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Dynamic model for isopropanol production by *Cupriavidus necator*

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Abstract: The Hybrid Cybernetic Model (HCM) enables the simulation of metabolic fluxes by using Elementary Modes Analysis and taking into account of selected cellular regulations. These latter are represented by cybernetic control variables. In this study, a simplified metabolic network was established in order to isolate a subset of Elementary Modes, representative of the main phenotypic capabilities of the microorganism. An innovative classification of the modes was introduced in the dynamic model, which permitted the selection of the active modes based on the microbial kinetics. The case study presented here is a genetically modified strain of *Cupriavidus necator*, engineered to produce isopropanol. Available experimental data were used for identification of parameters in the dynamic model. This model can be used in order to predict the value of maximal and minimal product yields when other substrates will be tested.

***Keywords**:* Hybrid Cybernetic Model (HCM), Elementary Modes (EMs), Yield Analysis, Isopropanol production, Biofuel.

1. INTRODUCTION

This new century presents crucial environmental challenges such as decreased water supplies, global warming and limited fossil fuels. Carbon dioxide (CO_2) emissions and fossil fuel usage for transportation are closely connected with greenhouse effects.

Currently, ethanol is one of the two main biofuels used in Europe. However, it presents numerous technical problems: it has a lower energy content than gasoline, is corrosive towards ferrous metals, and is difficult to transport across traditional pipelines because it degrades elastomers and flexible transfer lines in fuel systems (Bruno *et al.* 2009). These problems could be vanquished by the adoption of higher alcohols as biofuels, since they are compatible with storage and transportation infrastructures and have higher energy content than ethanol.

Among these higher alcohols, isopropanol is noteworthy because it has a very high research octane number (129) and is already being used as a gasoline and diesel additive (Peralta-Yahya and Keasling 2010). Moreover, isopropanol can be dehydrated to form propylene which is a petroleum-based product (Inokuma *et al.* 2010). Propylene is currently used as a material in many industrial products and it is expected that the world demand for propylene will continue to increase in the future (Molenda 2004).

Several microorganisms have been evaluated for isopropanol production. This work focuses on the genetically modified bacterium *C. necator*. This prokaryotic organism, also known as *Ralstonia eutropha,* is able to grow heterotrophically on multiple carbon sources and autotrophically on carbon dioxide. This microorganism is a natural producer of biopolymers (polyhydroxyalkanoates or PHAs) and the carbon flux towards PHAs can be diverted towards isopropanol after genetic modifications (Grousseau *et al.* 2014).

Since it is the first time that *C. necator* has been engineered to produce isopropanol, it is interesting to understand and quantitatively predict the phenotypic capabilities associated with the genetic modifications. Mathematical modeling enables one to assess the behaviour of a system by capturing its salient features (Song *et al.* 2013). In this way, it is possible under certain conditions to obtain predictive modeling results regarding the production system.

This paper focuses on dynamic modeling, which aims to capture the temporal evolution of a system. Dynamic optimization of a biological process needs to use robust model components, with known parameters (i.e. yields,

growth constants, etc.). The first step was to establish a mass balance model of the process. Furthermore, the mass balance model needs information on the biological kinetics via a yield matrix or stoichiometric matrix. The approach for estimating the latter was based on a metabolic model and the use of a reduced set of Elementary Modes (EMs) (Provost *et al.* 2007 ; Provost and Bastin 2004 ; Provost *et al.* 2006). An Elementary Mode is a set of non-decomposable pathways consisting of a minimal set of reactions that functions at steady state (Schuster *et al.* 2002). Elementary Mode Analysis has been used for: interpreting metabolic function networks, predicting gene expression patterns, and improving strain performance (Trinh *et al.* 2009).

However, a striking simplification of the metabolic model is required. This can be achieved by introducing the quasi-steady-state approximation for intracellular metabolites. Intracellular reactions usually show smaller time constants than extracellular reactions (Song *et al.* 2009). Thus, only parameters relative to external metabolites are considered, which constitute the basic postulate of Hybrid Cybernetic Model (HCM). However, the number of parameters remains important because it is directly linked to the number of Elementary Modes. The Elementary Modes Analysis shows a combinatorial explosion of the number of EMs (Schuster *et al.* 2002), which necessitates a step of metabolic network reduction.

To overcome the problems addressed above, this work first proposes a rational simplification of the metabolic network of *C. necator*. Secondly, the reduction of the set of modes was carried out using Yield Analysis (Song and Ramkrishna 2009) and the innovative modes classification based on the microbial kinetics of the available culture. Finally, model parameters were identified in order to affect a dynamic model, which enables a more realistic, mechanism-based simulation of cellular reactions. The advantage of using a reliable metabolic model of this engineered bacterium is to not only reduce the time, cost, and effort in experimental work, but also to find a breakthrough strategy for exceeding the existing limitations in the current biofuel production.

2. MATERIAL AND METHODS

2.1 Experimental data

C. necator Re2133 (Budde *et al.* 2011) was used as the parent strain for isopropanol production since the genes coding for the synthesis of PHB from acetoacetyl-CoA (*phaB1B2B3* and *phaC*) were deleted from the wild type strain H16. An inducible isopropanol production plasmid was constructed and incorporated in Re2133 resulting in the strain Re2133/pEG7c (Grousseau *et al.* 2014).

Re2133/pEG7c was cultivated in a flask (100 mL in 1 L flask). The minimal medium used in this study was previously described by Lu *et al.* (2012) with addition of gentamycin (10 mg/L) and kanamycin (100 mg/L). Concentrations of 20 g/L for fructose and 0.38 g/L for NH_4Cl were used as carbon and nitrogen sources respectively. The flask cultures were continuously shaken in a 30°C incubator at 200 RPM. Isopropanol production was induced with L-Arabinose (0.1%) at 9 h of cultivation time (Grousseau *et al.* 2014).

The residual substrate and product concentrations were quantified by High Performance Liquid Chromatography (HPLC). Biomass growth was monitored by measuring the optical density at 600 nm (OD_{600nm}) using a visible spectrophotometer. Culture results are detailed in Grousseau *et al.* (2014).

The case study was a pure and mono-substrate batch culture ; experimental data are presented in Fig. 1. The culture can be divided into three phases: phase 0 (P0) of latency ; phase I (PI) where fructose, the carbon source, is converted to biomass and isopropanol simultaneously ; and phase II (PII) where fructose was still being consumed while the nitrogen source (Ammonium Chloride) was exhausted and therefore no new cells were produced. During the phase II, isopropanol was produced concomitantly with a low acetone excretion. In this study, the phase 0 (P0) was not taken into account and, will be subject of future work.

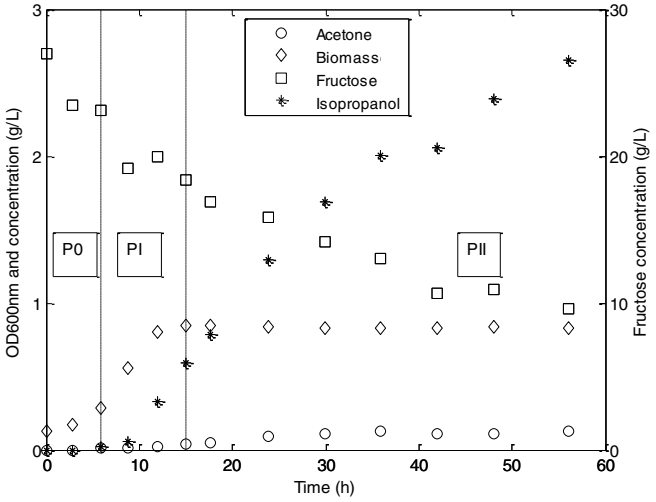

Fig. 1. Experimental data (fructose, biomass, isopropanol, and acetone): (P0) latency, (PI) growth and isopropanol production, and (PII) isopropanol and acetone production.

2.2 Metabolic Network

In this study, the metabolic network of Franz *et al.* (2011) was used as an initial network, which included a fructose uptake pathway, glycolysis, gluconeogenesis, a detailed pentose-phosphate-pathway, the tricarboxylic acid cycle, and a PHB production and consumption pathways.

Some characteristics of *C. necator*, which were not considered by Franz *et al.* (2011), were considered because they are necessary in order to implement complementary metabolic pathways. The existence of the enzyme named gluconate 6-phosphate dehydrogenase, which metabolizes "Glu6P" into "RI5P" (Table 1) has not been proven (Gottschalk *et al.* 1964). Thus, fructose is hypothesized to be catabolized via the Entner-Doudoroff pathway (Schlegel and Eberhardt 1972), which was added to the metabolic network by Grousseau *et al.* (2013). PHB production pathway was

deleted and replaced by the isopropanol production pathway, which has the same precursor as the production of PHB.

Altogether, the network contains 48 metabolites (39 internal and 9 external species) and 40 reactions. All reaction equations are listed in Table 1. Note that stoichiometry coefficients are given in units of *mmol*, except for the biomass which is given in units of *g*.

2.3 Mass Balance Model

The culture was carried out in batch mode. The mass balance model can be written according to the equation system (1):

$$\begin{cases} \dfrac{dB}{dt} = \mu \cdot B \\ \dfrac{dS}{dt} = -N_S \cdot r \cdot B \\ \dfrac{dP}{dt} = N_P \cdot r \cdot B \\ \dfrac{dC}{dt} = N_C \cdot r - \mu \cdot B \end{cases} \quad (1)$$

where B, S and P represent respectively the biomass, substrate and products; C is the intracellular metabolites vector; μ and r (dim = $n_C \times 1$) represent the constant specific growth rate and the intracellular specific reaction rates. The matrix N_S, N_P and N_C are the stoichiometric matrix of the metabolic network for the substrate, the products and the internal metabolites (i.e. metabolic pathway of isopropanol production) with dimensions $n_S \times n_r$, $n_P \times n_r$, and $n_C \times n_r$. If a quasi-steady-state for the intracellular metabolites is assumed (Stephanopoulos *et al.* 1998; Stephanopoulos 1999):

$$\frac{dC}{dt} = N_C \cdot r - \mu \cdot B \approx 0, \quad (2)$$

furthermore $\mu \cdot C \ll N_C \cdot r$, then $N_C \cdot r = 0$.

Having thermodynamic constraints on r, convex algebra can be used. The intracellular specific reaction rates can be expressed as a non-negative linear combination of the elementary vectors e_k:

$$r = \lambda_1 e_1 + \lambda_2 e_2 + \ldots + \lambda_k e_k \text{ with } \lambda_k \geq 0. \quad (3)$$

Finally, by defining a stoichiometric matrix K like $K = \left[-\frac{N_S}{N_P}\right].E$, where E is the reduced matrix of Elementary Modes (Provost *et al.* 2007; Provost and Bastin 2004; Provost *et al.* 2006). The classical dynamic model (for the substrate and products) can be represented as a function of metabolic flux as follows:

$$\frac{d}{dt}\left[\frac{S}{P}\right] = K.r_M.B \quad (4)$$

After determining the Elementary Modes matrix E_0 and using the equation (4), the equation (5) is obtained:

$$\frac{d}{dt}\left[\frac{S}{P}\right] = \left[-\frac{N_S}{N_P}\right].E_0.r_M.B \quad (5)$$

External metabolites are decomposed in $S = [FRU\ AMC\ O_2]^T$ and $P = [SUCx\ Form\ CO_2\ BIOM\ ISOP\ ACETONEx]^T$. r_M represents the vector of fluxes.

In this work, the E_o matrix will be reduced by using yield analysis. It is assumed in this study that this matrix is normalized with respect to a reference substrate.

2.4 Cybernetic variables

The Hybrid Cybernetic Model (HCM) aims to take into account, metabolic regulations (Song *et al.* 2009). Thus, the vector of fluxes r_{M_j} and the vector of inducible enzyme synthesis rates r_{ME_j} through EMs are controlled by the cybernetic variables $v_{M,j}$ and $u_{M,j}$ respectively. $v_{M,j}$ controls the enzyme activity and $u_{M,j}$ controls the enzyme level.

$$r_{M_j} = v_{M,j}.e_{M,j}^{rel}.r_{M_j}^{kin}, \quad (6)$$

$$r_{ME_j} = u_{M,j}.r_{ME_j}^{kin}, \quad (7)$$

r_{M_j} is catalysed by a vector of key enzyme $e_{M,j}$ determined from the following dynamic equation:

$$\frac{de_{M,j}}{dt} = \alpha_{M,j} + r_{ME_j} - \beta_{M,j}.e_{M,j} - \mu.e_{M,j}, \quad (8)$$

where the four terms on the right hand side represent: the vector of constitutive enzyme synthesis $\alpha_{M,j}$, the vector of inducible enzyme synthesis rates r_{ME_j}, the term $\beta_{M,j}.e_{M,j}$ represents the enzyme degradation, and the term $\mu.e_{M,j}$ represents the dilution rate induced by growth.

$e_{M,j}^{rel}$ is the enzyme level relative to their maximum value $e_{M,j}^{max}$ expressed as follows:

$$e_{M,j}^{rel} = \frac{e_{M,j}}{e_{M,j}^{max}} \text{ with } e_{M,j}^{max} = \frac{\alpha_{M,j} + k_{E,j}}{\beta_{M,j} + \mu_j^{max}}, \quad (9)$$

where $\mu_j^{max} = R_{\frac{Biom}{FRU},j}.k_j^{max}$ is the maximal growth rate of the j^{th} mode in which $R_{\frac{Biom}{FRU},j}$ represents the yield of biomass of the j^{th} mode.

A general form of the cybernetic control laws (Young and Ramkrishna 2007) $u_{M,j}$ and $v_{M,j}$ is given by:

$$u_j = \frac{p_j}{\sum p_k} \text{ and } v_j = \frac{p_j}{max(p_k)} \quad (10)$$

where p_j is the return on investment which can be calculated from a metabolic objective function. In this study, it was assumed that the organism maximized carbon source uptake and p_j have been defined (Song and Ramkrishna 2009) as:

$$p_j = f_{C,j}.e_{M,j}^{rel}.r_{M_j}^{kin} \quad (11)$$

where $f_{C,j}$ is the vector of uptaken carbon units.

2.5 Kinetic reactions

Kinetic equations relative to the vectors of unregulated rates $r_{M_j}^{kin}$ and $r_{ME_j}^{kin}$, follow a Michaelis-Menten formalism $S_1/(K_{a,j} + S_1)$. A term dedicated to the isopropanol inhibitor effect $K_{I,j}/(K_{I,j} + P_1)$ was included. Indeed, the toxicity of

this alcohol was presented in Nicolaou *et al.* (2010) and also proved experimentally (this work, data not shown). $K_{a,j}$ is the affinity constant and $K_{I,j}$ is the inhibition constant of the j^{th} mode.

$$r_{M_j}^{kin} = \begin{cases} k_j^{max} \cdot \frac{S_1}{K_{a,j}+S_1} \cdot \frac{K_{i,j}}{K_{I,j}+P_1} & \text{Phase I} \\ k_j^{max} & \text{Phase II} \end{cases}, \quad (12)$$

$$r_{M_{Ej}}^{kin} = \begin{cases} k_{E,j} \cdot \frac{S_1}{K_{a,j}+S_1} \cdot \frac{K_{i,j}}{K_{I,j}+P_1} & \text{Phase I} \\ k_{E,j} & \text{Phase II} \end{cases}, \quad (13)$$

where k_j^{max} is the rate constant and $k_{E,j}$ is the enzyme synthesis rate constant of the j^{th} mode.

Table 1. Reactions of metabolic network for isopropanol production (= and => mean reversible and irreversible reactions respectively)

v1 : FRU + PEP + ATP => F16P + PYR + ADP
v2 : F16P => F6P
v3 : F16P => 2 G3P
v4 : AMC => NH3
v5 : RI5P = R5P
v6 : RI5P = X5P
v7 : X5P + R5P = S7P + G3P
v8 : S7P + G3P = E4P + F6P
v9 : X5P + E4P = G3P + F6P
v10 : F6P => G6P
v11 : G3P + NAD + ADP = 3PG + NADH + ATP
v12 : 3PG = PEP
v13 : PEP + ADP => PYR + ATP
v14 : OXA + ATP => PEP + ADP + CO2
v15 : PYR => AcCoA + Form
v16 : PYR + NAD => AcCoA + NADH + CO2
v17 : AcCoA + OXA => ISC
v18 : ISC + NADP => AKG + NADPH + CO2
v19 : AKG + NAD => SucCoA + NADH + CO2
v20 : SucCoA + ADP = SUC + ATP
v21 : SUC + FAD = MAL + FADH
v22 : MAL + NAD => OXA + NADH
v23 : PYR + ATP => OXA + ADP
v24 : ISC => SUC + GOX
v25 : AcCoA + GOX => MAL
v26 : NH3 + AKG + NADPH => GLUT + NADP
v27 : GLUT + NH3 + ATP => GLUM + ADP
v28 : 2 AcCoA = AcAcCoA
v29 : AcAcCoA + SUC => CO2 + ACETONE + SucCoA
v30 : ACETONE => ACETONEx
v31 : SUC => SUCx
v32 : 2 NADH + O2 + 4 ADP => 2 NAD + 4 ATP
v33 : 2 FADH + O2 + 2 ADP => 2 FAD + 2 ATP
v34 : 0.21 G6P + 0.07 F6P + 0.9 R5P + 0.36 E4P + 0.13 G3P + 1.5 3PG + 0.52 PEP + 2.83 PYR + 3.74 AcCoA + 1.79 OXA + 8.32 GLUT + 0.25 GLUM + 41.1 ATP + 8.26 NADPH + 3.12 NAD => BIOM + 7.51 AKG + 2.61 CO2 + 41.1 ADP + 8.26 NADP + 3.12 NADH
v35 : G6P + NADP = GLU6P + NADPH
v36 : GLU6P + NADP = RI5P + CO2 + NADPH
v37 : GLU6P = KDG
v38 : KDG = PYR + G3P
v39 : ATP + RI5P + CO2 = ADP + 2 G3P
v40 : NADPH + ACETONE => NADP + ISOP

3. RESULTS AND DISCUSSION

3.1 Metabolic Yield Analysis

The stoichiometric matrix was obtained from the metabolic network. The next step was to calculate the set of Elementary Modes with the publicly program METATOOL 2005 (Kamp and Schuster 2006). A total of 865 EMs was obtained. As explained previously, the fluxes of Elementary Modes were normalized by the preferred substrate (fructose in this case), so that the yield space is a bounded convex hull in a two-dimensional space. Theoretically, the yield space vertices are supposed to span the whole range of phenotypes.

In this study, the EMs were classified into two groups, which correspond respectively to the two phases (PI) and (PII) identified in section 2.1. Since experimental data were available, it was possible to determine a reduced set of Elementary Modes. For each phase, yields of measured external metabolites (Table 2) were calculated by a linear regression. Yields are given in units of *mmol*/*mmol* except the biomass which is expressed in units of $g_{Biomass}/mmol$. The considered biomass formula was $C_1H_{1.77}O_{0.44}N_{0.25}$, 4% ashes, MW=25.35 *g*/*Cmole* (Aragao 1996).

Table 2. Yields and uncertainties of experimental data

Phase I		Phase II	
$R_{FRU,BIOM}$	$R_{FRU,ISOP}$	$R_{FRU,ACETONEx}$	$R_{FRU,ISOP}$
(*g*/*mmol*)	(*mmol*/*mmol*)	(*mmol*/*mmol*)	(*mmol*/*mmol*)
0.06±0.02	0.29±0.05	0.03±0.01	0.69±0.06

Thus, theoretical and experimental yields were located within the yield space. The selection of active modes (Elementary Modes used for the dynamic model), is not a straightforward task because several solutions are possible.

When the experimental yield is inside the convex hull, the phenotypic state can be expressed as a convex combination of the polygon vertices. In this work, the active modes chosen for best-enclosing the data are the vertices of a triangle. Actually, the triangle which possesses the largest area is calculated in order to maximize the phenotypic states taken into account around the experimental data.

As shown in Fig. 2, the measured experimental yields for the first phase (PI) are located within the convex hull bounded by the three selected active modes represented by black discs.

During the second phase (PII), among the available experimental data, only acetone and isopropanol are produced, which constituted the constraints for reducing the set of Elementary Modes. As previously done, experimental yields were calculated and positioned in the yield space. In order for the experimental data to be located within the convex hull, 3 boundary active modes were identified (Fig. 3).

Finally, in this work, active modes were selected in two stages ; a first reduction of the whole set based on the microbial kinetics of the culture and a second selection based on the experimental data. In the end, six active modes have been selected for the HCM.

3.2 The Hybrid Cybernetic Model (HCM)

The full HCM was described by the equations (5) to (13). After having selected the active modes, model parameters were identified. Indeed, experimental data of fructose, biomass, isopropanol, and acetone concentrations (11 data points) enabled calculation of the 13 kinetic parameters of the model. Since 2 phases and 6 active modes were identified, two parameters k_j^{max} and $k_{E,j}$ for characterizing each mode were identified. The inhibitor kinetic parameters $K_{I,j}$ were taken identical for every mode and the resulting K_I was also identified. The other parameter values $\alpha_{M,j}$ and $\beta_{M,j}$ were taken from Song *et al.* (2009) and $K_{a,j}$ was taken from Franz *et al.* (2011) (Table 3). $\alpha_{M,j}$, $\beta_{M,j}$, and $K_{a,j}$ were assumed to

be identically for every mode and then named α, β, and K_a respectively.

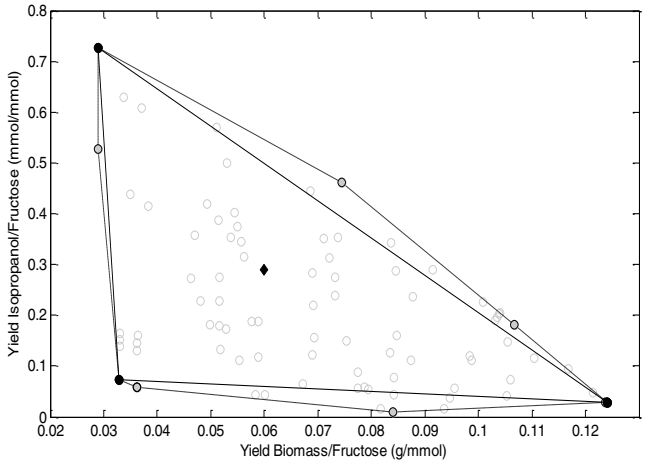

Fig. 2. Phase I: Selection of active modes in the yield space for the establishment of the dynamic model. Here are shown: the convex hull (- -), the experimental data (♦), Elementary Modes (○), Active Modes (●), and the largest area triangle (-).

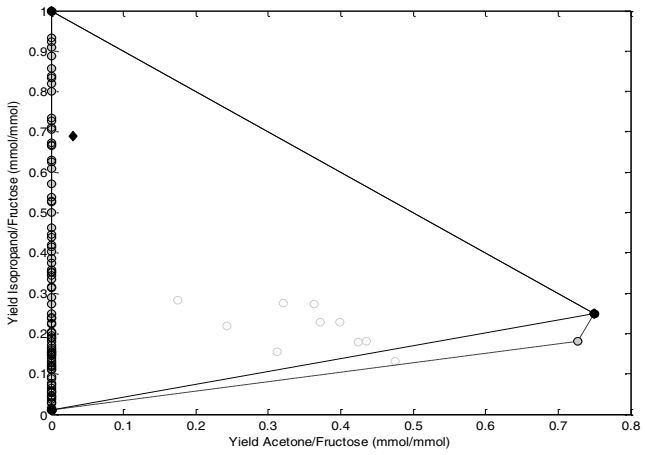

Fig. 3. Phase II: Selection of active modes in the yield space for the establishment of the dynamic model. Here are shown: the convex hull (- -), the experimental data (♦), Elementary Modes (○), Active Modes (●), and the largest area triangle (-).

The state vector $X = [S|P|e_M]^T$ was constituted by $S = FRU$, $P = [BIOM\ ISOP\ ACETONEx]$, and $e_M = [e_{M1}\ e_{M2}\ e_{M3}\ e_{M4}\ e_{M5}\ e_{M6}]$. The initial enzyme levels $e_{0M,j}$ were set to be the same for each active mode and was calculated by the following formula (Song *et al*. 2009) taking into account the preculture:

$$e_{0M,j} = \left[\alpha + k_{E,j}.\frac{FRU_0}{K_a + FRU_0}\right] / (\beta + \mu_j^{max}) \qquad (14)$$

The set of optimized parameters was estimated using the Rosenbrock function implemented in a toolbox of MATLAB®. The resulting parameter values are summarized in Table 3 and the corresponding simulated data are presented in Fig. 4.

Table 3. Values of identified parameters (k_j^{max}, $k_{E,j}$ and K_I) and parameters taken from Song *et al.* (2009) (α and β) and Franz *et al.* (2011) (K_a)

Parameter	Value
k_j^{max}	$[0.97\ 1\ 1\ 0.1\ 0.85\ 0.82]^T\ [L/h]$
$k_{E,j}$	$[0.0075\ 0.0092\ 0.035\ 0.19\ 0.53\ 0.14]^T\ [L/h]$
K_I	1.00 $[mmol/L]$
α	0.1 $[L/h]$
β	0.2 $[L/h]$
K_a	0.33 $[mmol/L]$

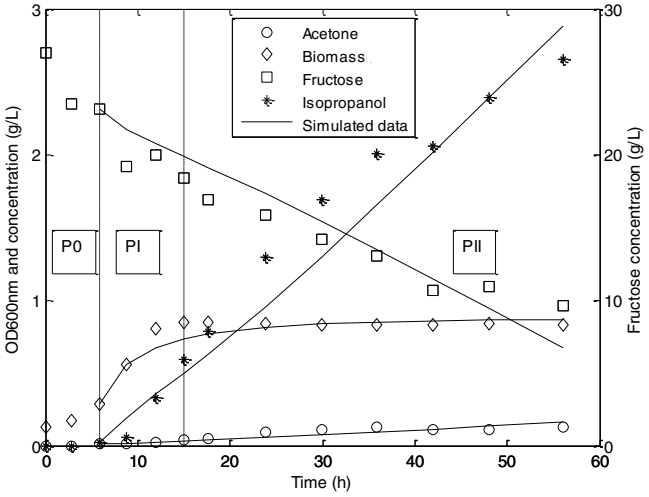

Fig. 4. Comparison between experimental results and simulated data for the phases PI and PII.

4. CONCLUSIONS

This work demonstrates for the first time the use of a Hybrid Cybernetic Model based on Elementary Mode Analysis to describe the dynamic metabolic behavior of *C. necator* for the production of isopropanol. Selection and classification of Elementary Modes took into account the microbial kinetics from batch-mode cultures. An engineered *C. necator*, capable of producing isopropanol, was used as a case study. A set of kinetic parameters was identified using an optimization technique. In this study, the available experimental data presented measurements for only four external metabolites. In the near future, experiments will be scheduled in a controlled environment in bioreactors, which will provide measurements for numerous additional external metabolites. On top of that, it will also be informative to incorporate the phase of latency (P0) into our calculations to further validate the model.

Several new perspectives have emerged from this work. It will be important, however, to test the sensitivity of the parameters and, in particular, ensure that the fixed parameters have no significant influence on the model's behavior. The expanded experimental data will be used to refine and validate the modeling technique. We believe the hybrid modeling is a very promising method with which to predict and evaluate the capabilities of newly engineered strains. Specifically, this method holds the promise of predicting the

value of maximal and minimal product yields for other proposed experimental substrates.

5. ACKNOWLEDGEMENTS

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