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DEVELOPMENT OF A PHENOMENOLOGICAL KINETIC MODEL FOR BUTANOL PRODUCTION USING *Clostridium beijerinckii*

CONSTRUCCIÓN DE UN MODELO CINÉTICO FENOMENOLÓGICO PARA LA PRODUCCIÓN DE BUTANOL USANDO *Clostridium beijerinckii*

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Abstract

At the present work the construction of an unstructured kinetic model guided by a phenomenological perspective was carried out to reproduce and simulate experiments regarding butanol production by *Clostridium beijerinckii* considering the ABE metabolic pathway reported for this genre as a biochemical basis for it. Fit and parametric sensitivity analyzes were performed to ensure the model could reproduce experimental kinetics at an overall correlation coefficient of 0.9882 and determined its high sensitivity thereof to the determination of the specific cell growth and death rates (μ_{maxX} and k_d) butanol production (μ_{maxBut}) and biomass / substrate yield calculation ($Y_{X/Sg}$). Finally, *in silico* batch analyzes were conducted at 60, 100, 150 and 200 g / L of initial glucose concentration values obtaining final butanol titers of 12.97, 13.95, 14.2 and 14.3 g / L respectively, which are consistent with the reported in the literature for *in vitro* experiments.

Keywords: modeling, biofuels, *Clostridium*, butanol, phenomenological.

Resumen

En el presente trabajo se llevó a cabo la construcción de un modelo cinético no estructurado desde la perspectiva fenomenológica para reproducir y simular experimentos de producción de butanol por *Clostridium beijerinckii* tomando como base bioquímica la ruta metabólica ABE reportada para este género. Se realizaron análisis de ajuste y sensibilidad paramétricos los cuales permitieron que el modelo reprodujera cinéticas experimentales con un coeficiente de correlación global de 0.9882 y determinaron la alta sensibilidad del mismo a la determinación de las velocidades específicas de crecimiento y muerte celular (μ_{maxX} y k_d), producción de butanol (μ_{maxBut}) y a el cálculo del rendimiento biomasa / sustrato ($Y_{X/Sg}$). Finalmente se condujo un análisis *in silico* de cinéticas en lote a 60, 100, 150 y 200 g/L de glucosa inicial prediciéndose valores de concentración de butanol final de 12.97, 13.95, 14.2 y 14.3 g/L respectivamente, demostrando ser consistentes con lo reportado en la literatura para experimentos *in vitro*.

Palabras clave: modelado, biocombustibles, *Clostridium*, butanol, fenomenológico.

1 Introduction

Biofuels can be defined as any solid, liquid or gas generated through the metabolism of living beings and includes from biomass itself to molecules resulting from the degradation of various substrates (Qureshi *et al*., 2008). Butanol is an alcohol comprised by four carbon atoms which can be used industrially as a solvent for the manufacture of explosives or as fuel in internal combustion engines (Berezina *et al*., 2009). Unlike ethanol which is currently produced in high volume to be used as biofuel, butanol has a lower hygroscopicity and increased caloric content that allows it to be used as an additive to gasoline

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or as a 100 % replacement for it without harming conventional engines (Mayank *et al*., 2012).

It has been known for over 100 years that butanol can be obtained by fermentation culturing strict anaerobic Gram-positive bacteria of the genus *Clostridium*, which have a metabolic pathway known as ABE which allows them to transform sugars or CO_2 to acetic and butyric acids in a first phase called acidogenesis and subsequently to butanol, acetone and ethanol in step known as solventogenesis, such molecular mechanisms are linked to the sporulation process and it depends on the generation of a pH gradient between the cell and the culture medium due accumulation of molecules produced in the acidogenic stage (Lee *et al*., 2008).

ABE fermentation industry was established since the late nineteenth century and remained as one of the primary sources for obtaining organic solvents until 1960's decade when it faced competition from the emerging petrochemical industry, which struck down production costs; however, the over exploitation of oil fields coupled with the increase in raw material prices and the negative impact of this industry over the environment have led to renewed interest in studying alternative energy sources to satisfy global demand (Chen *et al*., 2013).

Within the range of species belonging to the genus *Clostridium* the so-called *C. acetobutylicum* is capable of fermenting glucose to acetone, butanol and ethanol in the approximate proportions of 3:6:1 solvent moles per mole of sugar respectively. This species has been modified by molecular biology techniques to prevent the sporulation and increase its tolerance to high concentrations of solvents in order to increase productivity, however this strain hardly produces butanol in a titer above 14 g/L and its efficiency is limited by the need of glucose as its sole carbon source (Chen *et al*., 2013). Thus the scientific community has sought to isolate and characterize new species presenting beneficial attributes for the production of biofuels.

One species that has aroused most interest in recent years is *C. beijerinckii*, which not only has the ability to ferment glucose but also a wide range of poly and disaccharides, so it can be used in processes for obtaining the so-called second generation biofuels, which aim to utilize a wider spectrum of sugar sources like crop related residues (Ezeji *et al*., 2007).

The kinetic modeling of fermentation processes can be used to establish relationships between inputs and outputs of a system using mathematical tools that could unveil and predict its behavior under different operating conditions. They are the first step for the design and implementation of bioprocesses that aim to exploit the ability of microorganisms in order to obtain high quality goods and services (Mayank *et al*., 2012).

The mathematical modeling of biological systems can addressed with a structured or unstructured model, where the first attempt to predict the kinetics of intracellular reactions as closer as possible, while the latter considers the system as "black box" (Sweere *et al*., 1988). Although structured modeling represents the ideal scenario often requires a thorough understanding of the biochemical mechanisms within the cell to represent the system with greater certainty, which usually involves investing a lot of time and financial resources, so the creation of unstructured models remains a viable alternative for implementing biofuel production processes (Grosfils *et al*., 2007).

However most unstructured kinetic models have mathematical forms that make no sense within the biological point of view therefore the phenomenological development of unstructured kinetic models aims to find a middle ground between complexity and practicality to create systems that take into account as much substrates and products involved in a metabolic pathway as possible, which in turn results in better predictive models with a wider range of applicability to peer systems (Letisse *et al*., 2003).

As consequence this paper proposes an unstructured kinetic model from a phenomenological perspective for the production of butanol by fermentation by *Clostridium beijerinckii*, which was characterized and validated using fitting techniques and parametric sensitivity with respect to a set of experimental data and its predictive capacity was evaluated on a set of different initial conditions.

2 Methodology

2.1 Assumptions

For the generation of the kinetic model state equations the *Clostridium* genus ABE metabolic pathway was assessed from the one reported by (Haus *et al*., 2011), which is presented as follows:

It was proceeded to identify the input and output flows of the same, where were identified the five main states that make up the structure of the model: glucose, biomass, butanol, acetone and ethanol, however it is important to include the formation and consumption kinetics of intermediaries acetate and butyrate as Chang (2010) has reported that modulation of those

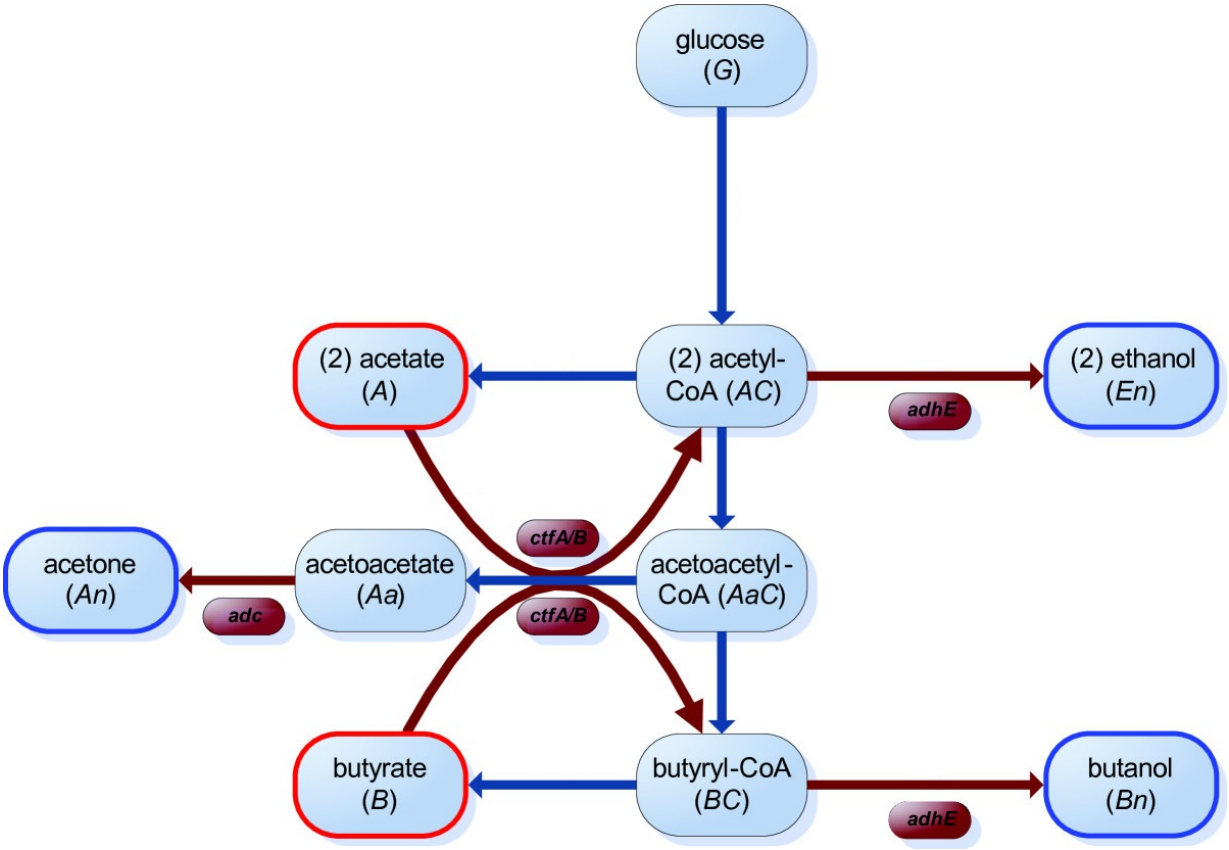

Figure 1. Simplified *Clostridium* genus ABE metabolic pathway reported by Haus (2011). It indicates the principal routes and enzymes involved on ethanol, acetone and butanol production and its intermediaries.

compounds through metabolic engineering techniques can potentially increase butanol production.

Shown in Fig. 1 the carbon flow from glucose is guided in a first stage to acetyl-CoA production which can be directed to the biosynthetic routes of ethanol, acetate and butyrate, therefore it was assumed that the state equations for the ethanol and acetate production were only dependent on the cellular growth rate and glucose consumption.

In a second stage it is appreciated that acetone and butanol concentrations depend on the generation and supply to the system of acetate and butyrate respectively, said states need to be modeled considering them as both substrates and products wherefore state equations for butanol and acetone were proposed to be dependent on cell growth and glucose and acetate or butyrate consumption as the case may be.

Additionally it was considered that biomass growth only depended on glucose consumption and that carbon source represented the limiting substrate, this due to the anaerobic nature of the microorganism.

Finally, it was known beforehand that the ABE fermentation process is characterized by presenting growth inhibition by product accumulation, which in this particular case is butanol, being reported an average inhibitory concentration of 14 g/L for this strain (Lee *et al.*, 2008).

2.2 *Model development*

With all the above considerations it was proceeded to generate the following set of differential equations, which is based on classical mass balance approach, that comprise the proposed kinetic model:

$$\mu_X = \mu_{maxX}\left(\frac{Sg}{Ksg+Sg}\right)\left(1-\frac{But}{Kbut}\right) \quad (1)$$

$$\mu_{But} = \mu_{maxBut}\left(\frac{Sg}{Ksg+Sg}\right)\left(\frac{Sb}{Ksb+Sb}\right) \quad (2)$$

$$\mu_{Sb} = \mu_{maxSb}\left(\frac{Sg}{Ksg+Sg}\right) \quad (3)$$

$$\mu_{Ace} = \mu_{maxAce}\left(\frac{Sb}{Ksb+Sb}\right)\left(\frac{Act}{KsAct+Act}\right) \quad (4)$$

$$\mu_{Et} = \mu_{maxEt}\left(\frac{Sg}{Kse + Sg}\right) \quad (5)$$

$$\frac{dSg}{dt} = -\left(\frac{X\mu_X}{Y_{X/Sg}} + \frac{But\mu_b}{Y_{But/Sg}} + \frac{Ace\mu_{Ace}}{Y_{Ace/Sg}} + \frac{Et\mu_{Et}}{Y_{Et/Sg}}\right) \quad (6)$$

$$\frac{dX}{dt} = (\mu_X - Kd)X \quad (7)$$

$$\frac{dBut}{dt} = X\mu_{But}Y_{But/X} \quad (8)$$

$$\frac{dSb}{dt} = X\mu_{Sb}Y_{Sb/X} - \frac{X\mu_{But}Y_{But/X}}{Y_{But/Sb}} \quad (9)$$

$$\frac{dAct}{dt} = X\mu_X Y_{Act/X} \quad (10)$$

$$\frac{dAce}{dt} = X\mu_{Ace}Y_{Ace/X} \quad (11)$$

$$\frac{dEt}{dt} = X\mu_{Et}Y_{Et/X} \quad (12)$$

See the notation section for variables description.

Eqs. (1) - (4) represent specific biomass growth and butanol, butyrate and acetate production rates respectively. The structure of the growth rate model obeys a product inhibition one proposed by Levenspiel (1980), with which it was tried to represent the influence of high concentrations of butanol in the system in accordance with those reported by most existing publications (Lee *et al*., 2008).

As stated in the assumptions the Eq. (2) represents butanol production rate based on a dual substrate kinetics involving glucose and butyrate concentrations considering the information extracted from the metabolic pathway, since it has been shown that adding the latter into the culture medium can redirect carbon flow into butanol production (Chang, 2010).

To provide the substrate and product dual characteristic to the model describing butyrate concentration it was necessary to specify a production rate for it described by Eq. (3) to use as a positive term in Eq. (8).

The acetone production process is described in the pathway as acetate and butyrate concentration dependent therefore Eq. (4) incorporates the influence of both in the rate at which it is carried out.

Eqs. (5) - (11) represent the system state equations, where Eq. (5) describes glucose concentration versus time and includes terms of substrate consumption from the major products of fermentation: biomass, ethanol, butanol and acetone.

Eq. (6) describing the biomass concentration incorporates Eq. (1) as a term of cell growth and includes a cell death constant parameter, which is justified as several authors (Chong, 2010; Lee *et al*., 2008) reported a drop in biomass concentration once the solventogenic phase had started.

Eq (8) which predicts butyrate concentration was planned in a way it could represent it as both a substrate to generate butanol and as a fermentation product in accordance with the metabolic pathway mentioned above, while Eqs. (7), (9-11) describe the production trend of butanol, acetate, acetone and ethanol respectively.

3 Results and discussion

3.1 Parametric identification and sensitivity

Once built the state equations for the kinetic model it was proceeded to perform the identification and parametric adjustment for each of these, the operation was carried out from a set of experimental data reported by Chang (2010), considering the following initial conditions of the corresponding mass concentrations: 55, 0.2, 2 and 0.5 g L^{-1} for glucose, biomass, butyrate and acetate respectively; who used metabolic engineering tools to increase butanol production by supplementing the culture medium with butyrate, this in order to minimize the metabolic flux shunt towards the production of acetone and ethanol.

ModelMaker ® 3.0.3 software was used to perform a non-linear parameter adjustment with the experimental data by the Levenberg-Marquardt algorithm; the values obtained for each one are presented in Table 1.

Having identified the set of parameter values that maximized model fitting into experimental values it was proceeded to conduct a local sensitivity analysis based on the application of the Fisher information matrix (Gunawan *et al*., 2005), which allows to quantify the inference degree of each parameter numerical variation at any simulation time and their confident intervals into their numerical determination at different stages of the fermentation.

As expected Fig. 2 shows that the model is highly sensitive to the precise determination of cell growth rates, butanol generation, cell death constant and biomass/substrate yield, this kind of information could provide a foundation for making adjustments to analytical or numerical techniques for the calculation of these parameters in future experiments.

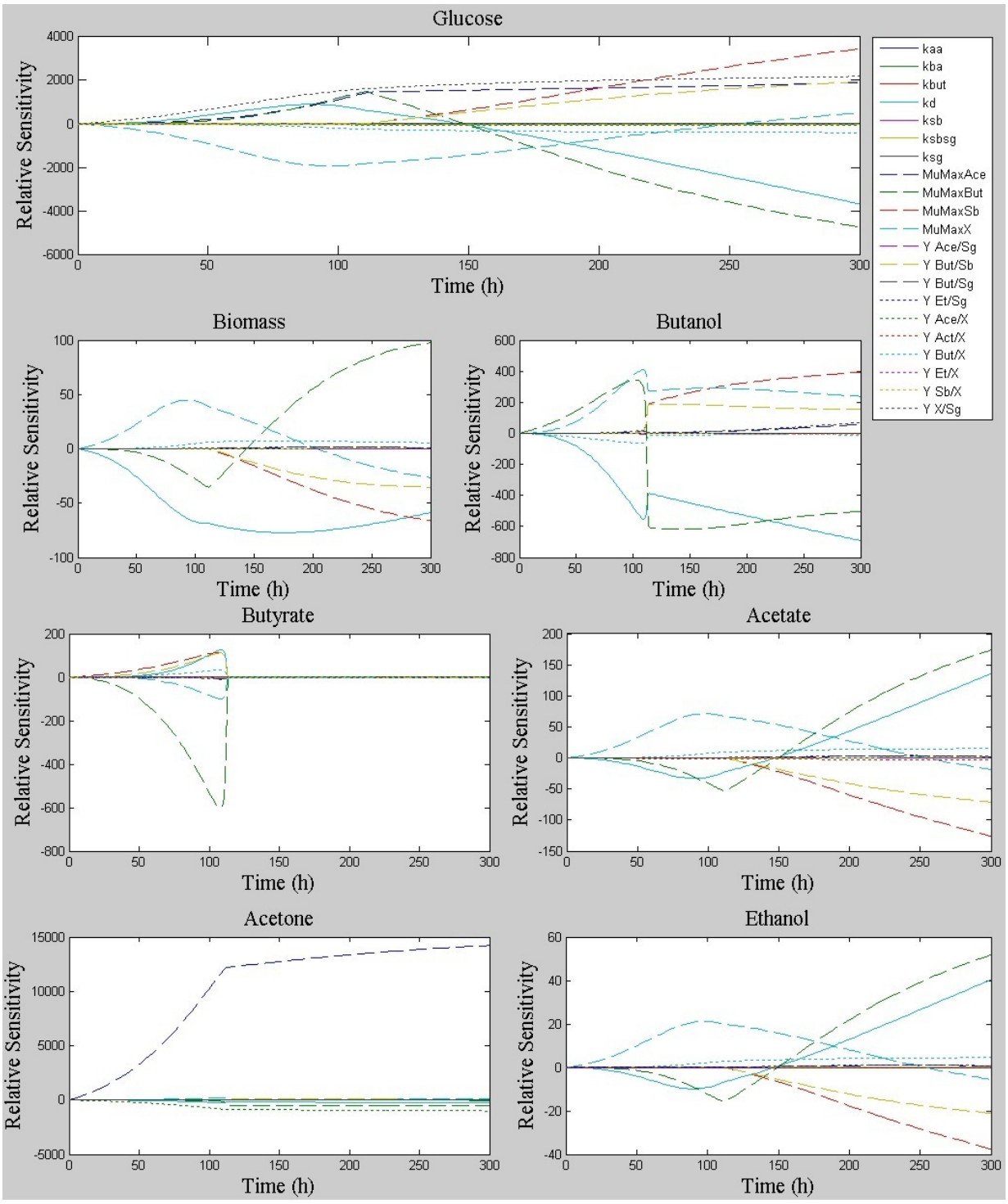

Fig. 2. Local parametric sensitivity analysis curves for all the modeled states versus fermentation time: Glucose, Biomass, Butanol, Butyrate, Acetate, Acetone and Ethanol.

Table 1. Numerical values for each model parameter resulted from the non-linear parametric identification.

Parameter	Value	Units
k_{aa}	9.2254×10^{-5}	g L^{-1}
k_{ba}	0.0364	g L^{-1}
K_{But}	13.3803	g L^{-1}
K_d	0.0040	h^{-1}
K_{Sb}	0.0296	g L^{-1}
K_{SbSg}	1.0219	g L^{-1}
K_{Sg}	0.9477	g L^{-1}
μ_{maxAce}	0.0004	h^{-1}
μ_{maxBut}	0.0196	h^{-1}
μ_{maxSb}	0.0058	h^{-1}
μ_{maxX}	0.0270	h^{-1}
$Y_{Ace/Sg}$	0.2597	g/g
$Y_{But/Sb}$	0.0361	g/g
$Y_{But/Sg}$	0.3352	g/g
$Y_{Et/Sg}$	0.1005	g/g
$Y_{Ace/X}$	0.0053	g/g
$Y_{Act/X}$	0.7493	g/g
$Y_{But/X}$	0.1130	g/g
$Y_{Et/X}$	2.5189	g/g
$Y_{Sb/X}$	0.4887	g/g
$Y_{X/Sg}$	0.0249	g/g

3.2 Simulation and bibliographical comparison

Numerical simulations using the constructed model were conducted in ModelMaker ® 3.0.3 software. Figure 3 shows the kinetics simulated by the model, compared with the one obtained experimentally by Chang (2010).

To quantify the reliability of the model there was performed a linear regression analysis for each state and calculated a global correlation value with respect to the fit of the model to the experimental data based on the protocol reported by Tejada (2007). The results of this study are reflected in Table 2.

As shown in Table 2 parametric adjust yielded correlation coefficients above 0.95 for most modeled states, the dynamics of butyrate and acetate could not be represented with greater accuracy presumably due to the structure of the differential equation describing them, since the experimental data exhibit that butyrate formation during the fermentation conducted by Chang (2010) is inhibited at the stage of exponential butanol production and instead the cells incorporate the butyrate present in the culture medium to carry out its transformation to alcohol, which can be seen in Fig. 3.

Table 2. Linear regression correlation indexes for each individual state and global model one versus Chong (2010) experimental data.

State	R^2
Glucose	0.9952
Biomass	0.9572
Butanol	0.9802
Butyrate	0.5503
Acetate	0.6614
Acetone	0.9932
Ethanol	0.9677
Global	0.9882

Chang's (2010) experimental data shows the culture doesn't display any "lag phase" as its growth rate is almost maxed out since the beginning of the fermentation; however growth seems to be severely diminished once butanol concentration increases, as high solvent values on the bacterial surrounding media can interfere with its vegetative reproduction cycle. There is biochemical evidence that suggest *Clostridial* strains lack an internal pH regulation mechanism, therefore their membrane and cell wall dependent processes become less efficient under these conditions (Gottwald *et al*., 1985).

The parametric identification conducted on this work allowed the proposed model to reproduce biomass dynamic efficiently, granted by the linear correlation index obtained on this particular state that is displayed on Table 2. Levenspiel (1980) structure for specific growth rate under product inhibition conditions combined with the implementation of a death constant "k_d" (refer to Nomenclature) on Eqs. (1) and (7) respectively proven to be enough to keep the model performance close to the experimental data.

Also it should be noted that the butanol inhibition constant value "K_{but}" (refer to Nomenclature) shown on Table 1, which was obtained by the parametric identification algorithm proves to be consistent with the initial assumption of the average inhibitory butanol concentration for *Clostridium beijerinckii* reported by Lee *et al*. (2008).

Once validated the model's predictive ability was assessed for different substrate feed initial conditions, as this parameter is generally the easiest to manipulate during experimental essays.

Therefore in an attempt to evaluate the model's performance versus a wider substrate concentration spectrum *in silico* essays were conducted, this time the goal was try to reproduce a previously reported

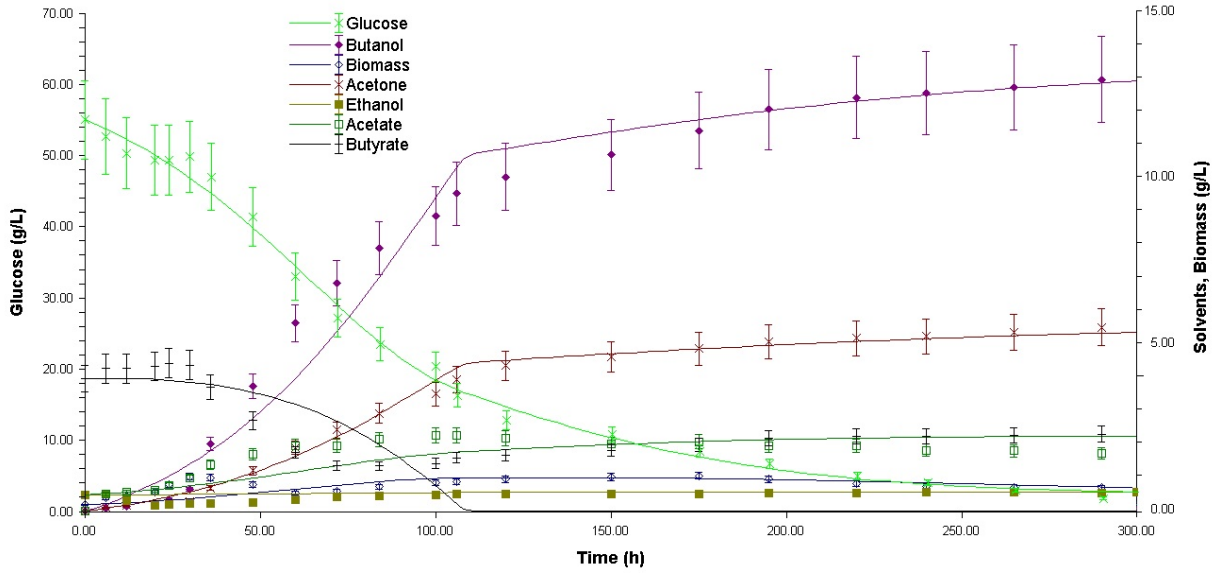

Fig. 3. Experimental and simulated *C. beijerinckii* ABE fermentation kinetics. Continuous line represents the modeled concentrations and crosses indicate experimental values (Chang, 2010).

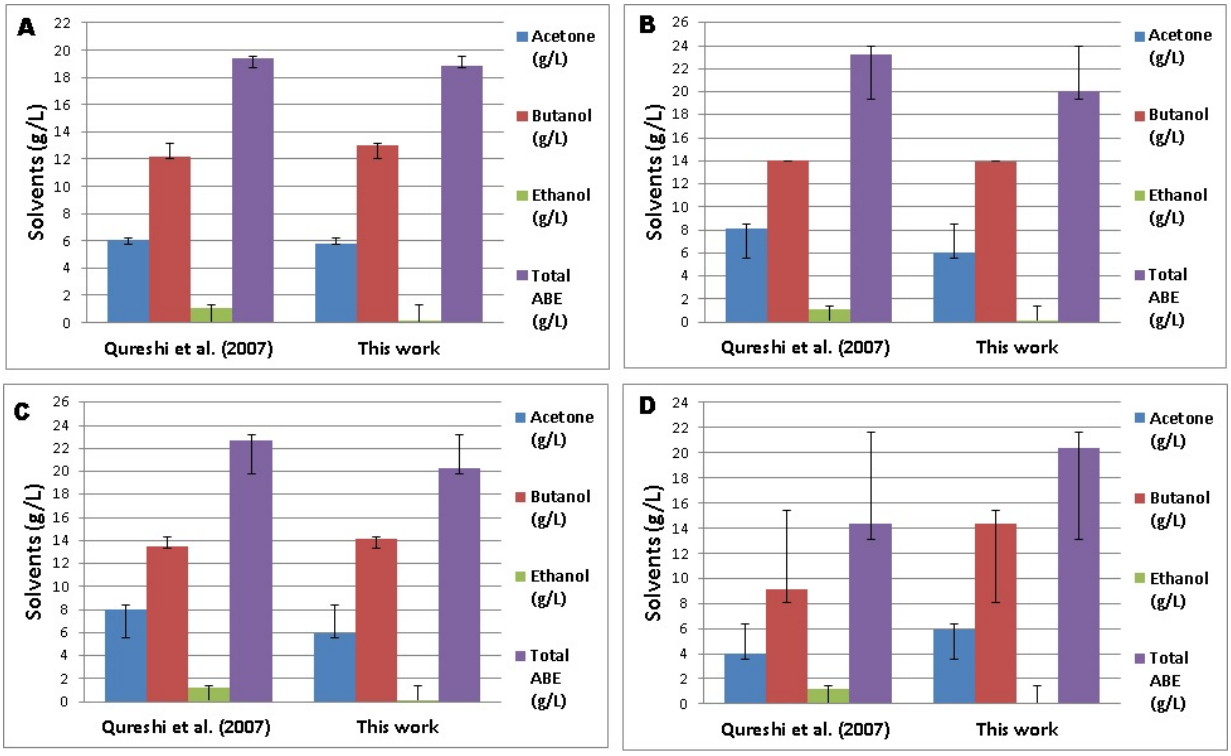

Fig. 4. Qureshi *et al.* (2007) experimental data versus proposed model *in silico* analysis comparison showing predicted final solvent titers after fermentation of glucose at an initial concentration of A) 60, B) 100, C) 150 g/L and D) 200 g/L respectively.

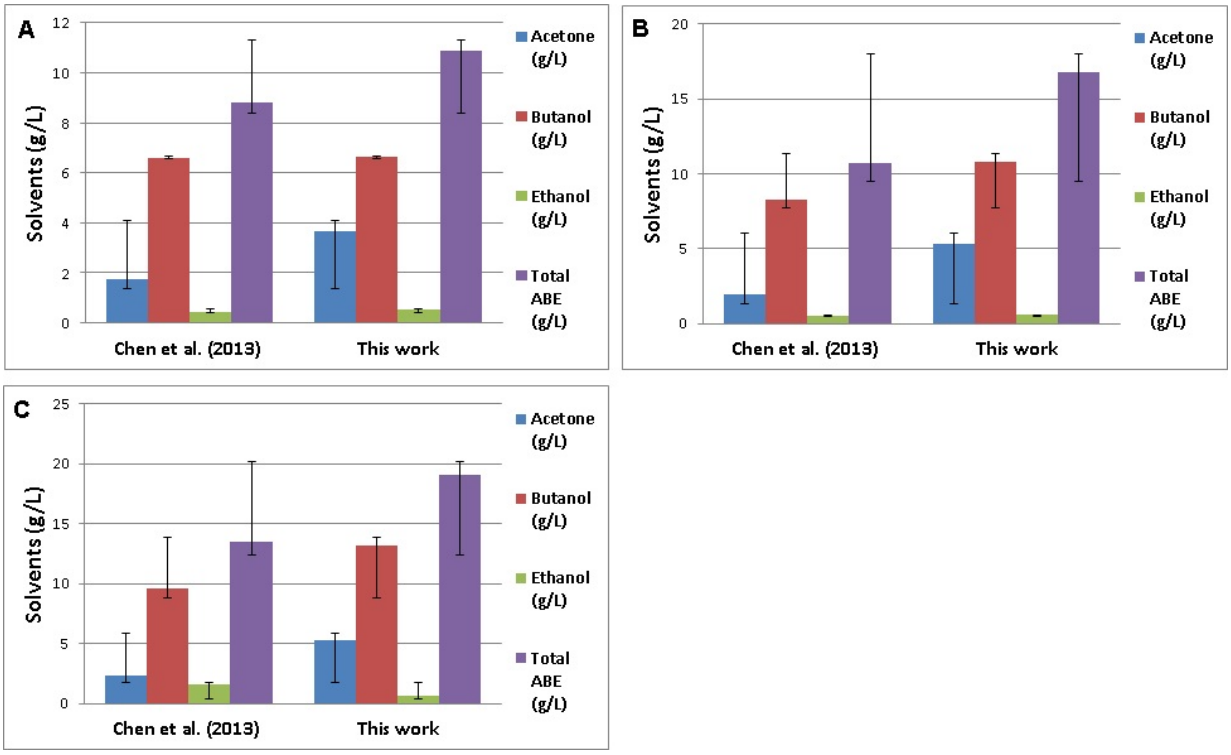

Fig. 5. Cheng *et al.* (2013) experimental data versus proposed model *in silico* analysis comparison showing predicted final solvent titers after fermentation of glucose at an initial concentration of A) 30, B) 40 and C) 60 g/L respectively.

experiment by Qureshi *et al.* (2007) where they use *Clostridium beijerinckii* on fermentations with initial glucose concentrations ranging from 60 to 200 g/L.

As shown in Fig. 4 the proposed model can simulate final acetone and butanol titers on the same magnitude order but struggles with the ethanol one, because as was mentioned earlier at the model development section ethanol production is altered on Chong's (2010) kinetic due metabolic engineering done to the culture in order to deviate carbon flow from ethanol synthetic pathway to butanol generation; this trend is consistent with the one displayed on Fig. 5 as all the final butanol titers were predicted to be higher than the ones reported by Qureshi *et al.* (2007).

Also the final product's titers were compared between the ones predicted by the model and the results obtained by Cheng *et al.* (2013) in batch cultures of Clostridium acetobutylicum so the *Clostridium beijerinckii* strain theoretical efficiency could be evaluated under the same operating conditions.

With the results expressed in Fig. 5 can be inferred that the *Clostridium beijerinckii* strains have theoretical yields superior to those observed on *Clostridium acetobutylicum* but within the same order of magnitude, which can validate the constructed model and ensure that can be used for *in silico* studies of biofuel production generated by the metabolism of this species.

Conclusion

The biotechnological industry involved on biofuels production demands finding new strains that enable the implementation of competitive bioprocesses at the medium term, so the study of the metabolic pathways of these organisms should influence the construction of kinetic models that can predict with a greater detail degree these mechanisms with the goal of reducing the margin of uncertainty that is generated by transferring knowledge of biological area to industrial applications.

It was demonstrated that the unstructured phenomenological kinetic modeling approach presented in this paper could reproduce experimental data from an ABE fermentation with *Clostridium beijerinckii* with a global correlation index over

0.98, making it suitable for experimentation *in sillico* exercises to predict biofuels titers at different initial conditions, this corroborated by the results of comparative tests with experiments conducted by various research groups currently.

Moreover a wide test set was applied to the model to let know it's sensitivity degree to the numerical variation of their parameters, which can be used to obtain confidence intervals within which the model is valid and at which points of the analytic techniques conducted in fermentations of this nature should be paid more attention, contributing not only within the theoretical scope but also in the process of designing experimental strategies with less disruption by mistake and greater efficiency in resource use.

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Nomenclature

Kaa	acetate-acetone affinity constant, g L^{-1}
Kba	butyrate-acetone affinity constant, g L^{-1}
$Kbut$	butanol growth inhibition constant, g L^{-1}
k_d	specific cell death rate, h^{-1}
Ksb	butanol-butyrate affinity constant, g L^{-1}
$Ksbsg$	butyrate-glucose affinity constant, g L^{-1}
Ksg	glucose affinity constant, g L^{-1}
$Y_{Ace/Sg}$	acetone per glucose mass yield, g/g
$Y_{But/Sb}$	butanol per butyrate mass yield, g/g
$Y_{Et/Sg}$	ethanol per glucose mass yield, g/g
$Y_{Ace/X}$	acetone per biomass yield, g/g
$Y_{Act/X}$	acetate per biomass yield, g/g
$Y_{But/X}$	butanol per biomass yield, g/g
$Y_{Et/X}$	ethanol per biomass yield, g/g
$Y_{Sb/X}$	butyrate per biomass yield, g/g
$Y_{X/Sg}$	biomass per glucose yield, g/g

Greek symbols

μ_{maxAce}	maximum acetate production rate h^{-1}
μ_{maxBut}	maximum butanol production rate h^{-1}
μ_{maxSb}	maximum butyrate production rate h^{-1}
μ_{maxX}	maximum specific cell growth rate h^{-1}

References

Chen, Y., Zhou, T., Liu, D., Li, An., Xu, S., Liu, Q., Li, B. and Ying, H. (2013). Production of Butanol from Glucose and Xylose with Immobilized Cells of *Clostridium acetobutylicum. Biotechnology and Bioprocess Engineering 18*, 234-241.

Chong, W. (2010). Acetone-Butanol-Ethanol Fermentation by Engineered *Clostridium beijerinckii* and *Clostridium tyrobutyricum*. Food Science and Technology PhD Thesis, Ohio State University, USA.

Ezeji, T., Qureshi, N. and Blaschek, H. P. (2007). Butanol Production From Agricultural Residues: Impact of Degradation Products on *Clostridium beijerinckii* Growth and Butanol Fermentation. *Biotechnology and Bioengineering 97-6*, 1460-1469.

Gottwald, M. and Gottschalk, G. (1985). The Internal pH of *Clostridium acetobutylicum* and its Effect on the Shift from Acid to Solvent Formation. *Archives of Microbiology 143*, 42-46.

Grosfils, A., Wouwer, A. V. and Bogaerts, P. (2007). On a general model structure for macroscopic biological reaction rates. *Journal of Biotechnology 130*, 253-264.

Gunawan, R., Cao, Y., Petzold, L. and Doyle, F J. (2005). Sensitivity Analysis of Discrete Stochastic Systems. *Biophysical Journal 88*, 2530-2540.

Haus, S., Jabbari, S., Millat, T., Janssen, H., Fischer, R-J., Bahl, H., King, J. R. and Wolkenhauer, O. (2011). A systems biology approach to investigate the effect of pH-induced gene regulation on solvent production by *Clostridium acetobutylicum* in continuous culture. *BMC Systems Biology 5*, 5-10.

Lee, S. Y., Park, J. H., Jang, S. H., Nielsen, L. K., Kim, J. and Jung, K. S. (2008). Fermentative Butanol Production by Clostridia. *Biotechnology and Bioengineering 101- 2*, 209-228.

Letisse, F., Lindley, N.D. and Roux, G. (2003). Development of a Phenomenological Modeling Approach for Prediction of Growth and

Xanthan Gum Production Using *Xanthomonas campestris. Biotechnology Progress 19*, 822-827.

Levenspiel, O. (1980). The Monod Equation: A Revisit and a Generalization to Product Inhibition Situations. *Biotechnology and Bioengineering 22-8*, 1671-1687.

Mayank, R., Ranjan, A., and Moholkar, V. S. (2012). Mathematical models of ABE fermentation: review and analysis. *Critical Reviews in Biotechnology*, 1-29.

Qureshi, N., Ezeji, T.C., Ebener, J., Dien, B.S., Cotta, M.A. and Blaschek, H.P. (2008). Butanol production by *Clostridium beijerinckii*. Part I. Use of acid and enzyme hydrolyzed corn fiber. *Bioresources Technology 99*, 5915-5922.

Qureshi, N., Saha, B.C., J. and Cotta, M.A. (2007). Butanol production from wheat stray hydrolysate using *Clostridium beijerinckii*. *Bioprocess Biosystems Engineering 30*, 419-427.

Sweere, A. P. J., Giesselbach, J. R., Barendse, de Krieger, R., Honderd, G. and Luyben, K. C. A. M. (1988). Modelling the dynamic behaviour of Saccharomyces cerevisiae and its application in control experiments. *Applied Microbiology and Biotechnology 28*, 116-127.

Tejada, A. (2007). *Estadística para ciencias sociales, del comportamiento y de la salud*. 3ª Edición, CENGAGE Learning, Mexico.