A MULTI-OBJECTIVE OPTIMIZATION OF THE BI-ENZYMATIC BATCH REACTOR USED FOR MANNITOL PRODUCTION

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Multi-enzymatic reactions can successfully replace complex chemical syntheses, using milder reaction conditions, and generating less waste. Among others, Batch Reactors (BR) are widely used in the biosynthesis industry, being simple to operate and control, flexible, and suitable for multi-product use. The paper aims to optimize a bi-enzymatic BR used for the synthesis of high purity mannitol (M) by using the enzymatic reduction of fructose (F) with nicotinamide adenine dinucleotide (NADH) as a cofactor, in the presence of mannitol dehydrogenase (MDH). The NADH is continuously regenerated in-situ, by means of the enzymatic decomposition of ammonium formate in the presence of formate dehydrogenase (FDH). By using a process kinetic model from the literature, experimentally validated, the BR optimal operating policy is determined by simultaneously minimizing the consumption of the expensive enzymes, and of NADH, while maintaining a high productivity in mannitol.

Keywords: D-fructose reduction with NADH to mannitol, enzymatic batch reactor optimization; MDH; FDH; NADH in-situ regeneration

Notations

c_j	species *j* concentration
c_j^*	« species *j* saturation level
k_j, K_j	rate constants
Min / Max	minimum / maximum
r_j, R1, R2	species *j* reaction rate; reaction rates
t	time

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t_f	the batch time
V	the BR volume
W	the objective function of the optimization problem
Greek Symbols	
ν_{ij}	stoichiometric coefficient of species j in reaction i
Index	
o	initial
f	final
Abbreviations	
arg	the argument of a function
BR	batch reactor
E	enzyme
F	D-Fructose
FBR	Fed-batch reactor
FDH	Formate dehydrogenase
HFCS	fructose/glucose syrup
$HCOO^-$	formate
M	Mannitol
MDH	Mannitol dehydrogenase
NAD(P)H	nicotinamide adenine dinucleotide (phosphate)
NAD, NAD^+	Nicotinamide adenine dinucleotide (oxidized form) »
NLP	Nonlinear (programming) optimization problem
[X]	Concentration of X
Λ	« and », that is the mathematical operator

1. Introduction

Mannitol is a natural hexitol with important applications in medicine and the food industry [1]. ”The present global market of mannitol is around $100 million in 2013, of an average price of $42-80 per kg., and with a production growth rate of 5%-6% annually. Around 50,000 tons/year of mannitol are produced currently by the costly chemical hydrogenation alone” [2], and the rest by the less expensive enzymatic routes [3]. The main routes to produce mannitol at a large scale are the followings [4]: a). *The chemical catalytic process* meaning the catalytic hydrogenation of fructose, sucrose (inverted sugar), or of the syrups containing equal parts of glucose and fructose (HFCS) coming from the enzymatic hydrolysis of starch in the presence of calcium ions [5,6]. This chemical route is very costly because it requires high pressures (50-80 atm), high temperatures (120-160°C), and a costly catalyst, such as the Raney nickel [2,6,10]. Combined enzymatic routes with the chemical catalytic conversion were also reported, but are still expensive technologies [6]. b). *Biological routes* are seeking

for the conversion of glycerol to mannitol on *Candida magnoliae* culture with 50% yield [7]. If HFCS is used instead, the yield reaches 83%., or even higher if mutants are used instead [8]. A review of biotechnological ways to produce mannitol from fructose and/or glucose by using various cell cultures was presented by [9,10].

However, the yield of the biological routes is incomparably lower than those of the enzymatic alternatives [11], as below underlined by the following disadvantages [11]:

- Significant fraction of fructose (10-15%) is converted into by-products such as lactate, acetate, ethanol, and carbon dioxide, leading to a costly purifying of the main product;
- The most selective among strains is *Lb. sanfranciscensis* which converts almost 100% fructose to mannitol. However, its culture is too sensitive to the environmental perturbations;
- Productivity of most of *Lb. sp.* is of only 6-32% if the reaction medium is lacking Mn(2+);
- Temperature influences extremely strongly the bioprocess productivity, especially in the case of *Lb. fermentum* for which a doubling of productivity is observed if it switches from 25 to 35°C;
- The best results are obtained for the bacterium *Leuconostoc pseudomesenteroides*, which completely converts fructose after 11-12 hours with a selectivity of 80-85% in batch cultures at 40°C.

c). *The bi-enzymatic route with NADH cofactor continuous regeneration.* One of the most effective technologies (studied in the present paper) is bi-enzymatic. "Mannitol is produced by the enzymatic reduction of fructose in the presence of MDH, and cofactor NADH as a proton donor. The resulted NAD(+) is continuously regenerated in-situ by the expense of the enzymatic decomposition of ammonium formate in the presence of FDH"[11], according to (Fig. 1):

The use of another cofactor, such as NADPH is not recommended, being much more expensive [12], and very unstable [13]. This bi-enzymatic technology presents a large number of advantages: i) It is less expensive, while requiring mild conditions (normal pressure, pH= 7, 25°C); ii) The selectivity is practically 100%, and thus separation of mannitol at the batch end is less costly; iii) Optimization of the BR operation (this paper) leads to a very much reduced consumption of costly enzymes (FDH, MDH); iv) The continuous in-situ regeneration of the NADH cofactor considerably reduces the production costs. v) The production costs could be even more reduced if immobilized enzymes on a suitable solid support will be used instead of free enzymes; vi) The use of the regenerable NADH was proved to be the most less expensive way to hydrogenate the fructose [10, 14-15].

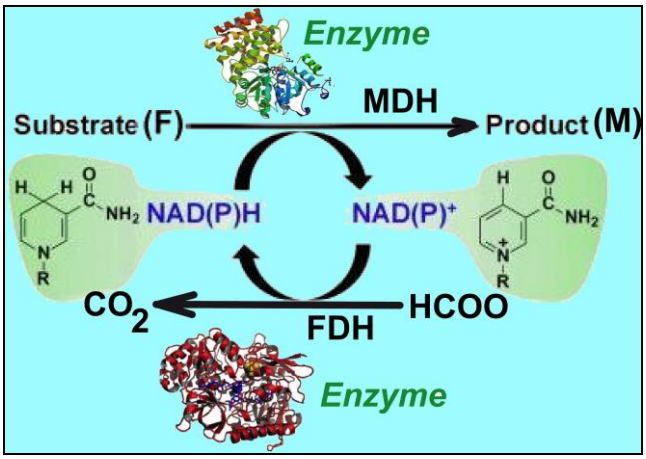

Fig. 1. "The simplified reaction scheme of the two coupled enzymatic reactions: (Up) D-fructose (F) reduction to mannitol (M) by using suspended MDH, and the cofactor NADH. (Down) NADH continuous regeneration by the expense of formate (HCOO) degradation in the presence of suspended FDH" [11].

A general analysis of the enzymatic process performances, compared to those of the biological processes (cell cultures in specialized bioreactors, such as fermenters), and those of the chemical catalytic processes was performed by [16], and presented in (Table 1). Such an analysis points-out the high potential of enzymatic processes, and their advantages compared to the classical chemical and biological syntheses.

Over the last decades, "remarkable progresses made in the development of new enzymes and in realizing complex coupled multi-enzymatic systems, able to in-situ recover the main reaction cofactor(s), reported important applications in the industrial bio catalysis, with important advantages, by integrating genetic and engineering methods" [17,18]. Due to their multiple advantages, "multi-enzymatic systems with parallel or sequential reactions, using milder reaction conditions, are successfully applied in the biosynthesis industry, by covering several alternatives reviewed by [19,21]. In the bi-enzymatic system here analysed, the second enzymatic reaction regenerates the co-factor of the main enzymatic reaction." [22]

Table 1

Comparison of main technologies for chemicals production [16].

Parameter	Classical fermentation (cell cultures)	Enzymatic processes	Chemical catalysis
Catalyst	Living cells	Enzymes	Metals, acids, etc.
Catalyst conc. (kg/m^3)	10-200	50-500	50-1000, or even higher
Specific reactions	Sometimes	Often	Often
Reaction	Moderate	Moderate	Moderate to extreme

conditions(*)			
Sterility	Yes	Yes	No
Yield (%)	10-95	70-99	70-99
Cost item	Cooling water	Enzyme(s)	Varies
Problems	Microorganism too high sensitivity, inactivation, reuse	Stability, reuse	Selectivity, stability

(*) referring to the temperature, pressure, pH, the presence of additives, catalyst immobilization requirements, etc.

By using a kinetic model taken from the literature [22], validated against the experimental data of [11], the present study is aiming at deriving the optimal operating conditions of a BR that minimize the enzymes (MDH, FDH), and cofactor (NADH) consumption, concomitantly with fructose conversion maximization under imposed technological constraints for the control variables.

A review of the objective criteria used in the literature for bioreactor optimization is given by [21]. In the (multi-)enzymatic reactor case, there are not many studies in the literature dealing with the BR multi-objective optimization. Some of them are given by [23,27]. That is because, solving such an engineering multi-objective problem is not a trivial one. "Even if the developed multi-enzymatic system is advantageous, its engineering part is not an easy task because it must account for the interacting reactions, differences in enzymes optimal activity domains and deactivation kinetics, the presence of multiple and often contrary objectives, nonlinear technological constraints, and an important degree of uncertainty coming from multiple sources: model inaccuracies (due to lack of enough structured experimental data), constraint uncertainty, presence of inherent random disturbances in the operating (control) variables, and the dynamic process of a high nonlinearity. Crucial engineering decisions should be taken based on the available information on the process kinetics, enzyme characteristics [activity, stability / half-life, temperature and pH optimal activity range, interactions among products and intermediates, carrier loading capacity if immobilized, enzyme recovery possibilities" [24]. Therefore, such an optimization problem for multi-enzymatic systems should be solved for every particular system [25,26].

The paper presents a significant number of novelty aspects, such as: a) The in silico (math model based) engineering analysis of a complex bi-enzymatic process, leading to the optimization of the related industrial BR. The derived operating policy was proved to be superior to other operating modes. b) There are few papers in the literature dealing with the BR multi-objective optimization for this process. c) The used numerical methodology to solve this multi-objective engineering problem (involving four contrary objectives) presents a high degree of originality. d) The scientific value of this paper is not "virtual", as long as the numerical analysis is based on a kinetic model [22] constructed and validated by using the extensive experimental data sets of [11].

2. Dynamic models for the bi-enzymatic process and BR

The approached BR is those of [11] used to study the bi-enzymatic process kinetics. The BR constructive scheme is presented by [26]. The BR characteristics presented in (Table 2) reveal a quite flexible operating domain, including large ranges for the initial concentrations of substrate (fructose [F]o), cofactor ([NADH]o), and enzymes ([MDH]o, [FDH]o). Such an observation opens a large number of optimization options. To simulate the BR dynamics, the simple mathematical model of (Table 3 in [26]) was adopted. This classic ideal model of the BR assumes the following simplifying hypotheses [28]: i) isothermal, iso-pH; ii) "additives (for the pH-control) are added initially and during the BR operation in recommended quantities; iii) perfectly mixed liquid phase (with no concentration gradients)" [22].

By using this BR, an extended experimental program was conducted by [11] at 25°C, pH 7.0, and under the large ranges of initial conditions of (Table 2).

The collected kinetic data allowed [22] to build-up a kinetic model of the bi-enzymatic process. "A simple Michaelis-Menten model of Ping-Pong-Bi-Bi type was proposed for both reaction rates R1 and R2 (Figs. 1-2). For simplicity, this model includes a non-competitive inhibition with respect to reactants. Inactivation of MDH and FDH enzymes during the batch has been neglected, due to the lack of available data" [22]. The rate constants have been estimated by using an effective nonlinear least squares procedure [29,30], with adopting a simple dynamic model for the BR (Table 3 in [26], and Fig. 2). The adequacy of the resulted kinetic model of (Fig. 2) was proved to be very good vs. the experimental data of [11], for all tested large number of initial conditions [22].

Table 2

"Nominal reaction conditions for the bench-scale BR of [11], used to investigate the enzymatic reduction of D-fructose to mannitol using MDH and NADH cofactor, with the in-situ continuous regeneration of the cofactor at the expense of formate degradation in the presence of FDH. The used FDH (EC 1.2.1.2) from *Candida boidinii* has a specific NAD-dependent activity of 2.4 U/mg, measured at 25°C and pH 7.0. The MDH (EC 1.1.1.67) from *Pseudomonas fluorescens DSM 50106* was over-expressed in *E. coli* JM 109. The NADH-dependent FDH and MDH typical activity in D-fructose reduction varies within the range of 0.1-2 kU/L. [11]

Parameter	*Value* [11]
Temperature / Pressure / pH (buffer solution)	25°C / Normal / 7
Molar initial concentrations	
Fructose, [F]o	0.1-1 M (tested by [11]) 0.1-4 M (to be optimized)
[NADH]o	0.1-0.5 M, (to be optimized)
[NAD]o	0.0005 M
Formate [HCOO]o	Identical to [F]o [11]
Others: [M]o = [CO2]o = 0	None
$c^*_{CO_2}$ = CO_2 saturation at 25°C and pH= 7	0.0313 M [34,35]

Reaction time	48 h
Initial FDH (referred to the reactor liquid)	0.1-2 kU/L, (to be optimized)
Initial MDH (referred to the reactor liquid)	0.1-2 kU/L, (to be optimized)

“

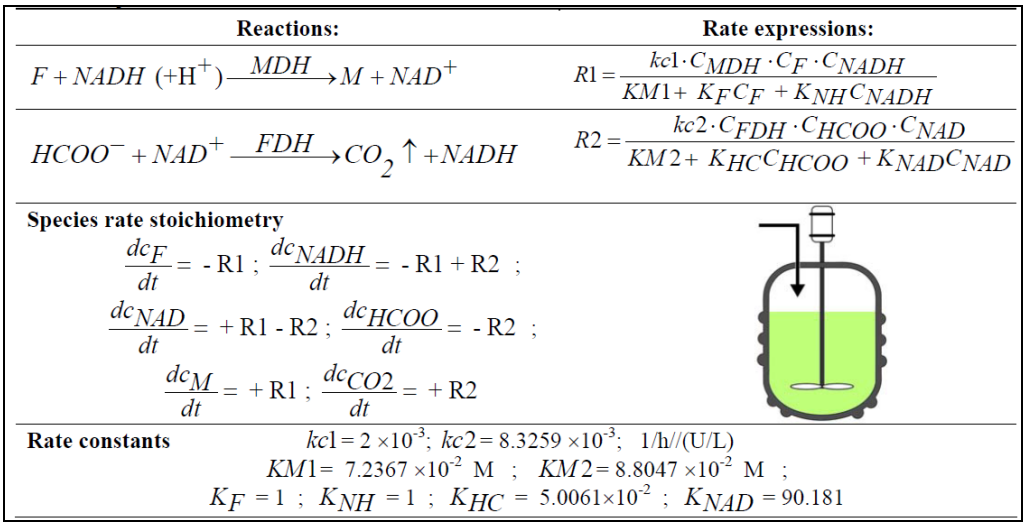

Fig. 2. “The kinetic model of [22] referring to the two coupled enzymatic reactions, that is: (R1) reduction of D-fructose to mannitol by using MDH enzyme and NADH cofactor and, (R2) in-situ continuous regeneration of the cofactor NADH at the expense of formate degradation in the presence of FDH (Fig. 1). Rate constants have been estimated under the nominal conditions of Table 2 to match the experimental kinetic data of” [11].

3. Optimization problem formulation

By repeatedly simulate the BR dynamics, with using the above presented process and reactor model, [22] performed a BR optimization on a quite tight range of initial [F]o and [NADH]o while concomitantly fulfilling three optimization criteria: (i-ii) *minimizing* the enzymes (MDH and FDH) consumption, while (iii) *maximizing* the fructose conversion at the batch end. To solve this multi-objective (i–iii) BR optimization problem, a simple exhaustive numerical algorithm has been used by [22]. As a general conclusion [22,26], in all the tested alternatives, the model-based predicted performances of the optimally operated BR are *much better* in terms of enzymes consumption (2x-5x less for FDH, and MDH), compared to the experimental trials of [11] to obtain high conversions in a optimally operated BR.

Later, for the same bi-enzymatic process, [26] compared an optimally operated serial sequence of identical BRs (SeqBR) with an optimal BR (repeatedly operated of the same number of cycles as the number of BRs in the SeqBR). The superiority of SeqBR vs. BR is proven not only in terms of economic efficiency (much less consumption of both enzymes), but also the

production flexibility offered by the adjustable number of BRs, and by the different initial load of each BR in the SeqBR series. In fact, a SeqBR is even more economic than the repeatedly operated BRs. Not only is the enzymes consumption much smaller to get the same high productivity, but the cumulated reaction time is also smaller. From a practical point of view, if the material handling is done quickly enough, the number of BRs in the SeqBR series can be even smaller than the adopted maximum number, by keeping the same productivity. Thus, once the first BRs from the series remain empty, as soon as the working front moves forward to the reactors from the tail of the series, they can become again part of the material moving front, thus leaving the last BRs from the series unused.

The present paper is aiming to explore how much the efficiency limits of the BR of (Table 2) can be "forced", by testing an even larger number of economic objectives, as followings:

i-ii).- minimum consumption of the costly enzymes MDH, and FDH;

iii).- minimum consumption of the cofactor NADH. Even if this compound is not very expensive [12], its separation over the product purification is relatively costly [31].

iv).- maximum BR productivity in mannitol;

v) an adjustable substrate [F]o consumption, to ensure its maximum conversion in the BR.

Consequently, the control variables of the optimization problem are the initial concentrations of the following compounds: [F]o, [NADH]o, [MDH]o, and [FDH]o. These variables are adjusted to match the optimal values in respect to the (i-v) objectives. The initial concentrations of the other compounds are null (that is, [M]o, and $[CO_2]$o), or at values recommended by [11] (that is [NAD]o, and [HCOO]o = [F]o).

Here, it is to observe that there are many other operating variables that could be considered when optimizing the BR (e.g., [HCOO]o ≠ [F]o , or the batch time t_f). However, if the number of control variables becomes too large, the associated nonlinear optimization problem (NLP) will become too multi-modal, which would not only greatly increase the computation time for finding a feasible problem solution but would also greatly increase the difficulty of finding the global optimum of the NLP problem.

In mathematical terms, the multi-objectives (i-v) problem translates in the following NLP problem:

Given [NAD]o , Find: { [F]o, [NADH]o ; [FDH]o ; [MDH]o } =
= arg W (c, c_o, k); (1A)
with the following composite objective function:

□□□□□□□□W = Max(Fobj1) Λ Min(Fobj2) Λ Min(Fobj3) Λ Min(Fobj4),

Where the component objective functions are the followings:

Fobj1 = $\left[M\left(t_f \right) \right]$, with [M] in M units.

Fobj2 = $\left[MDH \right]_o$, with MDH conc. in kU/L units.

Fobj3 = $\left[FDH \right]_o$, with FDH conc. in kU/L units.

Fobj4 = $\left[NADH \right]_o$, with NADH conc. in M units.

Eq. (1A) can be re-written in a form much more accessible to the numerical calculus, as follows:

Given [NAD]o , Find: { [F]o, [NADH]o ; [FDH]o ; [MDH]o } =
= arg W (c, c_o, k);
with the following composite objective function:
□□□□□□□□W = (Fobj2 + Fobj3 + Fobj4) / Fobj1. (1B)

As an observation, the formulated composite objective function W eqn. (1B) corresponds to the so-called "inverted utility function method" [32]. Because the four component objective functions in W are opposed, other optimization procedures can be applied as well, for instance the Pareto-front method [27]. The adopted optimization rule in the present study implicates the advantage of simplicity, and easy application.

Minimization of the composite objective function "W" in eqns. (1A-B) implicitly ensures consumption minimization of the two enzymes (MDH, FDH), and of the cofactor (NADH), concomitantly with obtaining a high production of mannitol.

However, the NLP problem eqns. (1A-B) is highly non-convex and nonlinear, being subjected to the following constraints:

$\frac{dc_j}{dt} = \sum_{i=1}^{n_r} \nu_{ij} r_i$ (dynamic model of the process [22,31]); J = species index (F, M, $HCOO^-$, NADH, NAD^+, CO_2, MDH,FDH)

With the initial conditions of:

$c_{j,o} = c_j\left(t = 0\right)$; where j = (F, NADH, MDH, FDH) are to be optimized; (2i)

$c_{j,o}$ = 0 , for j = (M, CO_2) ; $c_{j,o}$, for j = (NAD^+) is given in (Table 2);

$c_{j,o}$, for j = (HCOO) is [HCOO]o = [F]o , as recommended by [11].

$c_j(t) \geq 0$, for all *t* (physical significance constraints);	(2ii)
Searching ranges suggested by [11] (experimentally validated) are: [MDH]o ; [FDH]o Є [0.1-2] kU/L; 0.1 ≤ [F]o ≤ 3 M (extended to 4 M); 0.01 ≤ [NADH]o ≤ 0.5 M;	(2iii)
V = constant; (BR liquid volume);	(2iv)
The main reaction R1 occurs quantitatively, that is [M(*t*)] = [F]o − [F(*t*)], at any moment of the BR, that is t Є [0- t_f], with t_f = 48 h.	(2v)
One excludes the trivial solution (unfeasible): W = Fobj1 = Fobj2 = Fobj3 = Fobj4 = 0	(2vi)

Concerning the formulation of the optimization problem eqn. (1A-B), some observations are necessary: (i) the chosen units for M, MDH, FDH, and NADH allow the direct comparison of Fobj1, Fobj2, Fobj3, and Fobj4, and their concomitant use in the W composite objective function, because they present the same order of magnitude; (ii) the way in which W was built implicitly ensures the simultaneous realization of the component objectives formulated in eqn. (1A) ; (iii) other formulations of the W function are also possible, depending on the weight given to each component objective (Fobj1-Fobj4) [21].

The resulted NLP optimization problem eqn. (1B+2) includes 4 searching variables, which are subjected to multiple nonlinear implicit and explicit constraints. To avoid local sub-optimal solutions, a very effective multi-modal adaptive random search procedure has successfully applied, that is the MMA of [29,30].

4. Results and discussions

The results of the BR optimization problem eqn. (1B+2) with accounting for 4 opposed objectives are presented in (Table 3), in the area marked with thick line. For comparison, this table also includes several operating policies of the same BR of (Table 2), but obtained in different ways, namely: (a) optimal policies obtained by [22,26] for [F]o {0.15, 1, or 3 M} by solving a similar NLP optimization problem but adopting only 3 opposed objectives (MDH, and FDH enzymes consumption minimization, and F-conversion maximization).

Besides, the used optimization rule (an exhaustive search) is not very effective, compared with those used in this paper; (b) optimal BR operating policies obtained by [11] following an extended, and very costly experimental program, with more than 10 trials of initial conditions for the BR.

Table 3

Optimal BR policies obtained in this study by using 4 opposed objective functions (in the area marked with thick lines), compared to the optimal BR policies predicted by [22,26] by using only 3 opposed objective functions, and also compared to some sub-optimal BRs experimentally derived by [11]. Initial conditions for other species are those recommended by [11], that is: [HCOO]o = [F]o ; [NAD]o = 0.0005 M; Batch time = 48 h.

[F]o = 0.15M				
Enzyme	Optimal BR of [22,26] [NADH]o = 7.91×10^{-3} M [11]			Experimental [11]
FDH (U/L)	500	500	500	1000
MDH (U/L)	214	380	800	1000
F conv.	0.67	0.76	0.87	0.98
[M]f (M)	0.67	0.76	0.87	0.98
[F]o = 1M				
Enzyme	Optimal BR of [22,26] [NADH]o = 7.91×10^{-3} M [11]			Experimental [11]
FDH (U/L)	500	1000	1000	1000
MDH (U/L)	1388	1000	1500	1000
F conv.	0.61	0.62	0.80	0.68
[M]f (M)	0.61	0.62	0.80	0.68

[F]o = 3 M					
Enzyme	Optimal BR of [22,26] [F]o = 3 M		Optimal BR (this paper)		Experimental [11]
			[F]o = 3 M	[F]o = 4 M	
	[NADH]o = 0.5 M [26]		[NADH]o = 0.215 M	[NADH]o = 0.246 M	No data
FDH (U/L)	500	300	274.82	297.72	
MDH (U/L)	500	100	158.59	184.15	
F conv.	0.999	0.805	0.67	0.687	
[M]f (M)	2.997	2.415	2.014	2.748	

These results of (Table 3) lead to several observations:

i.- The F-conversion decreases with the [F]o increase, and with the [NADH]o decrease.

ii.- In all the alternatives, the model-based predicted optimal performances of the studied BR are *much better* in terms of enzymes consumption (2x less for FDH, and 3x-5x less for MDH) compared to the experimental trials of [11] to obtain a similar F-conversion. Besides, in-silico derivation of all the optimal BR operating policies is much less costly than the lab-scale large experimental program used to find an optimal BR operating alternative. The reader is referred to [22,26] to visualize the species dynamic trajectories during the batch for several optimal BR operations.

iii.- The species dynamics plots of [22,26] indicated "a close connection between the two coupling reactions, enzyme concentrations, and the quasi-stationary of the NADH/NAD ratio over the batch. For all the investigated optimal operating policies, the two enzymatic reactions are well coupled. Thus,

the high reaction rates R1 and R2 ratio reaches a quasi-stationary level, leading to a quasi-constant NADH / NAD ratio much higher than 10, thus maintaining the process efficiency." [22]

iv.- The same plots indicated that "the cofactor NADH regeneration is very efficient, the format decomposition being quasi-complete and leading to saturation $[CO_2]$* in short time (after ca. 10 h, or even earlier), with removal of the CO_2 excess from the system over the rest of the batch." [22,26].

v.- As revealed by the repeated simulations of [22,26] (not presented here), and the results of (Table 3), the BR performances are more sensitive to the [MDH]o, and [NADH]o than to the [FDH]o.

vi.- The BR optimal policies derived in this study (using 4 opposite objective functions) indicates a high reactor productivity for high [F]o of 4 M. This best BR policy ensures a high M-production with using 3x less costly FDH, and MDH compared to all the others sub-optimal policies of the (Table 3) derived with using only 3 opposite objectives. Even if the F-conversion (0.687) is not very high, the M-production (2.748 M) in absolute terms is the best, achieved with using a small recoverable NADH amount.

5. Conclusions

Despite being computationally intensive, the numerical/engineering analysis of this paper, based on an experimentally validated kinetic model from literature, demonstrates that an optimally operated BR in respect to a larger number of objectives (four here) can lead to a high reactor productivity with a substantially lower consumption of costly enzymes compared to the (repeatedly) use of a sub-optimally operated BR.

As proved, the model-based engineering analysis is not only less costly but can save a lot of expensive experimental effort to identify the optimal operating conditions for a multi-enzymatic process with also using cofactors.

"The relatively simple but relevant case study analysed in this paper proves that, for the coupled multi-enzymatic systems, derivation of the optimal operating conditions (minimum enzyme consumption, with maximum reactor productivity) is not a trivial engineering problem even for a simple BR case." [22,33].

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