ACADEMIA ROMÂNĂ
Revue Roumaine de Chimie
https://www.icf.ro/rrch/

Rev. Roum. Chim.,
2022, *67*(4-5), 241–249
DOI: 10.33224/rrch.2022.67.4-5.02

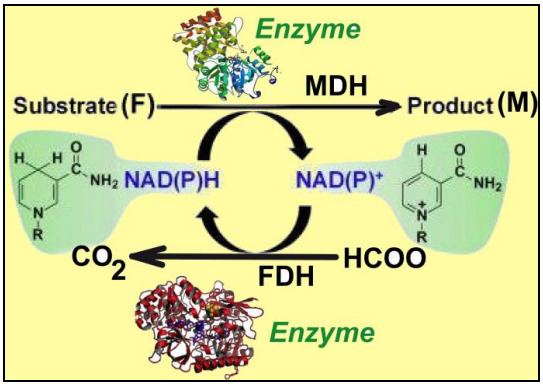
IN-SILICO OPTIMIZATION OF A BI-ENZYMATIC BATCH REACTOR FOR MANNITOL PRODUCTION WITH CONTINUOUS REGENERATION OF NADH COFACTOR

Gheorghe MARIA,[a,*] Laura RENEA,[a] Ioana Mirela PEPTĂNARU[a] and Cristina MARIA[b]

[a]Dept. of Chemical and Biochemical Engineering, Politehnica University of Bucharest,
Polizu Str. 1-7, 011061, Bucharest, Roumania
[b]National Institute for Research and Development in Environmental Protection,
Splaiul Independenței 294, Sector 6, Bucharest – 060031, Roumania

Received August 11, 2021

The paper aims to optimize a discontinuous bi-enzymatic reactor (BR), with a given production capacity, used for the synthesis of mannitol of 99% purity by using the enzymatic reduction of fructose with nicotinamide adenine dinucleotide (NADH) as a co-factor, in the presence of mannitol dehydrogenase (MDH). The NADH is continuously regenerated in-situ, using the enzymatic decomposition of ammonium formate in the presence of formate dehydrogenase (FDH). By using a kinetic model of the bi-enzymatic process, validated against the experimental data from the literature, as well as a standard mathematical model for the batch reactor, the optimal operating policy of the BR is determined by minimizing the consumption of the expensive enzymes (MDH and FDH), while maintaining a high fructose conversion (over 0.9).



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,[2] 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]

The chemical catalytic process implies the catalytic hydrogenation of fructose, sucrose (inverted sugar), or of the syrups containing 50% glucose and 50% fructose (HFCS) coming from the enzymatic hydrolysis of starch in the presence of calcium ions.[5,6] The chemical route is very costly because it requires the use of high pressures (50-80 atm), of high temperatures (120-160°C), and a Raney nickel catalyst,[2] or another costly catalyst.[6,7,11] Combined enzymatic routes with the chemical catalytic conversion were also reported, but are still expensive technologies.[7]

Various biological routes. (a) Khan *et al*.[8] uses a cell culture of *Candida magnoliae* to convert glycerol to mannitol with more than 50% yield. By contrast, if a mixture of fructose and glucose (HFCS) is used instead, the yield reaches 83%. If mutants are used instead,[9] then higher yields are reported. (b) Similarly, Loesche and Kornman[10] reported quantitative conversions of glucose, or

* Corresponding author: gmaria99m@hotmail.com

sucrose by using a culture of *Streptococcus Mutans*. (c) A review of biotechnological ways to produce mannitol from fructose and/or glucose by using various cell cultures was presented by Saha and Racine.[11]

However, the yield of the biological process is incomparably much lower than those of the enzymatic alternatives of Slatner *et al.*[12] [below process]. By comparing the performances of using various bacteria, several disadvantages of these biological routes are worth noting:

Significant fraction of fructose (10-15%) is converted into by-products such as lactic acid, acetic acid, 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.

For instance, the productivity of *Lb. brevis*, *Lb. fermentum*, *L. mesenteroides*, and *L. pseudomesenteroides* decreases by 6, 32, 9, and respectively 17% in the case of a reaction medium lacking Mn(2+).[12]

The 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.

The bi-enzymatic process with the NADH cofactor continuous regeneration. One of the most promising technology is those proposed by Slatner *et al.*[12] Mannitol is produced by the enzymatic reduction of fructose in the presence of mannitol dehydrogenase (MDH) and the 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 format dehydrogenase (FDH), according to (Figure 1): The use of another cofactor, such as NADPH is not recommended, being much more expensive,[13] and very unstable.[14] 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%, separation of mannitol at the batch end being easy and less costly; (*iii*) Optimization of the BR operation (this paper) leads to a much reduced consumption of costly enzymes (FDH, MDH); (*iv*) The continuous in-situ regeneration of the NADH cofactor reduces very much the production costs; (*v*) The production costs could be further reduced if immobilized enzymes on a suitable solid support would be used instead of the free enzymes; (*vi*) The use of the regenerable NADH was proved to be the most less expensive way to hydrogenate the fructose.[11,15,16]

A general analysis of the enzymatic process performances, compared to those of the biological processes (cell cultures in specialized bioreactors), and those of the chemical catalytic processes was performed by Moulijn *et al.*,[17] and presented in (Table 1). Such an analysis points-out the high potential of enzymatic processes, and their advantages compared to the classical 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 biocatalysis, with important advantages, by integrating genetic and engineering methods".[18,19]

"Thus, multi-enzymatic reactions are modern alternatives which often can successfully replace complex chemical syntheses, by using milder reaction conditions, and generating less waste. Multi-enzymatic systems with parallel or sequential reactions are successfully applied in this respect, by covering several alternatives:[20]

(*i*) Co-immobilizing two (or more) enzymes on the same support so that the substrate for the second enzyme is generated in situ as the first reaction takes place (that is for successive reactions);[21]

(*ii*) The second enzymatic reaction regenerates the co-factor of the first enzymatic reaction (*e.g.* regeneration of NADH or NADPH) in the enzymatic hydrogenation reactions (as proved in the present study);

(*iii*) The second enzymatic reaction shift equilibrium of the main reaction by removing the intermediate or by-product from the system (*e.g.*, removal of pyruvate as lactate by lactate dehydrogenase in the presence of NADH, during the Cori cycle in the liver);[22]

(*iv*) The second enzyme removes excess of biomass by hydrolysis or prolongs the life (duration of activity) of the first enzyme by a particular mechanism (*e.g.*, catalase prolongs the life of pyranose-2-oxidase used to oxidize D-glucose by decomposing the resulted hydrogen peroxide by-product)".[23]

Table 1

Comparison of technologies for chemicals production[17]

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, Even higher
Specific reactions	Sometimes	Often	Often
Reaction conditions(*)	Moderate	Moderate	Moderate to extreme
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.

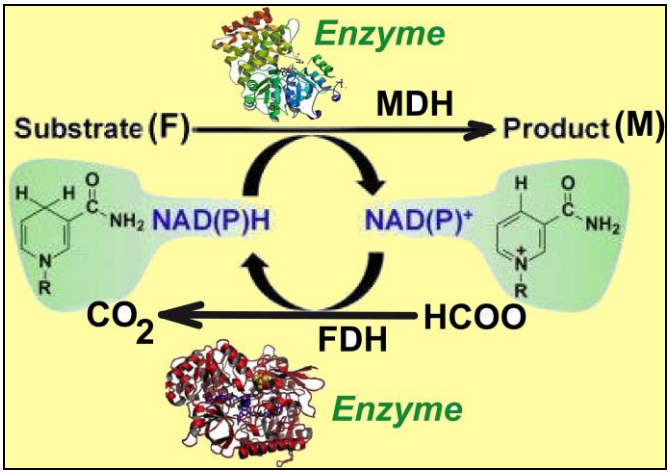


Fig. 1 – Simplified "reaction scheme of the two coupled enzymatic reactions: (Up) D-fructose (F) reduction to mannitol (M) by using suspended MDH (mannitol dehydrogenase), and the cofactor NADH (Nicotinamide adenine dinucleotide). (Down) NADH cofactor continuous regeneration by the expense of formate (HCOO) degradation in the presence of suspended FDH (Formate dehydrogenase)".[12]

By using a kinetic model taken from the literature,[24] validated against the experimental data of[12] for the approached bi-enzymatic process, the present study is aiming at deriving the optimal operating conditions of a batch reactor (**BR**) that minimize the enzymes (MDH, FDH) consumption, concomitantly with fructose conversion maximization. (>90%) for an imposed production capacity of the BR (7500 t/yr).[25]

Solving such an engineering multi-objective problem is not a trivial one. "Even if the multi-enzymatic system is advantageous, when developing such a process, the 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, 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) parameters and the dynamic process of a high nonlinearity. These 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".[26] This is why, such an optimization problem for multi-enzymatic systems should be solved for every particular system.[27,34]

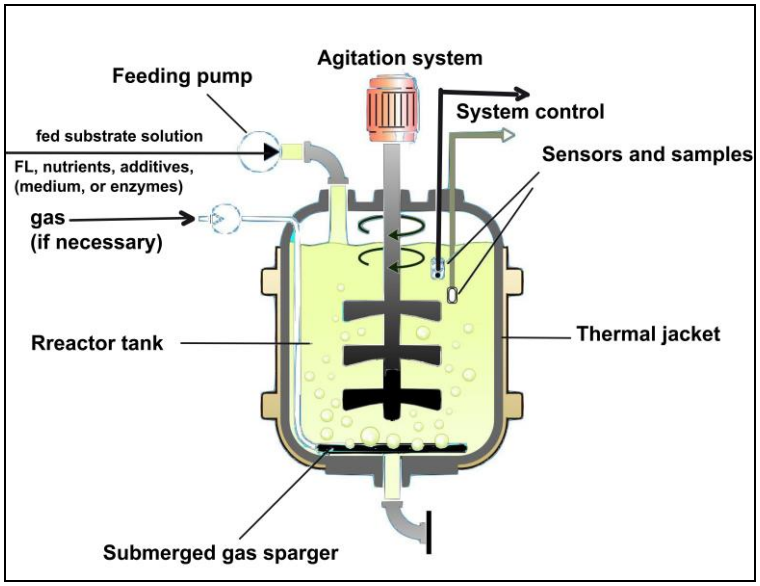


Fig. 2 – Simplified scheme of a BR, or a FBR used to conduct enzymatic or biological processes. In the BR operating mode, substrate(s), biocatalyst, and additives are initially loaded in the recommended amounts (concentrations). In the FBR operating mode, the substrate(s)/ biocatalyst(s) and additives (nutrients, pH-control substances) are continuously fed, following a certain (optimal) policy.[29]

Table 2

"Nominal reaction conditions of Slatner *et al.*[12] for the enzymatic reduction of D-fructose to mannitol using MDH, and NADH cofactor in an experimental BR, 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 0.5-2 kU/L."[12]

Parameter	**Value and remarks[12]**
Temperature / Pressure / pH (buffer solution)	25°C / Normal / 7
Molar initial concentrations	
Fructose, [F]o	0.1-1 M (tested by Slatner *et al.*[12]) 0.1-3 M (this paper)
[NADH]o	0.008 M (0.1-0.5 M)(this paper)
$[NAD^+]o$	0.0005 M
Formate [HCOO]o	Identical to [F]o
Others: [M]o = [CO2]o = 0	None
$c^*_{CO_2}$ = CO_2 saturation level at 25°C and pH= 7	0.0313 M[29,30]
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)

Table 3

The ideal model (species mass balances) for the enzymatic BR:[32] Indices: "o" = initial; "f" = final (at the batch time); "i" = reaction; "j" = species. Superscript "*" = saturation level. Notations are given in the abbreviation list

$\frac{dc_j}{dt} = \sum_{i=1}^{n_r} \nu_{ij} r_i$; " j = species index (F, M, HCOO, NADH, NAD^+, CO2). Reaction stoichiometry ν_{ij} is given in Figure 3.

t_f = 48 h (batch time).

Initial conditions $c_{j,o} = c_j\ (t{=}0)$ are given in Table 2.

$\frac{dc_E}{dt} = 0$, (negligible inactivation of MDH and FDH); *E* = enzymes (MDH and FDH);

If $c_{CO_2} > c^*_{CO_2}$, then $c_{CO_2} \approx c^*_{CO_2}$ (excess being removed from the liquid phase)".[32]

The paper presents a significant number of novelty aspects. Among these, are to be mentioned the followings:

The in silico (math-model-based) engineering analysis of a complex bi-enzymatic process, leading to the optimization of the related industrial BR. The previously experimentally validated dynamic model of the bi-enzymatic process allows determining the optimal operating policy of BR which, was proved to be superior to other operating modes (not presented here).

Before this paper, there are very few bi-, or multi-enzymatic processes analysed in the literature from the engineering point of view.

This bi-enzymatic process is already known, but the associated engineering analyses are missing in the literature, as well as the ways to maximize the productivity of the related reactors.

The scientific value of this paper is not "virtual", as long as the numerical analysis is based on a kinetic model[24] constructed and validated by using the extensive experimental data sets of.[12]

DYNAMIC MODELS FOR THE BI-ENZYMATIC PROCESS AND BR

The approached BR in this study is those of Slatner *et al.*[12] used to study the bi-enzymatic process kinetics. The characteristics of this BR 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. The BR constructive scheme is presented in (Figure 2). To simulate the BR dynamics, the simple mathematical model of (Table 3) was adopted. This classic ideal model of the BR assumes the following hypotheses:[32] (*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).[29]

By using this BR, Slatner *et al.*[12] conducted extended experiments at 25°C, pH 7.0, under large ranges of initial conditions: [F]o ∈ [0.1 – 3] M; [NADH]o ∈ [0.008 – 0.5] M; [HCOO]o = [F]o; [NAD]o = 0.0005 M.

The collected kinetic data by Slatner *et al.*[12] allowed Maria[24] to build-up a kinetic model of the bi-enzymatic process. Based on these experimental data sets, and other qualitative observations, a simple "Michaelis-Menten kinetic model of Ping-Pong-Bi-Bi type was proposed by Maria[24] for both reaction rates R1 and R2 (Figure 3) by analogy with a similar process of pseudo 2-nd order kinetics.[23] For simplicity, this model includes a non-competitive inhibition with respect to reactants even if the mannitol inhibition might be significant.[12] Enzymes MDH and FDH inactivation during the reaction have been neglected due to lack of available data." The rate constants have been estimated by using an effective nonlinear least squares procedure,[28,33] with adopting a simple dynamic model for the BR (Table 3, Figure 3). The adequacy of the resulted kinetic model (Figure 3) was proved to be very good vs. the experimental data,[12] for the all tested large number of initial conditions.

OPTIMIZATION PROBLEM FORMULATION

By far, the bi-enzymatic alternative proposed by Slatner *et al.*,[12] also approached in this paper, is the most advantageous technology to produce mannitol at an industrial scale, the process occurring under mild conditions (pH= 7, 25°C) and generating negligible waste. "However, due to the costly enzymes, reflected in the product cost, a favorable solution of the engineering part (this study) is very important. Recent advances try coupling the two reactions, not in the same BR, but in the same genetic modified micro-organism (*Bacillus megaterium*) used as host for both enzymes synthesis, and cofactor regeneration".[30] However, this last route is not yet available at an industrial scale.

In brief, the main goals for the present BR optimization are the followings: (*i*) For the main reaction R1: selectivity > 99%; fructose conversion: > 90%; minimum consumption of the costly MDH; (*ii*) For the regeneration reaction R2: formate conversion: > 90%; minimum consumption of the costly FDH; (*iii*) The investigated range of BR initial conditions (control variables) are: [MDH]o, [FDH]o ∈ [100, 2000] U/L; [F]o ∈ [0.1, 3] M; [NADH]o ∈ [0.008, 0.5] M; [HCOO]o = [F]o; [NAD^+]o = 0.0005 M.

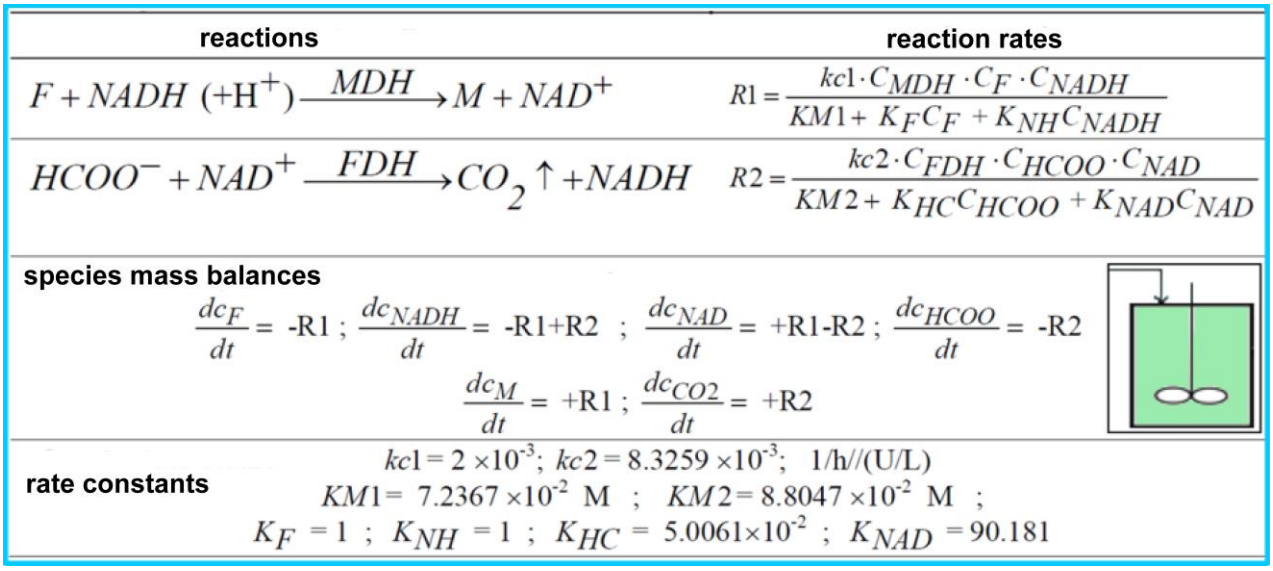


Fig. 3 – "The kinetic model of[24] referring to the two coupled enzymatic reactions, that is: (R1) reduction of D-fructose to mannitol by using MDH enzyme and NADH cofactor and, (R2) in-situ continuous regeneration of the cofactor NADH at the expense of formate degradation in the presence of FDH (Fig. 1). Rate constants have been estimated under the nominal conditions of Table 2 to match the experimental kinetic data of".[12]

In mathematical terms, all these objectives translate in the following nonlinear optimization problem:

For a given [F]o, [HCOO]o = [F]o ; [NAD^+]o ,
Find:
{[NADH]o ; [FDH]o ; [MDH]o} = arg Min W (c, c_o, k);
with the following composite objective function:
W = (Fobj2 + Fobj3) / Fobj1, where:
Fobj1 = $\left[\mathrm{M}\left(\mathrm{t_f}\right)\right]$, with [M] in M units;
Fobj2 = $\left[\mathrm{MDH}\right]_\mathrm{o}$, with MDH conc. in kU/L units.
Fobj3 = $\left[\mathrm{FDH}\right]_\mathrm{o}$, with FDH conc. in kU/L units. **(1)**

Minimization of the objective function "W" implicitly ensures minimization of the two enzymes consumption, and a high fructose conversion.

The optimization problem (1) is subjected to the following constraints:

(*i*) The dynamic model of the process, given in Table 3, and Fig. 3); J = species index (F, M, $HCOO^-$, NADH, NAD^+, CO_2, MDH,FDH);
(*ii*) The initial conditions $[c_j](t=0)$ are the searching (control) variables, that is {[NADH]o; [FDH]o; [MDH]o}. Except for the "J"-species corresponding to the given [F]o; [NAD^+]o; [HCOO]o; **(2)**

(*iii*) $c_j(t) \geq 0, \forall\, t$ (physical significance constraints);

(*iv*) Searching ranges suggested by Slatner *et al.*,[12] are: [MDH]o; [FDH]o ∈ [0.1-2] kU/L; [F]o ∈ [0.1, 3] M; [NADH]o ∈ [0.008,0.5] M;

(*v*) V = constant (for the BR during the batch);

(*vi*) The main reaction R1 occurs quantitatively, that is [M(*t*)] = [F]o - [F(*t*)], at any moment during the batch. t_f = 48 h;

(*vii*) One excludes the trivial solution (infeasible): **W** = Fobj2 = Fobj3 = Fobj1 = 0

(2)

"To not complicate calculations when solving the optimisation problem (1+2) a simple exhaustive search of the optimal [MDH]o, [FDH]o, and [NADH]o have been used in the experimental ranges mentioned in eq. (2), with a step resulted by dividing the search range of unknown [FDH]o, [MDH]o, and [NADH]o to NdivF, NdivM, and NdivN trial points respectively" {see Maria[24] for computing details}. Small search steps (corresponding to Ndiv > 50-100) ensure localization of the problem global optimum, but with the cost of a considerably computational effort.

RESULTS AND DISCUSSION

Some of the resulted optimal operating policies for the BR corresponding to several initial [F]o, and [NADH]o are presented in (Table 4). The marked optimal policies concomitantly fulfil the two main optimization criteria, that is: minimizing the enzymes (MDH and FDH) consumption, with realizing a high fructose conversion (0.80-1) at the batch end. These results lead to several comments:

(*i*) As a general conclusion, in the all alternatives, the model-based predicted performances of the optimally operated 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 Slatner *et al.*[12] to obtain a high conversion in a non-optimally operated BR. The species dynamics during the batch are presented by Maria[24] for several optimal BR operations.

(*ii*) The optimal BR, for [F]o = 0.1 M, and for [F]o = 1 M have been experimentally validated by Slatner *et al.*[12]

(*iii*) "There is a close connection between the coupling reactions, enzyme concentrations, and the quasi-stationary of the NADH/ NAD^+ ratio over the batch. For all the optimal conditions, the two enzymatic reactions are well coupled. Thus, the high reaction rates R1 and R2 ratio reach a quasi-stationary level, leading to a quasi-constant NADH /NAD^+ ratio much higher than 10, thus maintaining the process efficiency."[24]

(*iv*) The "cofactor NADH regeneration is very efficient, the formate 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-time." See Maria[24] for details.

(*iv*) As revealed by the repeated simulations of Maria[24] (not presented here), and the results of (Table 4), the BR performances are more sensitive to the [MDH]o, and [NADH]o than to the [FDH]o.

CONCLUSIONS

The numerical/engineering analysis of this paper, based on an experimentally validated kinetic model from literature, demonstrates that optimally operated BR can lead to high productivities with a substantially lower consumption of costly enzymes compared to the (repeated) use of simple BR sub-optimally operated.

Generally, in the all tested alternatives, the model-based predicted performances of an optimally operated 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 Slatner *et al.*[12] to obtain a high conversion in a non-optimally operated BR.

Table 4

Some optimal BR operating policies predicted by using the kinetic model of Maria,[24] and Crisan and Maria[35] compared to the experimental data of Slatner *et al.*[12] Initial conditions: [HCOO]o = [F]o ; [NAD^+]o = 0.0005 M; Batch time = 48h

Enzyme	**[F]o = 0.15M ; [NADH]o = 0.00791 M[12]**				**Experimental[12]**
FDH (U/L)	500	500	500		1000
MDH (U/L)	268	380	744		1000
F conv.	**0.85**	**0.90**	**0.94**		**0.98**
Enzyme	**[F]o = 1 M ; [NADH]o = 0.00791 M[12]**				**Experimental[12]**
FDH (U/L)	500	1000	1000	1000	1000
MDH (U/L)	1388	1246	1000	1388	1000
F conv.	**0.61**	**0.78**	**0.68**	**0.82**	**0.98**
Enzyme	**[F]o = 3 M ; [NADH]o = 0.5 M** (this paper)				**Experimental[12]**
FDH (U/L)	500	500	500	300	100	
MDH (U/L)	500	100	50	100	100	No data
F conv.	**1**	**0.99**	**0.80**	**0.87**	**0.50**	

"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.[24,31]

Solving this optimization engineering problem by only using an experimental approach, as tried by Slatner *et al.*,[12] may not be the best choice because it involves high costs and a large number of experimental separate tests.

The use of an adequate process model can offer an approximate if not exactly solution to the problem (depending on model quality), with a moderate computational effort. In addition, the paper proves in a simple, yet suggestive way how a lumped but adequately dynamic model can successfully support *in silico* engineering evaluations aiming to optimize the BR or other reactors operation, thus saving considerable experimental effort".

ABBREVIATIONS

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
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 the reaction **i**
o	initial
f	final
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) »
[X]	Concentration of X

REFERENCES

1. Wikipedia, "Mannitol", 2021, URL: https://en.wikipedia.org/wiki/Mannitol,(last accessing Aug. 07, 2021).
2. S. M. Bhatt, A. Mohan and S. K. Srinivastava, *Biotechnology*, **2013**, Article ID 914187.
3. N. Von Weymarn, "Process development for mannitol production by lactic acid bacteria", Technical report, *PhD Diss.*, Helsinki University of Technology, Laboratory of Bioprocess Engineering, **2002**. URL: http://lib.tkk.fi/ Diss/2002/isbn9512258854/ (last accessing Aug. 07, 2021).
4. S. M. Ghoreishi and R.G. Shahrestani, *Trends Food Sci. Tech.*, **2009**, *20*, 2009, 263-268.
5. A. Liese, K. Seelbach, and C. Wandrey, "Industrial biotransformations", Weinheim, Wiley-VCH, 2006.

6. M. Makkee, A. P. G. Kieboom and H. Van Bekkum, *Carbohydr. Res.*, **1985a**, *138*, 225-236.
7. M. Makkee, A. P. G. Kieboom and H. Van Bekkum, *Carbohydr. Res.*, **1985b**, *138*, 237-245.
8. A. Khan, A. Bhide and R. Gadre, *Bioresource Technology*, **2009**, *100*, 4911–4913.
9. K. H. Song, J. K. Lee, J. Y. Song, S. G. Hong, H. Baek, S. Y. Kim and H. H. Hyun, *Biotechnology Letters*, **2002**, *24*, 9–12.
10. W. J. Loesche and K. S. Kornman, *Arch. Oral Biol*., **1976**, *21*, 551-553.
11. B. C. Saha and F. M. Racine, *Appl. Microbiol. Biotechnol*., **2011**, *89*, 879–891.
12. M. Slatner, G. Nagl, D. Haltrich, K.D. Kulbe, and B. Nidetzky, *Biocatalysis and Biotransformation*, **1998**, *16*, 351-363.
13. H. K. Chenault and G. M. Whitesides, *Appl. Biochem. Biotechnol.*, **1987**, *14*, 147-197.
14. G. Maria and M. D. Ene, *Chem. Biochem. Eng. Q.*, **2013**, *27*, 385–395.
15. D. Peacock and D. Boulter, *Biochem. J.*, **1970**, *120*, 763-769.
16. T. Schmidt, C. Michalik, M. Zavrel, A. Spiess, W. Marquardt and M. B. Ansorge-Schumacher, *Biotechnol. Prog.*, **2010**, *26*, 73-78.
17. J. A. Moulijn, M. Makkee and A. van Diepen, "Chemical process technology", New York, Wiley, 2001.
18. K. Buchholz and D. C. Hempel, *Eng. Life Sci.* **2006**, *6*, 437–437.
19. D. C. Hempel, *Eng. Life Sci.*, **2006**, *6*, 443–447.
20. R. Xue and J. M. Woodley, *Bioresour Technol.*, **2012**, *115*, 183–195.
21. H. W. Blanch and D. S. Clark, "Biochemical engineering", New York, Marcel Dekker, 1997.
22. B. D. Hames, N. M. Hooper and J. D. Houghton, "Instant Notes in Biochemistry", New York, Springer Verlag, 1997.
23. G. Maria, M. D. Ene and I. Jipa, *J. of Molec. Catal. B: Enzymatic*, **2012**, *74*, 209-218.
24. G. Maria, *Computers & Chemical Engineering*, **2020a**, *133*, 106628-106635.
25. I. M. Peptanaru, "Optimization of the enzymatic batch reactor for the production of mannitol with the continuous in-situ regeneration of NADH", *MSc. Diss.*, University Politehnica of Bucharest, **2021**, (in Roumanian).
26. M. M. Domach, *The Chemical Engineering Journal*, **1984**, 29, B1–B8.
27. G. Maria and M. Crisan, *Asia-Pac. J. Chem. Eng.*, **2015**, *10*, 22-44.
28. G. Maria, "ARS combination with an evolutionary algorithm for solving MINLP optimization problems", in M.H. Hamza (Ed.), "Modelling, Identification and Control", Anaheim (CA), IASTED/ACTA Press, 2003, p. 112-118; URL: https://www.actapress.com/Content_of_Proceeding.aspx?proceedingID=213 (last accessing March-7, 2021).
29. G. Maria, *Molecules*, **2020b**, *25*, 5648-5674
30. C. Bäumchen, A. H. F. J. Roth, R. Biedendieck, M. Malten, M. Follmann, H. Sahm, S. Bringer-Meyer and D. Jahn, *Biotechnol. J.*, **2007**, *2*, 1408-1416.
31. P. Gajardo, H. C. Ramirez and A. E. Rapaport, *SIAM J. on Control and Optimization*, **2008**, *47*, 2827-2856.
32. A. Moser, "Bioprocess Technology: Kinetics and Reactors", New York, Springer-Verlag, 1988.
33. G. Maria, *Chem. and Biochem. Eng. Quarterly*, **2004**, *18*, 195-222.
34. G. Maria and I. M. Peptănaru, *MDPI-Dynamics*, *1*, 134-154, 2021, https://doi.org/10.3390/dynamics1010008
35. M. Crisan and G. Maria, *Rev. Chim.* (*Bucharest*), **2017**, *68*, 2196-2203.