EVALUATION OF SOME CONSTRUCTIVE AND OPERATING ALTERNATIVES WHEN DESIGNING A BIOREACTOR FOR A BI-ENZYMATIC PROCESS

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Due to the high selectivity and specificity of the enzymatic processes, consuming less energy, and producing less environmental pollution and byproducts, the industrial enzymatic biosynthesis reactors compete with those used for complex chemical synthesis which offen involve a large number of intermediate reaction steps and byproducts. However, chemical engineers have to consider that enzymes have limited applications due to some disadvantages: difficult control of the process due to its low reproductibility, and a too high enzyme activity sensitivity to operating conditions. Due to the overwhelming contribution of the enzyme cost in the final product selling cost for most of the industrial biosyntheses, the present study is aiming at applying a modular screening procedure for selecting and optimizing different operating policies for some enzymatic reactors in the case of a given bi-enzymatic process of known kinetic model. The case study refers to the complex oxidation of D-glucose to 2-keto-D-glucose, the optimal reactor policy corresponds to the minimum amount of required pyranose 2-oxidase and catalase that ensures an imposed reaction conversion and reactor productivity under various technological constraints.

Keywords: optimization, modular platform, operating policies, enzymes, D-glucose oxidation, pyranose 2-oxidase

Nomenclature

c_j	-	species *j* concentration
D	-	reactor content dilution rate
k_j, k_c, k_d, K_j	-	rate constants
$k_{oxl}a$	-	overall gas-liquid mass transfer coefficient
M	-	molecular weight
n	-	Yano-Koya exponent
N_{inj}	-	number of enzyme injections over the batch

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r_j	-	species *j* reaction rate
$r(S)$	-	production rate of species S
t	-	time
Δt	-	time interval
t_f	-	final batch time, or final running time
V	-	liquid volume
x, $\mathbf{x}$	-	conversion, or state variable vector
Y	-	stoichiometric coefficient

Greeks

μ_L	-	dynamic viscosity of the liquid
μ_m	-	turnover number of the main reaction
ρ	-	density

Index

app	-	apparent
f	-	final
in	-	inlet
inj	-	injected
L	-	liquid
max	-	maximum
min	-	minimum
o	-	initial
S	-	substrate
tot	-	total
w	-	water
- / +	-	just before / immediately after

Superscipts

*	-	saturation

Abbreviations

BR	-	batch reactor
BRP	-	BR with intermittent addition of enzyme solution
DG	-	D-glucose
DO	-	dissolved oxygen
kDG	-	2-keto-D-glucose
E	-	enzyme
NAD(P)H	-	nicotinamide adenine dinucleotide (phosphate)
NLP	-	nonlinear programming
P2Ox	-	pyranose oxidase
S	-	substrate
W	-	water

1. Introduction

A substantial progress has been achieved over the last decades in the area of biochemistry and correspondingly in biochemical engineering, beside improvements in the synthetic biotechnology leading to the production of modified enzymes of improved characteristics for a target process. All these have made simple enzymatic routes a real competitor against classical chemical synthesis complex pathways. There are currently over 5000 known enzymes and a large number of reactions catalyzed by these enzymes [1]. Their high selectivity and specificity have made them very attractive for different practical applications in several domains such as: pharmaceutical, detergent, textile industry, food processing, manufacture of bio-sensors, medical tests, etc. Unfortunately, under 5% of the known enzymes are used in significant technical and therapeutic applications [2]. New efforts are invested in protein engineering coupled with bioactive nanostructure fabrication to overcome most of the difficulties related to the use of biocatalysts at an industrial level.

A prerequisite for any successful industrial process is rational design and development. Therefore, when developing a new and improved enzymatic process, at an industrial level, one crucial engineering problem concerns determination of the optimal design and operation alternatives that extremise a performance index, called „cost function" in financial or engineering units [3,4,5]. The solution for such an optimization problem is difficult to be found knowing that the optimal operating policy depends not only on the reactor type, whether it is fluid mixing in batch reactors (BR) vs. plug flow in fixed-bed columns, or batch/semi-batch vs. continuous reactor, but also on the different enzymatic process characteristics, such as kinetics, range of operating parameters, raw-material purity, enzyme activity and stability domains, inactivation type and rate, interactions among enzymes for a multienzymatic process, etc. Even if a process kinetic model is available, the task is still challenging due to the presence of multiple and often contrary objectives, technological constraints, and an important degree of uncertainty of the model, parameters, constraints, raw-material quality, and enzyme quality [3,6]. Therefore, this analysis can be much better performed in a systematic way that allows model and reactor optimal operating policy updates, and by employing multi-objective performance criteria.

The reactor choice and optimization is even more laborious for multienzyme systems due to complex interaction among enzymatic reactions. Besides, usually the enzymes display different optimal activity domains of temperature and pH.

All of the discussed aspects have been taken into account when deriving the optimal operating policy of the selected reactor. Recently, Maria [7] proposed a systematic computing methodology based on a modular simulation platform that

is capable of optimizing and comparing the performances of the main types of enzymatic reactors for a given enzymatic process of known kinetics. This procedure can help in determining the level of enzyme stability that makes an alternative to be economically preferred. By using ideal reactor models, that include simple batch (BR), batch with intermittent addition of enzyme following certain addition policy (BRP), semi-batch with optimal enzyme continuous feeding policy (SBR), fixed-bed (FXBR), or mechanically agitated continuous reactors (MACR) with immobilized enzyme on a suitable porous support, this approach demonstrated that, the right choice of the operating alternative, can lead to significant savings in the enzyme consumption, by preserving an imposed conversion and reactor productivity [7].

This paper is aiming at investigating some alternatives to operate free-enzyme industrial BR/BRP for a given bi-enzymatic process of known kinetics in order to highlight how such an approach can lead to reduce both enzyme consumption. The examined process is the enzymatic oxidation of D-glucose (DG) to 2-keto-D-glucose (kDG) in the presence of pyranose 2-oxidase (P2Ox) and catalase.

2. Enzymatic process description

This paper is focused on studying the scale-up possibility for the enzymatic oxidation of β-D-glucose (DG) in 2-keto-D-glucose (kDG) in the presence if P2Ox (pyranose 2-oxidase) and catalase (Fig. 1). Generally P2Ox catalyzes oxidation of mono-and disacharides, especially at C-2 position leading to 2-keto-"sugars" [8].

This approached reaction is of particular importance in the sweeteners industry and for the production of some monosacharides derivates, being the first step of the Cetus technology for production of high purity fructose in two enzymatic steps [9] as displayed in Fig. 1.

The first step is the oxidation of DG to kDG using P2Ox at 25-30°C and pH=6-7. Being an enzymatic reaction, it displays high conversion and selectivity, leading to a product free of allergenic compound traces (such as aldoses). The second step of the Cetus technology is the kDG enzymatic hydrogenation to D-fructose using NAD(P)H proton donor (dependent on aldose reductase ALR) at 25°C and pH=7. This process is not currently an alternative for the production of fructose at a large scale, although it presents several advantages: mild operation conditions, high enzyme activity, high product purity. The process drawbacks are mainly related to the fast deactivating P2Ox, and to the costly regeneration of $NAD(P)^+$. To prolong P2Ox life, catalase (EC 1.11.16 from bovine liver) has been added in large [catalase]/[P2Ox] ratios (up to 300/1-1000/1 U/U) to decompose the reaction byproduct H_2O_2 (Leitner et al., 1998; Maria et al., 2012).

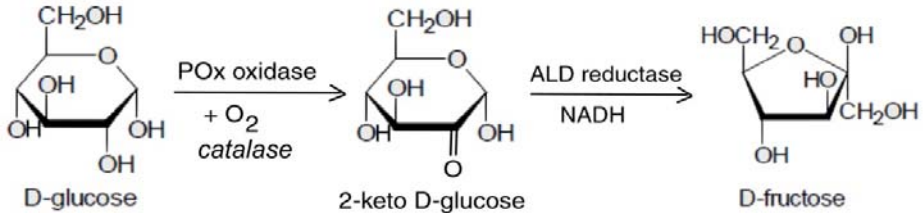

Fig 1. The Cetus technology for producing high purity fructose (adapted from Treitz et al., 2001[10])

For the investigated temperature of 30°C (optimal for P2Ox activity), Maria [8] proposed a kinetic model accounting for three main reactions (Table 1), with the estimated rate constants correlated with the catalase / P2Ox ratio.

Table 1

The kinetic model of Ene & Maria [12] for D-glucose oxidation using P2Ox, and for H_2O_2 decomposition using catalase[(a)]. Initial conditions: $[DG]_0$ = 100 mM; $[P2Ox]_0$ = 0.25 U mL^{-1}, pH= 6.5, temperature of 30°C, [Catalase]/[P2Ox]= 0-300 U U^{-1}. Notations: DG = D-glucose; kDG = 2-keto-D-glucose; DO = dissolved oxygen.

Reactions	Rate expressions
$DG + O_2 \xrightarrow[(water)]{P2Ox} kDG + H_2O_2$ $Y_{P2Ox} P2Ox + H_2O_2 \xrightarrow[(water)]{Fe\ traces} P2Oxox\ \ (inactive)$ $H_2O_2 \xrightarrow[(water)]{catalase} H_2O + 0.5O_2$	$r_{our} = \frac{\mu_m c_{P2Ox} c_{DG} c_{DO}}{K_{DG} c_{DO} + K_{DO} c_{DG} + c_{DG} c_{DO}}$ $r_d = k_d c_{P2Ox} c_{H_2O_2}$ $r_c = \frac{k_c c_{catalase} c_{H_2O_2}}{1 + K_{OH} c_{H_2O_2}^n}$
Mass balance equations in batch operation mode	**Operating parameters**
$\frac{dc_{DO}}{dt} = k_{oxl} a (c_{DO}^* - c_{DO}) - Y_{ox} r_{our} + 0.5\, r_c - D\, c_{DO}$ $\frac{dc_{DG}}{dt} = -r_{our} - D\, c_{DG}$; $\frac{dc_{kDG}}{dt} = r_{our} - D\, c_{kDG}$ $\frac{dc_{POx}}{dt} = -Y_{P2Ox} r_d - D\, c_{P2Ox}$ $\frac{dc_{H_2O_2}}{dt} = r_{our} - r_d - r_c - D\, c_{H_2O_2}$; $\frac{dV}{dt} = D\, V$	Content dilution: $D = \frac{1}{V}\frac{dV}{dt} = \frac{d[H_2O]_r}{dt}\frac{M_w}{\rho_w}$; M_w = 18 g mol^{-1}; ρ_w = 996 (30°C) g L^{-1} c_{DO}^* = 0.2484 mM (fed air, 30°C)

Parameter	°C	[Catalase]/[P2Ox] (U U^{-1})		
		0	100	300
μ_m , [mM s^{-1} (U mL^{-1})$^{-1}$]	30	0.988	0.369	0.049
K_{DG} , (mM)	30	0.519		
K_{DO} , (mM)	30	14.545		
k_d , [s^{-1} (U mL^{-1})$^{-1}$]	30	$1.42 \cdot 10^{-3}$	$6.07 \cdot 10^{-4}$	$1.15 \cdot 10^{-4}$
k_c , [s^{-1} (U mL^{-1})$^{-1}$]	30	-	$5.55 \cdot 10^{-5}$	$(10^{-4})^{(c)}$
K_{OH} , (mM^{-n})	30	-	0.58	

n	30	-	2.58
Y_{P2Ox} , (U mL^{-1} mM^{-1})	30	0.01019	
$k_{oxl}a$, (s^{-1}) (b)	30	0.01-0.02	

(a) recombinant P2Ox from *Coriolus sp.* expressed in *E. Coli*, EC 1.1.3.10, product number P4234 Sigma – Aldrich, activity of 10.4 U mg-protein^{-1}; Catalase EC 1.11.16 from bovine liver, product number C1345 Sigma – Aldrich, activity of 2860 U mg-protein^{-1};
(b) approximated from separate in-vitro tests;
(c) low confidence parameter.

This model assumes that DG oxidation to kDG follows a Ping-Pong-Bi-Bi kinetics, P2Ox deactivation by H_2O_2, and the H_2O_2 decomposition by catalase of a Yano-Koya kinetics. In further calculations, no significant change in rate constants by the co-immobilization of P2Ox and catalase on porous gel beads will be assumed as reported by some literature results [11].

3. Enzymatic reactor type and mathematical models

The simulation platform that screens among reactors includes a library of individual ideal reactor models of general structure [7] that can be used for every new application. In the present case study, a model-based analysis of process performance has been made when two reactor types have been used: BR and BRP.

The most commonly used reactor for slow processes in multi-purpose and multi-product plants is the batch reactor. This type of reactor is mainly employed because it exhibits a high flexibility and an easy operation. The design of perfectly mixed isothermal reactor has been considered for the following two operation alternatives:

(i) Batch operation with initial addition of enzymes (BR), in this case P2Ox and catalase. The reactor model presented in Table 2 shows that the species concentration dynamics is determined by integrating the differential mass balance equations, starting from the initial load of substrate and enzymes. Considering the process that takes place in the reactor, a dilution term (D) is also considered by accounting for the small amount of water resulting from the hydrogen peroxide in-situ decomposition, and also due to the variation of reactor liquid volume occurring during the batch due to enzyme solution addition [13].

(ii) Batch operation with intermittent addition of enzyme solution (BRP) of volumes $V_{inj,u}$ and given concentration $c_{E,inj,u}$ over N_{inj} uniformly distributed addition times $t_{inj,u}$ over the batch (u=1,... N_{inj}). The BRP model presented in Table 3 is the same as those of the BR model, the only difference being the enzyme addition times

$t_{inj,u}$. Therefore, species concentrations have to be calculated by using the solution mixing balance equations of Table 3 after each enzyme solution addition. Because the enzyme solution is added over the entire batch, every injected volume $V_{inj,u}$ is thus determined. Calculation of the injected volumes is made for the alternative of an uniform addition policy only (see Maria & Crisan [13] for other operation alternatives). Determination of $\{V_{inj,u}, t_{inj,u}\}$ requires the knowledge of final batch time t_f , number of injections N_{inj} , and the total injected volume $V_{inj,tot}$ (usually taken as 10% V_0 in industrial practice to not dilute too much the reactor content) [7].

Table 2

Batch reactor model [8,14]

Mass balance equations	Observations
BR – Batch reactor (isothermal, homogeneous, perfectly mixed): $\frac{dc_{DO}}{dt} = k_{oxl}a\left(c^*_{DO} - c_{DO}\right) + r_{DO} - D\ c_{DO}$ $\frac{dc_j}{dt} = r_j - D\ c_j$, j = species DG (S), kDG, P2Ox (E), H_2O_2 $D = \frac{1}{V}\frac{dV}{dt}$ (diluting water from reactions)	Added enzyme solution of maximum $0.1\,V_o$ at $t = 0$. At $t = 0_+$, $c_j = c_{j,o}$. $D = \frac{d[H_2O]_r}{dt}\frac{M_w}{\rho_w}$

Notations: M_w = water molecular weight; ρ_w = water density; c^*_{DO} = DO saturation concentration; $[H_2O]_r$ = water resulted from H_2O_2 decomposition.

Table 3

Batch reactor model with intermittent addition of P2Ox (E) solution at times $t_{inj,u}$ [8,14]

Mass balance equations	Observations
Species mass balance ibidem to BR. Mass balance after each enzyme addition at times $t = t_{inj,u}$: $\left.\frac{V}{V_o}\right\|_{t_{inj,j+}} = 1 + \sum_{u=1}^{i}\frac{V_{inj,u}}{V_o}$; $0 \le t_{inj,u} \le t_f$, $u = 1,...N_{inj}$ $c_j(t_{inj,u+}) = \left(1 - \frac{V_{inj,i}/V_o}{V/V_o}\right)c_j(t_{inj,u-}) + \frac{V_{inj,i}/V_o}{V/V_o}c_{j,inj}(t_{inj,u})$, $\Delta t_{inj,u} = t_{inj,u+1} - t_{inj,u} = t_f / N_{inj}$, $u = 1,...,(N_{inj}-1)$ Checked enzyme addition policies: Uniform addition policy, $V_{inj,u}/V_o$, $u = 1,...,N_{inj}$: $\frac{V_{inj,u}}{V_o} = \frac{V_{inj,tot}}{V_o}\frac{1}{N_{inj}}$; $t_{inj,u} = \frac{t_f}{N_{inj}}(u-1)$	At $t = 0$, $c_j = c_{j,o}$, $V = V_o$; $V_{inj,u}/V_o$ = enzyme solution added volumes, $u = 1,...,N_{inj}$ (no. of injections); Δt_{inj} = constant. Constraints: $N_{inj} > 1$; $c_{E,inj,u}$ = constant (given);

	$\sum_{u=1}^{N_{inj}} V_{inj,u} = V_{inj,tot} = 0.1\ V_o$

Notations: $t_{-/+}$ = just before/immediately after time t; t_f = final batch time; subscripts: inj= injected; in= inlet; o= initial; catalase is already loaded at initial reaction time. Notations: M_w = water molecular weight; ρ_w = water density; c_{DO}^* = DO saturation concentration; $[H_2O]_r$ = water resulted from H_2O_2 decomposition.

4. Derivation of optimal operating policies for the approached enzymatic reactors

The next step in the analysis, after selecting the two reactor models to be checked (BR and BRP here) and after including the process kinetic model with the reaction conditions, is to formulate an objective for reactor optimization which has to be solved. In general, optimization of a bioreactor implies derivation of operating conditions ensuring maximization of an economic /performance criterion (such as conversion, yield, productivity, benefit, operating time, utilities, etc.) in the presence of technological and safety constraints [7]. Optimization problem formulation: minimization of the amount of enzymes (P2Ox and catalase) that ensures an imposed reactor productivity and conversion specified in Table 4. To compare the process efficiency in terms of enzyme consumption for the different reactor operation alternatives certain nominal operating conditions have to be imposed for all alternatives (Table 4).

The optimal operating policies are determined using a random search MMA optimization procedure [15]. The rate constants for various [Catalase]/[P2Ox] tried ratios have been interpolated using the known values of Table 1. In the case of batch operation, on which this study is focused, the optimum cost function is actually the minimum amount of added enzyme (P2Ox and catalase) that is necessary to obtain a specified conversion over the imposed batch time for an imposed reactor capacity.

Table 4

Nominal operating conditions and pilot reactor characteristics for the D-glucose oxidation to 2-keto-D-glucose using P2Ox and catalase, corresponding to a capacity of 360t DG year^{-1} [(a)].

Operating conditions	Value
Reactor volume (liquid, or liquid with solid, V_0)	15 m^3
Temperature / pressure / pH (buffer solution)	30°C / normal / 6.5
Reaction (batch) time t_f	10 hrs.
Maximum volumetric dilution of the reactor content due to enzyme addition	10% V_0
Initial concentrations of species[(b)]	$c_{DG,o}$ = 0.25 M;

	$c_{P2Ox,o}$ and $c_{Catalase,o}$ are to be optimized
P2Ox concentration in the feed	to be established by optimization from matching the imposed problem constraints(c)
Catalase / P2Ox ratio in the reaction environment(c)	to be established by optimization from matching the imposed problem constraints(c)
Number of enzyme additions over the batch time (N_{inj})	1; 20
Imposed DG conversion	99.00 %

(a) Physical liquid properties correspond to a solution of 0.25 M D-glucose: aprox. water density, viscosity μ = 1.02 cP, diffusivity of glucose and H_2O_2 respectively, in water $D_{DG,L}$ = 3×10^{-11} m^2 s^{-1} [16], $D_{H_2O_2,L} = 14\times10^{-10}$ m^2 s^{-1} [17];

(b) Higher concentrations of DG are possible, but have not been checked due to the kinetic model limited validity range and due to oxygen transport rate limitations (see also Leitner et al., [9], for an extensive discussion on oxygen transport limitations vs. used DG initial concentration);

(c) P2Ox reported activity of ca. 10.4 U mg-protein^{-1} (recombinant P2Ox from Sigma-Aldrich from *Coriolus sp.* expressed in *E. Coli*), while the free P2Ox maximum concentration is of 70 U mL^{-1} (ca. 7 mg mL^{-1}) [9], with a recommended running value of ca. 0.5-5 U mL^{-1} [18]. The recommended free catalase concentration in the reaction environment is of maximum 5000 U mL^{-1} [1]. The running enzyme concentrations in the bioreactor usually must not overpass the Catalase/P2Ox = 300/1 U/U ratio [8,12], even if co-immobilized catalase/P2Ox ratios of 30/1–1000/1 U U^{-1} are also reported [8,19]. The problem constraints are: DG conversion of 0.99; 10 hrs. reaction time, imposed reactor productivity.

Table 5

Comparison between operating alternatives for D-glucose oxidation using suspended P2Ox and catalase on porous alginate beads, in various enzymatic reactor capacity of 360 t DG year^{-1}, under nominal conditions of Table 2. The imposed conditions correspond to an imposed (constant) output conversion of 99.00% over 10 hrs reaction time in the reactor (maximum liquid dilution of 10%).

Reactor	*operating policy*	*[Catalase] (U mL^{-1})*	*[P2Ox] in fed solution (U mL^{-1}) (a)*	*[P2Ox] range in the reactor (U mL^{-1})*	*Inactivation (%) (loaded m_{P2Ox})*
BR	Initial addition of P2Ox (N_{inj} =1)	0	27.5	0-3	**89**
		40	59.5	0-5	**39**
BRP (N_{inj}=20 injections)	Uniform (equal P2Ox injected quantities)	0	53.0	0-3	**5**
		40	158.5	0-13	**2**

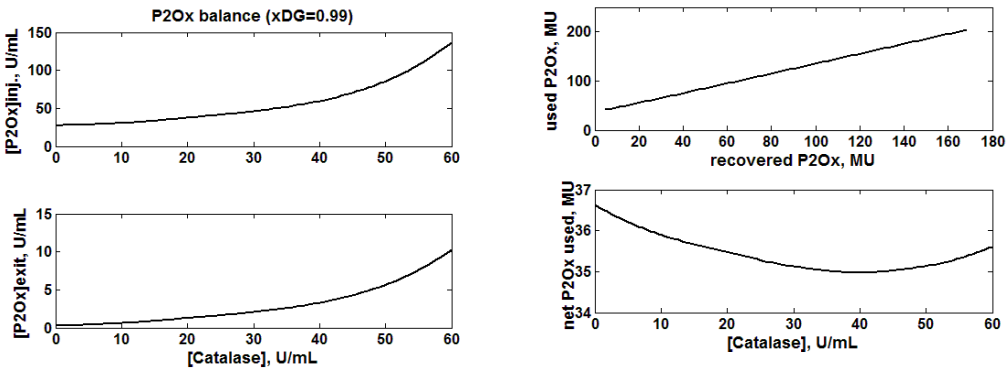

Fig. 2. Total and net consumption of P2Ox *in the BR* for a final imposed DG-conversion of 99.00% over 10 h runtime. Nominal conditions correspond to [DG]o = 0.25 M, 30°C, pH = 6.5, maximum volume dilution of 10%V_o.

The results of the applied optimization rule for the two batch reactor operation alternatives (BR and BRP with uniform enzyme addition over the batch) are presented in Table 5. The results indicate the uniform P2Ox addition in a BRP with initial [catalase] = 40 U/mL as being the optimal alternative, because of several reasons:

- the net P2Ox consumption is minimum for the minimum catalase consumption, that is inlet [catalase] = 40 U/mL as revealed by Fig. 2 and 4.
- The [P2Ox] is quasi-uniform over the batch as revealed by Fig. 3 and 5, thus ensuring a good enzyme utilization.
- The P2Ox net consumption is minimum, that is 158.5 x 0.02 = 3.2 U/ mL vs. 27.5 x 0.89 = 24.5 U/ mL for the BR case. Beside, it is to observe that the remaining P2Ox could be easily separated after the batch and reused over several cycles.

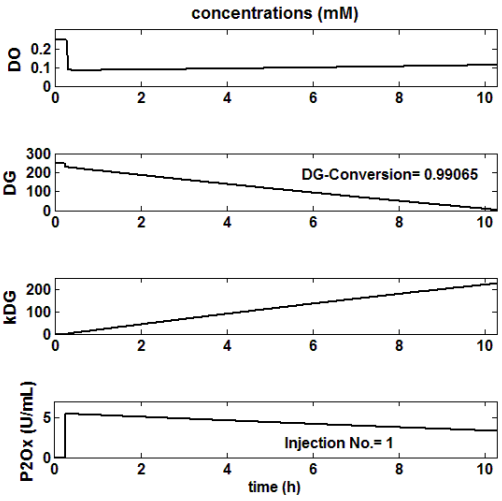

Fig. 3. Key species concentration dynamics and production rate *in the simple batch reactor BR* for a final imposed DG-conversion of 99.00% over 10 h runtime. The setpoint at 30°C corresponds to optimum $[P2Ox]_{inj}$ = 59.5 U mL^{-1}, [Catalase] = 40 U mL^{-1}.

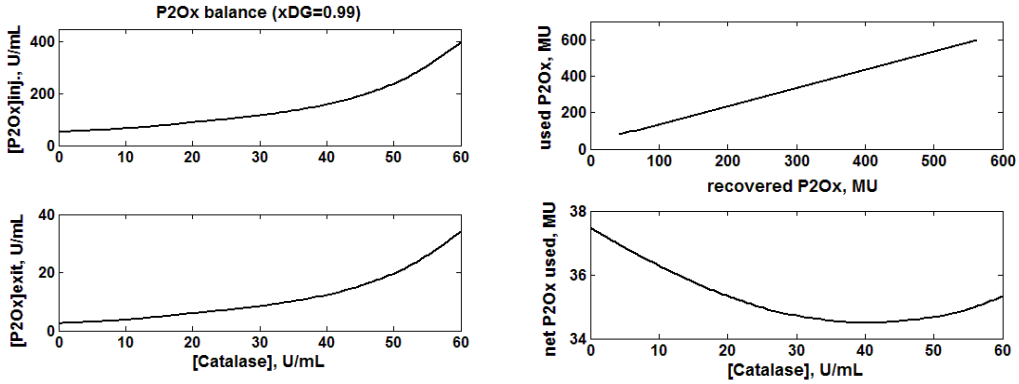

Fig. 4. Total and net P2Ox consumption *in the batch reactor BRP* for an intermittent and *uniform P2Ox enzyme solution addition policy*, and for a final imposed DG-conversion of 99.00% over 10 h runtime. Nominal conditions correspond to [DG]o = 0.25 M, 30°C, pH = 6.5, maximum volume dilution of 10%V_o.

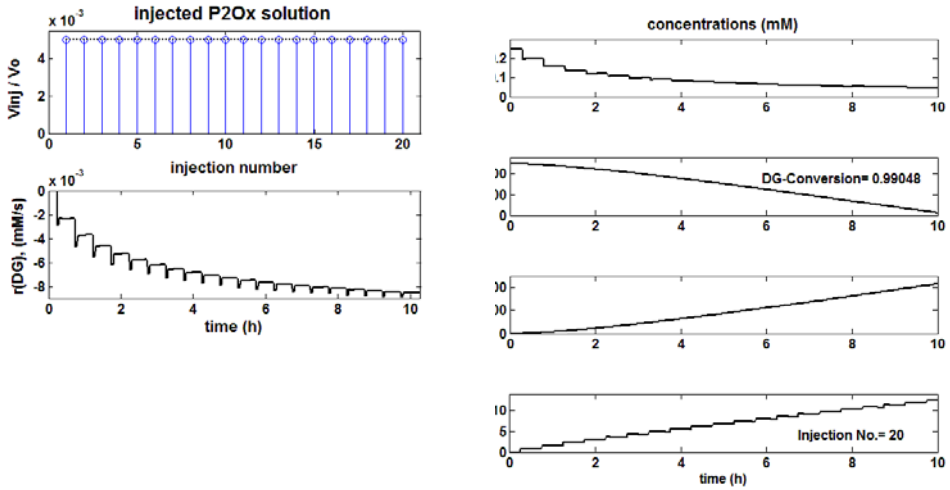

Fig. 5. Key species concentration dynamics and production rate *in the batch reactor BRP* for an intermittent and *uniform P2Ox enzyme solution addition policy* and a final imposed DG-conversion of 99.00% over 10 h runtime. The setpoint at 30°C corresponds to optimum $[P2Ox]_{inj}$ = 158.5 U mL^{-1}, [Catalase] = 40 U mL^{-1}.

5. Conclusions

The approached bi-enzymatic case study reveals the large possibilities offered by such a modular reactor simulation platform in assessing the operating alternatives for various enzymatic reactors, if the process kinetics and enzyme deactivation characteristics are specified. Such an approach allows selecting the best reactor type and operating policy for a defined case study based on consistently formulated economic objectives, but also checking the reactor performance when using different type of biocatalysts (from various sources) of specified activity, or checking the feasibility of increasing the reactor capacity by increasing the enzyme consumption and number of batches.

From a broader perspective, a decision on choosing the suitable enzymatic reactor type (BRP, SBR, MACR) for an imposed productivity depends on a large number of variables, which should be considered in a global analysis of the process efficiency. This economic assessment has to include not only reducing the production costs related to raw-materials and enzyme consumption, but also costs related to investment and reactor operation, implementation of a sophisticated control system to maintain the desired operating policy, costs of accidentally lost batches (with a known frequency), all being connected to the market requirement and product value.

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