Supplementary Information

Catalyst replacement policy on multienzymatic systems: theoretical study in the one-pot sequential batch production of lactofructose syrup.

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1. Materials and Methods

1.1. Determination of immobilized glucose isomerase activity

One international unit of isomerization activity (IU) is defined as the amount of enzyme that isomerizes 1 µmol of fructose per minute at 45 °C, pH 7 and 1 M initial fructose concentration of 1 M. The enzymatic activity was quantified by measuring the initial rate of isomerization at fixed temperature and pH. Fructose isomerization produces the release of glucose in the reaction medium, this product being quantified using the Glucostat GLUCOSE-LQ kit (SPINREACT).

1.2. Determination of β-galactosidase activity.

The enzymatic activity of β-gal was assayed using *o*-NPG as substrate and measuring the *o*-NP release spectrophotometrically at 420 nm using a thermostatized spectrum (Jenway 6715) with constant magnetic stirring. One international unit of β-gal activity ($IU_{\beta gal}$) was defined as the amount of enzyme that hydrolyzes 1 µmole of o-NPG per minute from a 45 mM o-NPG solution in 100 mM citrate-phosphate buffer pH 7 at 25°C. Molar extinction coefficient of o-NP was 2.21 $mM^{-1} \cdot cm^{-1}$.

1.3. Immobilization of β-galactosidase on heterofunctional support

Heterofunctional support was prepared from commercial methacrylate support activated with epoxy groups (Relizyme EP113, Resindion, Milano, Italy), as previously described by Mateo et al 2010. Immobilization of β-galactosidase was carried out as previously reported by Guerrero et al. 2017. First, the enzyme was ionically adsorbed into the support, which was done by contacting 10 mL of enzyme solution per 1 g of support at 25 °C. Second, a multi-point covalent attachment of the already adsorbed protein was carried out by means of the ε-amino groups in their lysine residues and the aldehyde groups of the support, which was done by filtration of the suspension and incubation in 0.05 M bicarbonate buffer pH 10. Then, to obtain stable amide bonds between enzyme and support 1 mg of sodium borohydride per mL of solution was added. After 30 min, the resulting immobilized biocatalyst was washed with abundant distilled water.

2. Supplementary Results

2.1. Immobilization of β-galactosidase in heterofunctional support

The immobilization of the enzyme in the heterofunctional support was carried out and the. immobilization kinetics is shown in **Figure S1**. The immobilization yields in activity and protein were 50% and 100%, respectively, and a specific activity of the catalyst was of 1200 $IU \cdot g^{-1}$.

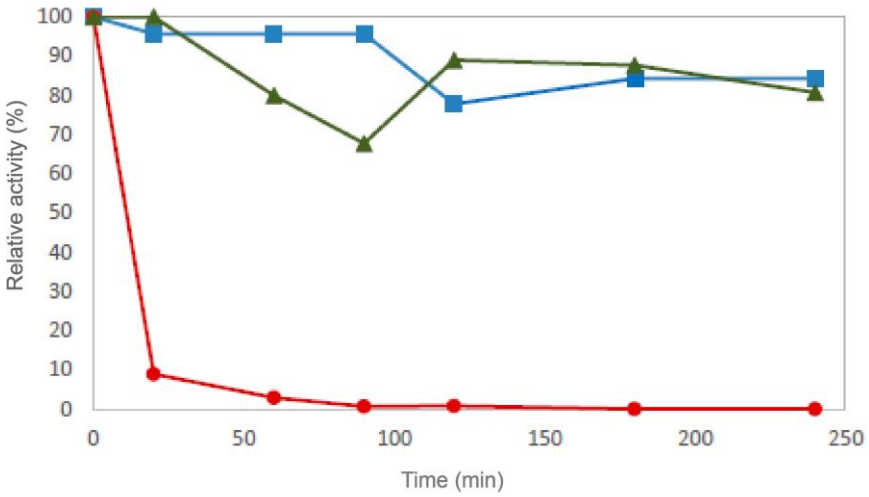

Figure S1. Immobilization kinetics on heterofunctional β-galactosidase methacrylate support at low protein load. (● Supernatant, ▲ Suspension, ■ Blank).

2.2. Stability study of β galactosidase under non-reactive conditions

The results obtained when performing the stability tests under non-reactive conditions for this biocatalyst was modeled according to the inactivation mechanisms proposed by Henley and Sadana (1985). The mechanism that better describes the inactivation kinetics of β-galactosidase (β-gal) at all temperatures is one-step first-order without residual activity (**Figure S2**).

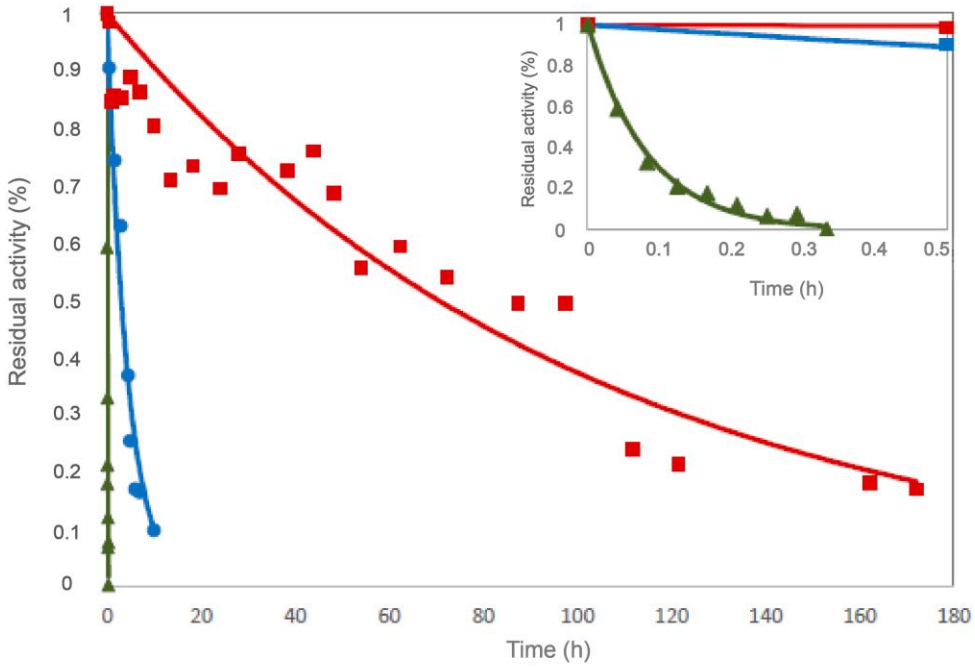

Figure S2: Effect of temperature on the stability of β-galactosidase at pH 7. Experimental data at 40°C: ●, 50°C: ■ and 60°C: ▲. Model: solid line.

Table S1. Inactivation parameters of beta-galactosidase at different temperatures at pH 7. k_1 first order inactivation constant, $t_{1/2}$ halftime.

Temperature (ºC)	k_1 (h^{-1})	$t_{1/2}$ (h)	R^2
40	0.001	70.90	0.955
50	0.228	3.00	0.985
60	11.888	0.06	0.997

The effect of temperature on the inactivation rate constant was modeled according to the Arrhenius equation, whose parameters are presented in **Table S3.**

Table S2. Activation energy, frequency factors and coefficient of determination of Arrhenius equation for the first order inactivation constant of β-galactosidase.

Parameter	Value
E_a (Kcal·mol^{-1})	73.461
K_0 (h^{-1})	1.64E+49
R^2	0.993

2.3. Glucose isomerase stability under non-reactive conditions

The inactivation kinetics obtained when performing the stability tests under non-reactive conditions at pH 6 and 7 are presented i**n Figure S3.** According to the results obtained when modeling the data, for the three temperatures studied, the inactivation mechanism that better fitted the experimental data is one-stage first order.

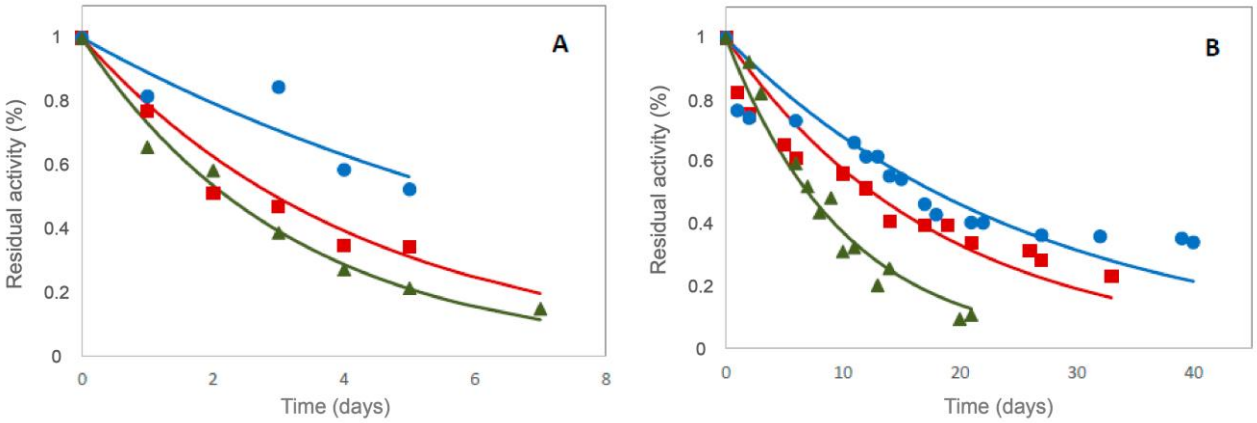

Figure S3: Effect of temperature on the stability of glucose isomerase at pH 6 (A) and pH 7 (B). Experimental data at 40°C: ■, 50°C: ● and 60°C: ▲). Model: solid line.

Table S3. First order inactivation parameters referred to glucose-isomerase at different pH.

pH	Temperature	k_1 (h^{-1})	$t_{1/2}$ (days)	R^2
6	40	0.0097	2.9	0.953
	50	0.0048	6.0	0.906
	60	0.013	2.2	0.992
7	40	0.0023	12.6	0.981
	50	0.0016	18.1	0.952
	60	0.0041	7.0	0.989

The effect of temperature on the inactivation rate constant was modeled according to the Arrhenius equation, whose parameters are presented in **Table S4.**

Table S5. Activation energy, frequency factors and coefficient of determination of Arrhenius equation for the first order inactivation constant of glucose-isomerase at different pH.

pH	K_0 (h^{-1})	E_a (Kcal·mol)	R^2
6	2.8248	0.6919	0.0706
7	23.2499	5.8709	0.3573

2.4. Selection of operating conditions for simulations

In order to select the GI/β-gal activity ratio that maximizes the production of fructose syrup, an analysis was carried out based on the productivity during one batch at different temperatures. Figure S4 shows the variation of the productivity with respect to the GI/β-gal activity ratio at different reaction temperatures. An optimum GI/β-gal activity ratio for each temperature resulting in 0.46, 0.25 and 0.18 (IUGI/IUβ-gal) corresponding to 40, 45 y 50ºC, respectively.

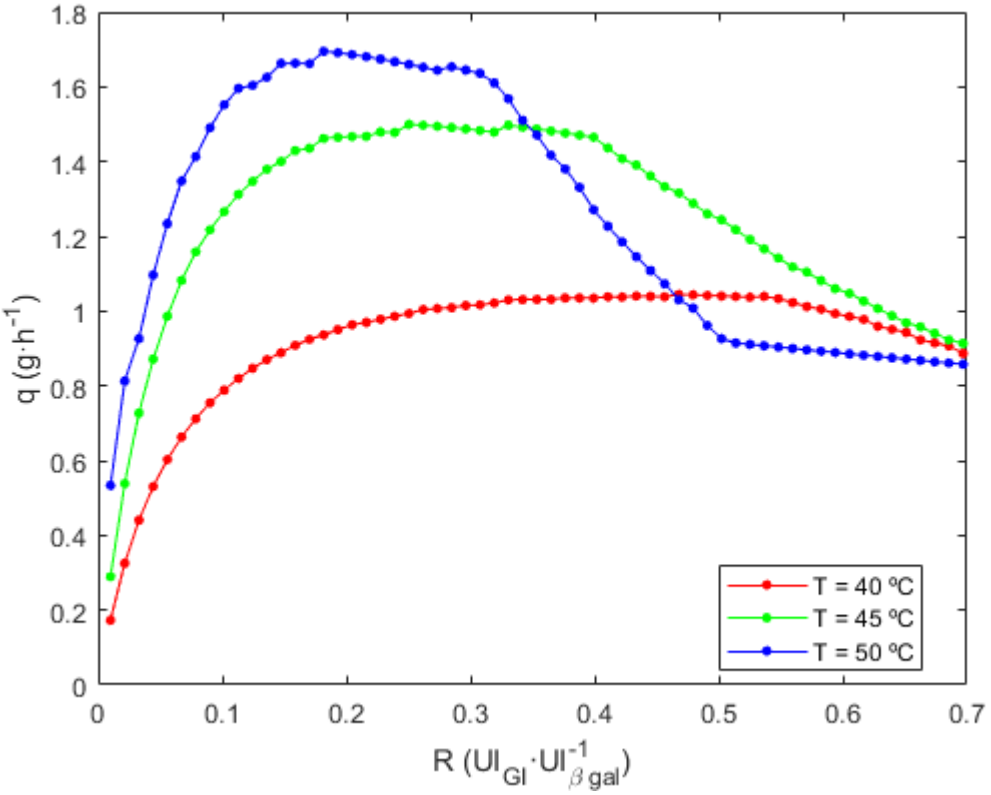

Figure S4: Effect of the GI/β-gal activity ratio on the productivity at different reaction temperatures.

The results obtained in the simulations performed of the 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 are presented in Table S5.

Table S5. Summary of results obtained in the simulation of the one-pot production of lactofructose syrup in sequential batch operation in one cycle of use of GI for different modulation factors (η).

η	Clycles of use β-gal	Numbers of Batches	Cycle Time (h)	Produced fructose (g)	$Q_{\beta\text{-gal}}$ ($g \cdot g^{-1} \cdot h^{-1}$)	Q_{GI} ($g \cdot g^{-1} \cdot h^{-1}$)	q_a ($g \cdot h^{-1}$)
0	4	19 17 14 11	487.3	279.9	0.51 0.44 0.36 0.28	0.054	0.574
0.2	4	23 20 15 13	608.9	320.1	0.50 0.42 0.31 0.23	0.056	0.525
0.4	3	31 24 17	608.1	323.3	0.50 0.37 0.25	0.050	0.532
0.6	2	46 28	610.0	331.3	0.48 0.27	0.051	0.543
0.8	1	77	607.8	344.33	0.40	0.054	0.567

3. References

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