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Double Substrate Limitation Model for the Experimental Scale-up of Succinic Acid Production from Biorefinery Glycerol

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A combination of experimental and computational work has been performed in order to assess and optimise the experimental conditions for the bio-production of succinic acid - one of the top-value added chemicals (Werpy et al., 2004) from crude glycerol, which is the major by-product of the bio-diesel production process. The kinetics of a single substrate (crude glycerol) model have been fully analysed and further optimisation based on both yield and productivity of succinic acid defined the decision parameters of a batch process. At the same time, the introduction of a double substrate limiting model has been suggested as it has been proved that both the uptake rate of glycerol and of dissolved CO_2 have a significant effect on succinic acid production using Actinobacillus Succinogenes (Der Werf et al., 1997; Binns et al., 2011). Process parameters that influence the transfer rate of gaseous CO_2 in the broth have been incorporated in the model. The developed model can be utilized to successfully predict the concentration profiles of six state variables (biomass, glycerol, succinic acid, formic acid, acetic acid and dissolved CO_2) for a range of initial glycerol concentrations and working volumes. Kinetic parameters of the model were estimated by minimizing the difference between experimental and predicted values (Vlysidis et al., 2011a) for a range of batch experiments.

1. Introduction

To be considered as a viable alternative to conventional petroleum oil, biodiesel needs to be competitive from an energy, financial, environmental and availability standpoint without imperilling the food crops. From an economic perspective, reductions in taxation and subsidies offered by the European and US Governments in conjunction with the increases in petroleum prices have contributed to that direction (Pagliaro and Rossi, 2010). One of the main costs from biodiesel production is that of the raw material utilised and alternative feedstocks, such as cooking oil wastes can minimise it (da Silva et al., 2009). However, to truly enhance biodiesel production the valorisation of glycerol which is the main by-product, through the co-production of high value chemicals and the opening to new markets seems more necessary than ever. In case these products are currently derived petrochemically, the release from fossil fuel dependence will be an extra advantage (Ferreira et al., 2012). Such is the case of succinic acid which constitutes a key building block for the formation of many other commodity and specialty chemicals (Werpy et al., 2004). Assessing the overall economic feasibility of the conversion of glycerol to value added chemicals via chemical and biochemical methods, showed that even if glycerol purification is included, succinic acid presents a ratio of sales price to total production cost greater than one. In order for crude glycerol conversion to succinic acid to be even more attractive improvements in the conversion yields and in downstream processes are needed (Posada et al., 2012). The integrated biorefinery concept was simulated by Vlysidis et al. (2011b), to assess the profitability of succinic acid fermentation in situ. Crude glycerol was directly used by A. Succinogenes without former purification and led to higher gross margin compared to the purification alternatives. However, it was suggested that the plant's capacity, and raw materials and succinic acid prices influence the sustainability of the whole scheme, thus improvements in the bioreactor performance and manipulation of the bacterial behaviour were suggested. Increasing

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productivity as well as products yield is quite important for the scaling up and industrialisation of a process. The current study investigates the ability of a single substrate model to represent moderate scale up conditions and focuses on providing the means for successful scale up. That is achieved through a new unstructured model incorporating dissolved CO_2 and scale up parameters.

2. Modified Unstructured Single Substrate Model

2.1 Determination of kinetic parameters

Improvement of an existing unstructured model (Vlysidis et al., 2011a) was performed by including in the glycerol consumption rate, the contribution of acetic and formic acid formation. Sensitivity analysis proved that when the production rate of one of the products changed, the rest of the products as well as glycerol changed unduly. Additionally, a further constraint was introduced to ensure conservation of the total mass (TMB). Theoretically the total mass balance of the five state variables should remain constant throughout the process, given that glycerol is consumed just for succinic, formic and acetic acid and nothing else converts into these products. In reality, the experimental total mass value varies due to the additional carbonate sources of the semi defined medium and the intracellular carbon content that might change throughout the experimental time. The bounds of the kinetic parameters of the new model - Eq(1) were defined based on screened experimental data. More specifically, bounds for K_{GLR} and I_{GLR} where estimated graphically since their physical meaning is the lowest and highest concentration at which specific growth rate equals one half of the maximum specific growth rate (Andrews, 1968). The bounds for yield factors for the biomass, the succinic, formic and acetic acids to glycerol as well as μ_{max} were also estimated throughout the experimental time for all the experiments used for fitting. The estimation of the new kinetic parameters was based on a combination of stochastic and deterministic optimisation methods. Simulated annealing, a stochastic algorithm, was used to compute a family of solutions near the (potentially) global optimum and Successive Quadratic

$$\frac{dX}{dt} = \left(\mu_{max} \frac{GLR}{GLR + k_{GLR} + {GLR^2}/{I_{GLR}}} \left(1 - \frac{P_{SA}}{P_{SA}^*}\right)^{n_{SA}} \right) X$$

$$\frac{dGLR}{dt} = -\frac{1}{Y_{\frac{X}{GLR}}}\frac{dX}{dt} - m_{GLR}X - \frac{1}{Y_{\frac{SA}{GLR}}}\frac{dP_{SA}}{dt} - \frac{1}{Y_{\frac{AA}{GLR}}}\frac{dP_{AA}}{dt} \qquad (1)$$

$$\frac{dP_i}{dt} = a_i\frac{dX}{dt} + \beta_i X \qquad i = SA,\ AA$$

$$GLR + P_{SA} + P_{FA} + P_{AA} - TMB = 0$$

Table 1: Kinetic Parameters

Symbol	Description	Units	Single Model	Double Model
μ_{max}	Maximum Specific growth	h^{-1}	0.1136	0.0900
K_{GLR}	Substrate saturation constant	g GLR L^{-1}	7.4980	0.5019
I_{GLR}	Substrate inhibition constant	g GLR L^{-1}	8.9254	25.4783
n_{SA}	Linearity of the product inhibition		1.2061	0.8053
$Y_{X/GLR}$	Yield factor of biomass X to substrate GLR	g X g^{-1} GLR	0.3122	4.0000
m_{GLR}	Specific maintenance coefficient on GLR	g GLR g^{-1} X h^{-1}	0.0234	0.0047
α_{SA}	Growth association constant for SA	g SA g^{-1} X	1.4920	3.4508
β_{SA}	Non-growth association growth SA	g SA g^{-1} X h^{-1}	0.0631	0.1000
$Y_{SA/GLR}$	Yield factor of SA to substrate GLR	g SA g^{-1} GLR	5.0983	1.0000
α_{AA}	Growth association constant for AA	g AA g^{-1} X	0.0878	0.0015
β_{AA}	Non-growth association growth for AA	g AA g^{-1} X h^{-1}	0.0015	0.0162
$Y_{AA/GLR}$	Yield factor of AA to substrate GLR	g AA g^{-1} GLR	0.4589	0.5450
K_{CO2}	CO_2 saturation constant	g CO_2 L^{-1}		0.0054
I_{CO2}	CO_2 inhibition constant	g CO_2 L^{-1}		1.2000
m_{CO2}	Specific maintenance coefficient on CO_2	g CO_2 g^{-1} X h^{-1}		0.0047
$Y_{X/CO2}$	Yield factor of biomass X to CO_2	g X g^{-1} CO_2		2.3274
$Y_{SA/CO2}$	Yield factor of SA to CO_2	g SA g^{-1} CO_2		4.9073
r_M	Constant reaction of MgCO3 to dissolved CO2			1e-5

Programming was subsequently implemented to estimate the final optimum (minimum) and the corresponding set of kinetic parameters (Table 1). The objective function was the sum of squared differences between the predicted and the experimental values of the five state variables at each sample point (Vlysidis et al., 2011a). Model predictions compared with an experiment conducted in Small Anaerobic Reactors (V_w = 50mL) used in parameter fitting are presented in Figure 1. Biomass (X), Succinic Acid (P_{SA}) and Acetic acid (P_{AA}) are well predicted with slight overprediction of the final biomass concentration. Formic acid (P_{FA}) is overpredicted as a result of the TMB overperdiction.

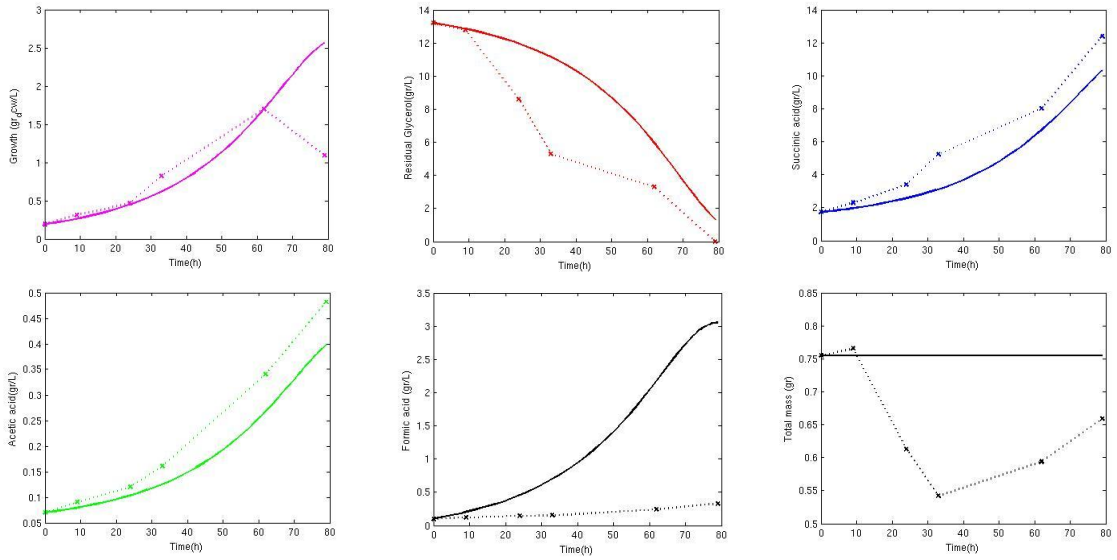

Figure 1: Experimental results (points) vs. model predictions (solid lines) of an experiment conducted in SAR and included in the parameter fitting procedure.

Validation

To validate the model, a batch experiment conducted in a Bench Top Reactor (V_w= 1.0 L) was plotted in Figure 2. As it can be seen, the model represents well at least four out of the five state variables of the model in larger scale without additional fitting.

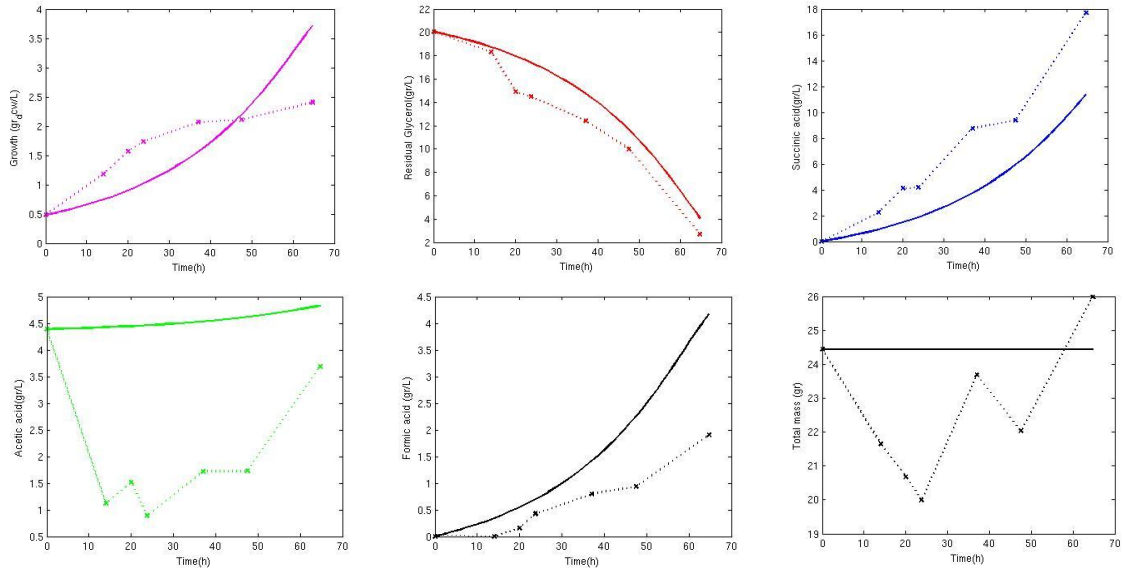

Figure 2: Validation of new parameters for an experiment conducted in BTR :Experimental data(points) vs model predictions (solid lines)

2.2 Batch Process Optimisation

Using the new set of kinetic parameters, batch model optimisation was performed to maximise productivity. The optimisation parameters selected are shown in Table 2. Results did not seem to change

significantly when the objective function was either the process productivity or the sum of yield and productivity. For narrower bounds of X_o (Opt.1), less GLR can be converted to SA and the maximisation of the combined function seemed to compute GLR_o and t_f up to their upper limits.

Table 2: Batch Optimisation

Decision Parameters	Description	Base Case	Opt 1	Opt.2
X_o (g_{DCW} L^{-1})	Initial biomass concentration	0.1900	0.3	0.5919
GLR_o (g L^{-1})	Initial substrate concentration	13.2000	28.2834	35.0153
t_f (h)	Fermentation time	79	119	119
Yield		0.6530	0.8045	0.8563
Productivity		0.1566	0.1903	0.2509

3. New Unstructured Double Substrate Model

3.1 Dissolved CO_2 as a second limiting substrate

Studies of the metabolism of A. Succinogenes have shown that the main steps affecting succinic acid production from glycerol are the glycerol uptake rate, the reduction of malate to succinic acid and the reaction from pyruvate to malate (Binns et al., 2011; De Barros et al., 2013). Additionally, sufficient CO_2 is necessary for H_2 to promote succinate production by increasing PEP carboxykinase activity to make Oxaloacetate and finally succinate through the reductive branch of TCA cycle (Der Werf et al., 1997; McKinlay and Vieille, 2008). Apparently the intracellular availability of CO_2 is related to the succinic acid production. Knowledge of how to increase its extracellular availability, can lead to optimisation of the fermentation. Additional carbonate (CO_3^{2-}) and bicarbonate (HCO_3^-) salts are therefore present in most anaerobic fermentations describing succinic acid production in order to complement the total carbonate content and maintain the dissolved CO_2 at high level. Although HCO_3^- and CO_3^{2-} cannot diffuse directly across cell membranes without the expense of ATP (Xi et al., 2011), they act as indirect CO_2 donors (Gutknecht et al., 1977). In the model suggested by Xi et al. 2011, when dissolved CO_2 is consumed by the cells, HCO_3^- and CO_3^{2-} are converted into dissolved CO_2. In particular, that takes place when the rate of CO_2 consumption is greater than the dissolution rate of gaseous CO_2 (gas transfer limitation). In the absence of additional carbonates, the transient concentration of dissolved CO_2 would decrease significantly imperilling the availability of CO_2 for the bacteria. Since CO_2 is considered to be a key substrate in the anaerobic fermentation of glycerol to succinic acid, sufficient gas-liquid mass transfer should be achieved. According to the two film model of Lewis and Whitman(1924), the total volumetric mass transfer rate of a gas is described by the following equation:

$$GTR = k_L a \, (CO_2^* - CO_2) \quad (2)$$

where CO_2^* is the gas solubility in equilibrium with the gas partial pressure in the gas phase, CO_2 is the dissolved gas concentration in the bulk liquid and $k_L\alpha$ is the overall mass transfer coefficient, typically expressed in h^{-1}. It is apparent from Eq(2) that the volumetric transfer rate of CO_2 and thus its availability for a given reactor system could be controlled either by causing changes in the solubility or by affecting the $k_{L\alpha}$ value. For the first control method, the concentration of solutes in the medium can be altered or the partial pressure of the sparging CO_2, given that the temperature is fixed for most fermentation processes. Changes in the medium composition are more significant when carbonates (such as $MgCO_3$) are included whereas partial pressure can be modified by supplying more than one gases. In the second control method, the mass transfer coefficient is affected by changing the rotational speed of the impeller or the flow rate of gaseous CO_2. Both these control actions are capable of affecting the initial conditions of any fermentation process but have a different effect during the course of the fermentation since the concentration of the medium changes, changing the solubility and the mass transfer coefficient via viscosity change. However, it's been observed that for concentrations up to 20 g L^{-1} DCW of the totally dispersed cell cultures E.coli, Pseudomonas & Candida, viscosity is not affected significantly (Operational Modes of Bioreactors, 1992). As a result, solubility must either be considered as a function of the carbonate content or be treated as a constant variable of a model including the carbon dioxide mass balance. The new model represented by Eq(4) consists of one additional differential algebraic equation and 6 more kinetic parameters as seen Table 1. The concentration of dissolved CO_2 changes in time due to formation of products (acids) as well as changes in the concentration of carbonates in the medium.

The same methodology for computing the kinetic parameters as described for the single substrate model is used for the double substrate model. In this case, however, experiments in Bench top reactors (BTRs)

with a concentration of 10 g L^{-1} $MgCO_3$ were used. The dissolved CO_2 concentration for each experiment was defined according to the initial medium concentration for 100 % gaseous supply and the saturation concentration was estimated maximum value (Rischbieter et al., 1996) as the one when no additional carbonate source is used (Weisenberger, 1996). The mass transfer coefficient was calculated according to the equation suggested by Fukuda et al., 1968. The predictions of the double substrate model (solid lines) compared with results from the experiment used (x symbols) for parameter fitting, which was also previously employed for the validation of the single substrate model, are presented in Figure 3.

$$\frac{dX}{dt} = \mu_{max} \frac{GLR}{GLR + k_{GLR} + {GLR^2}/{I_{GLR}}} \frac{CO_2}{CO_2 + k_{CO2} + {CO2^2}/{I_{CO2}}} \left(1 - \frac{P_{SA}}{P_{SA}^*}\right)^{n_{SA}} X$$

$$\frac{dGLR}{dt} = -\frac{1}{Y_{X/GLR}}\frac{dX}{dt} - m_{GLR}\,X - \frac{1}{Y_{SA/GLR}}\frac{dP_{SA}}{dt} - \frac{1}{Y_{FA/GLR}}\frac{dP_{FA}}{dt} - \frac{1}{Y_{AA/GLR}}\frac{dP_{AA}}{dt}$$

$$\frac{dP_i}{dt} = a_i\frac{dX}{dt} + \beta_i X \qquad i = SA, AA \tag{3}$$

$$\frac{dCO_2}{dt} = -\frac{1}{Y_{\frac{X}{CO2}}}\frac{dX}{dt} - m_{CO2}\,X - \frac{1}{Y_{\frac{SA}{CO2}}}\frac{dP_{SA}}{dt} + k_L a(CO_2^* - CO_2) + r_M MgCO_3$$

$$GLR + CO_2 + P_{SA} + P_{FA} + P_{AA} - TMB = 0$$

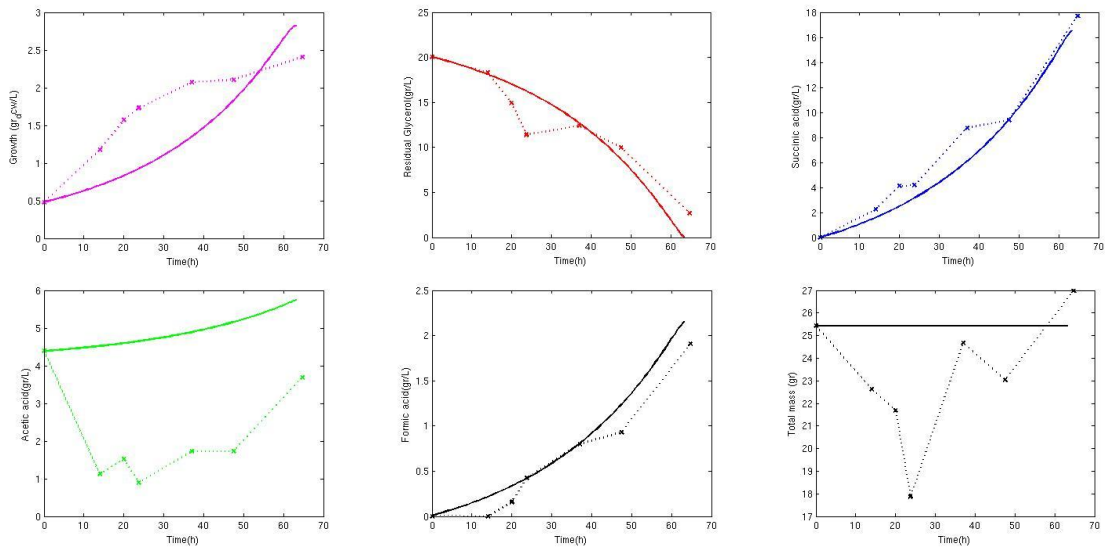

Figure 3: Experimental data (dotted) vs model predictions(line) of an experiment conducted in BTR and included in the parameter fitting procedure

As it can be seen the double substrate model can better simulate the dynamic concentration changes of succinic acid, the desired fermentation product, as well as that of formic acid. It is worthwhile to note here that experimental measurement of the dissolved CO_2 concentrations (currently implemented) will enhance the model and consequently its predictive capabilities.

4. Conclusions

In this work we have developed an improved single-substrate model for the bio-production of succinic acid from glycerol as well as a new double-substrate model, which includes the effect of dissolved CO_2 on the overall process. We have performed parameter estimation studies for both models utilizing experiments in small anaerobic reactors, for the single substrate model and in bench top reactors, for the double substrate one. We have also tested the performance of the single substrate model by making comparisons with experimental results from bench top reactors. Both models can predict the dynamic concentration changes of all state variables reasonably well as well as the corresponding performance indicators, i.e. process yield and productivity. The double substrate model can predict the production of succinic acid and of formic acid with slightly improved accuracy, however its main benefit is that it can be used for the design of

scaled-up experiments since it contains the effect of operational parameters such as, agitation speed, temperature and the supply rate of gaseous CO_2 (included in the kLa parameter) as well as the effect of carbonate content in the medium through the presence and the corresponding rate of dissolution of MgCO3. We are planning to improve the model by including dynamic measurements of dissolved CO_2 and to subsequently use it in conjunction with pilot scale experiments in 150L bioreactors currently underway.

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