _{m_f} :	maximum reaction rate for fructose isomerization into glucose (mol/(g·h))
V _{m_g} :	maximum reaction rate for glucose isomerization into fructose (mol/(g·h))
x _{Lac} :	lactose conversion
γ ₁ :	residual activity of β-gal
γ ₂ :	residual activity of GI
MW _F :	fructose molecular weight (g/mol)

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