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Mathematical modeling of recombinant *Escherichia coli* aerobic batch fermentations

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Abstract

In this work, three competing unstructured mathematical models for the biomass growth by recombinant *E. coli* strains with different acetate inhibition kinetics terms were evaluated for batch processes at constant temperature and pH.
The models considered the dynamics of biomass growth, acetate accumulation, substrate consumption, Green Fluorescence Protein (GFP) production and three metabolic pathways for *E. coli*. Parameter estimation and model validation was carried out using the Systems Biology toolbox for Matlab (The Mathworks) with different initial glucose concentrations (5g/kg to 25g/kg) in a 5dm^3 bioreactor. Model discrimination was based on the two model selection criterion (Akaike's information criterion and normalized quadratic difference between the simulated and experimental data criterion). The first model described by Jerusalimsky approach is an approximation to the non-competitive substrate inhibition. Cockshott approach describes the inhibition at high acetate levels and Levenspiel considers the critical inhibitory acetate concentration that limits growth. Within the studied experimental range, Jerusalimsky model provided a good approximation between real and simulated values and should be favored. The model describes the experimental data satisfactorily well.

1 Introduction

Acetate, when present in *Escherichia coli* fermentations during aerobic growth on glucose is a major obstacle to cell growth and recombinant protein production (Jensen and Carlsen 1990,Nakano *et al.* 1997) and a detailed documentation of acetate production becomes important.
Many different mathematical models were reported in the literature to describe the kinetics of primary metabolites and the acetate overflow metabolism from *E. coli* (Varma and Palsson 1994,Xu *et al.* 1999, Akesson *et al.* 1999). However, there have been few studies focused on the recombinant proteins (GFP) production multi-route models with acetate inhibition kinetics. Lee and Ramirez (1992) used a simple and unstructured model to simulate recombinant protein production that include inducer effect on cell growth and recombinant protein production. A similar model was used by Chae and co-workers (2000) with introduction product inhibition term on the specific growth rate. However, models from previous works for recombinant proteins production are based usually on simplified Monod kinetics description and have neglected the influence of inhibition effect of acetate on *E. coli* growth and glucose uptake although it is known that this play an important role.
The models evaluated here take into account three specific substrate uptakes kinetics with different acetate inhibition terms for the representation of the growth of recombinant *E. coli* strain HMS174AcGFP1. In this work, a set of batch data on fermentations of glucose has been also collected for a better understanding of the environmental effects (glucose concentration) of the acetate overflow phenomenon and

GFP production. The aim of this study was to test three well-established kinetic models for their suitability to describe recombinant *E. coli* growth using batch fermentations data. Subsequently, the best kinetic model can be applied to fed-batch processes in order to determine optimal operation conditions.

2 Material and Methods

Pre-cultures and batch cultures
E. coli HMS174(DE3)pet28aAcGFP1 strains were obtained originally from the stock centre and were used throughout the present study. In these strains, GFP proteins are induced by isopropyl-β-D-thiogalactopyranoside (IPTG). Cells from a glycerol stock culture stored at -80ºC were reactivated by inoculating a frozen aliquot (1ml) into 1L Erlenmeyer flasks with 300 ml of a defined inoculum media with kanamicin (0.03 g kg^{-1}). Flasks were then incubated at 37ºC and 150 rpm until the culture reached exponential phase. Finally, the culture was then transferred into the reactor, containing 3 L from defined medium as described previously in Rocha and Ferreira (2002) to give an initial optical density at 600nm (OD_{600}) in the bioreactor of 0.20-0.25.

Medium and Fermentation Conditions
Cells were grown in a defined medium, with kanamicin and ampicilin as described above. The different initial glucose solution (5.0, 10.0, and 25.0 g kg^{-1}) and the mineral solution were sterilized separately by autoclaving and were later added to the reactor together with a sterile filtered solution containing $MgSO_4$, $CaCl_2$, trace elements, vitamins and thiamine prior to inoculation.
All fermentations were carried out in batch-mode in a 5 L fermenter from B. Braun Biotech (Germany) with a working volume of 3 L equipped with a digital control unit (DCU). In addition, temperature, pH, dissolved O_2 and CO_2 sensors were connected to this DCU and to a data acquisition system. All the cultures were run at ca. 25h and with controlled temperature (at 37ºC) and dissolved oxygen (above 30% to maintain aerobic condition of growth at all times). Silicone (Merck) was used as antifoam reagent.

Analytic Methods
Cell growth was monitored by measuring the OD at 600nm on a UV-visible Jasco V-560 spectrophotometer. The OD_{600} unit was converted to the cell dry weight based on the relation: OD_{600} = 0.21 g kg^{-1}. Samples for acetate and the glucose measurements during the course of the batch fermentations were collected at regular intervals and centrifuged at 10000g in a microcentrifuge for 10 min. After filtration through a 0.2 mm syringe filter the samples were stored at -20ºC for subsequent analysis. Glucose and acetate concentration in samples were detected and measured by high performance liquid chromatography (HPLC) with a Metacarb 87H, 300x7.8mm column (Varian, Palo Alto, CA), a UV detector (Jasco) with a detection wavelength of 210 nm and a refractive index detector (Knauer). The mobile phase used was 0.01N H_2SO_4 with a flow rate of 0.7ml min^{-1} and at a column temperature of 60ºC. The glucose concentration was also measured using commercial enzymatic kits according to the manufacturer's instructions (Spinreact, Sant Esteve de Bas, Spain).

Kinetic Model Development
The presented model combines terms for cell growth, substrate consumption, acetate accumulation and green fluorescence protein (GFP) production for fed-batch cultures with constant pH and temperature. Glucose was assumed to be the only carbon source used for the oxidation and fermentation processes needed for biomass growth and GFP production (eq. 1 and 2). The oxidation of acetate is considered as well (eq. 3) but the oxygen and carbon dioxide dynamic are not. The potential effect of the induction with isopropyl-beta-Dthiogalactopyranoside (IPTG) on the decrease of the growth rate

and yield coefficients was not contemplated in the model. The substrate inhibition was also not considered in the kinetic model.

The reformulated model was based from Bastin and Dochain (1990) and considers three main metabolic pathways:

Oxidation of glucose,

$$k_1 S \xrightarrow{\mu_1} X + k_5 P \qquad \text{if } qs < qs_{cr} \text{ and } A > 0 \qquad (1)$$

fermentation of glucose,

$$k_2 S \xrightarrow{\mu_2} X + k_3 A \qquad \text{if } qs < qs_{cr} \text{ and } A \leq 0 \qquad (2)$$

oxidation of acetate,

$$k_4 A \xrightarrow{\mu_3} X \qquad \text{if } qs > qs_{cr} \text{ and } A > 0 \qquad (3)$$

and the following four ordinary differential equations (4-6), capable of describing glucose fermentation by *E. coli*:

The biomass growth is given by,

$$\frac{dX}{dt} = (\mu_1 + \mu_2 + \mu_3)X - \left(\frac{F}{W}\right)X \qquad (4)$$

and the substrate concentration by,

$$\frac{dS}{dt} = (-\mu_1 k_1 - \mu_2 k_2 - m_S)X - \frac{((\mu_1 + \mu_2 + \mu_3)k_5 + \beta)X}{K_6} - \left(\frac{F}{W}\right)(S_0 - S) \qquad (5)$$

that included the substrate conversion to product formation, maintenance and cell growth.

Finally, the acetate accumulation and product formation is described by equations 6 and 7 respectively,

$$\frac{dA}{dt} = (\mu_2 k_3 - \mu_3 k_4)X - \left(\frac{F}{W}\right)A \qquad (6)$$

$$\frac{dP}{dt} = (\mu_1 + \mu_2 + \mu_3)k_5 X + \beta X - \left(\frac{F}{W}\right)P \qquad (7)$$

where P (GFP production) includes a growth and non-growth associated term. The variable X, S, A and P represents biomass, substrate, acetate and product concentrations, respectively (g kg^{-1} and mg kg^{-1} for product); F and W are the mass flow feed rate and weight of the fermentation broth, respectively (for cultures operated in batch mode F is zero; the k_i are the yield coefficients; β, non-growth associated product constant; m_s maintenance coefficient and μ_i the specific growth rates.
As described in the introduction, it is known that acetate accumulation limits the productivity of the fermentation process. For this reason, three alternative equations for the specific substrate uptake rates (q_s) with different acetate inhibition terms tested in this study at initial glucose concentration 5 g kg^{-1}. Model I, according to Jerusalimsky (Roels, 1983) is an approximation to the non-competitive substrate inhibition. Model II (Cockshott and Bogle 1999) describes the inhibition at high acetate levels and model III (Han and Levenspiel 1988) considers the critical inhibitory acetate concentration that limits growth.

3 Results and Discussion

Parameter estimation and model evaluation

The simulated data are obtained by solving the differential equations (1-4), and the experimental state variables were taken from the experimental studies described in the Materials and Methods section. The kinetic parameters were estimated by fitting the experimental data using the simulated annealing algorithm from the Systems Biology toolbox running in Matlab v.7.1.0 (The Mathworks). For model discrimination, Akaike's information criterion (AIC) and normalized quadratic difference between the simulated and experimental data criterion (*dif*) were used.

Batch fermentations using *E. coli* for HMS174pacGFP strain were performed for estimation and validation of model parameters. Observation of the experimental data revealed that acetate was not metabolized (Figure 1). In this way, the metabolic pathway for the acetate oxidation (μ_3) was not considered in the developed model. Initial estimates for the yield parameter (k_5) were first calculated using the experimental data obtained from the batch fermentations. The initial estimates for the remaining parameters were taken from the literature for *E. coli* on a single substrate. Subsequently, some of the parameters were set to the values described on Table 1 (k_1, k_2, K_s, m_s, β_1, n, and A_{crt}), while all the others parameters were fitted using the experimental data.

Table 1. Initial parameter values (kinetics and yield coefficients) for the growth of *E. coli* K12 taken from the literature and determined from experimental data.

Parameter	Values	Units	Reference (s)
k_1	1.88	($g_S\ g_X^{-1}$)	(Galvanauskas *et al.* 1998)
k_2	20	($g_S\ g_X^{-1}$)	(Rocha, 2003)
$\boldsymbol{k_3}$	14	($g_A\ g_X^{-1}$)	(Rocha, 2003)
k_4	-	($g_A\ g_X^{-1}$)	-
$\boldsymbol{k_5}$	9.22	($mg_P\ g_X^{-1}$)	determined
$\boldsymbol{k_6}$	50	($mg_P\ g_S^{-1}$)	(Aucoin *et al.* 2006a)
$\boldsymbol{qs_{max}}$	1.8	($g\ g^{-1}\ h^{-1}$)	(Galvanauskas *et al.* 1998)
K_s	0.1	($g\ kg^{-1}$)	(Galvanauskas *et al.* 1998)
$\boldsymbol{K_{ai}}$	15.5	($g\ kg^{-1}$)	(Galvanauskas *et al.* 1998)
$\boldsymbol{qs_{cr}}$	0.15	($g\ g^{-1}\ h^{-1}$)	(Akesson *et al.* 1999)
m_s	0.02	($g\ g^{-1}\ h^{-1}$)	(Galvanauskas *et al.* 1998)
β	0.1	($mg\ g^{-1}\ h^{-1}$)	(Aucoin *et al.* 2006b)
A_{crt}	0.9	($g\ kg^{-1}$)	(Aristidou *et al.* 1999)
n	1.41	-	(Théodore and Panda 1999)

The estimated parameters are showed in Table 2. The parameters obtained from the experimental data fitting were in relatively agreement to the values observed in the literature reports for model I, contrary to the model II and III. However, the value estimated from the parameter k_3 and qs_{cr} was higher in comparison to the literature data. This discrepancy might be attributed not only to different operating conditions and/or *E. coli* strains, but also to different modeling strategies to parameter fitting.

The proposed Jerusalimsky and Cockshott approaches (models I and II, respectively) describe very well the experimental data for the time courses of glucose, acetate, biomass growth and GFP concentration (Figure 1). However, the model III curves were in significant disagreement with the experimental data for the acetate concentration. These observations can be confirmed by comparing the statistical analysis from selection models criterions AIC and *dif*. According to the Table 2, models I has the lowest AIC and *dif* values. As a consequence, it was favoured and selected for further analysis.

Table 2. Estimated values of parameters for the model I obtained by fitting the experimental data with 5 g kg^{-1} initial glucose concentration.

Parameter	Model I	Model II	Model III
k_3	33.45	69.89	70.00
k_5	12.78	12.73	12.02
k_6	51.95	25.73	25.00
qs_{max}	0.97	0.89	7.50
K_{ai}	7.75	7.75	25.60
qs_{cr}	0.61	0.63	0.63

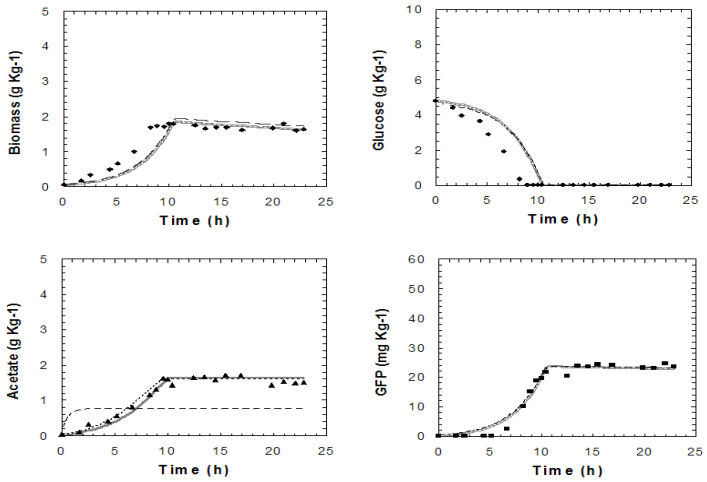

Figure 1. Comparison between experimental and simulated data (a) biomass growth, (b) glucose, (c) acetate and (d) GFP concentration in batch fermentation by HMS174AcGFP1 at 5g kg^{-1} initial glucose concentration. Solid lines represent the simulation data from model I, depicted lines the model II and dashed lines the model III, using the fitted parameters (Table 2). Lines from model I, II and III for glucose and GFP are overlapped for biomass, glucose and GFP concentrations.

Table 3. Model selection criterion Akaike's (AIC) and normalized quadratic difference between the simulated and experimental data (*dif*) at 5.0 g Kg^{-1} initial glucose concentration for model I, II and III.

Criterion	Model I	Model II	Model III
AIC	201.28	221.52	243.14
dif	2.32	2.34	3.45

* AIC and *dif* are the average values from the variable states

To validate the developed model it is necessary to use other experimental data set than that applied to identify initial parameters. Therefore, the selected kinetic model (model I) was validated and tested using the fitted parameters values with different fermentation conditions. Figure 2 compares the experimental data profiles and simulation data 10 g kg^{-1} concentration.

The better fitting with this initial glucose concentration to the experimental biomass, acetate and GFP concentration is observed and demonstrates the applicability of the presented kinetic model. However, the model was unable to predict the experimental data with 25.0 g kg^{-1} glucose concentration (data not shown). Additionally, the simulated glucose concentration at 10 g kg^{-1} glucose concentration was considerably lower than the one obtained by experimental methods, probably because there is a substrate consumption inhibition phenomenon that is not considered in the model.

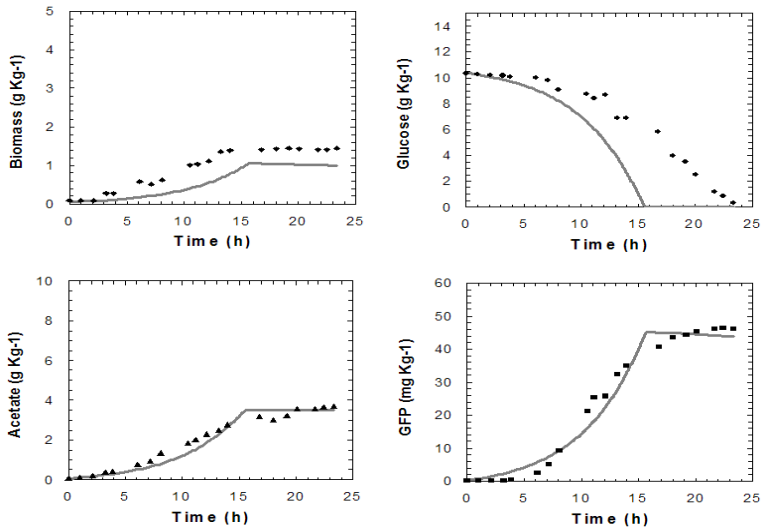

Figure 2 Comparison between experimental and simulated data (a) biomass growth, (b) glucose, (c) acetate and (d) GFP concentration in batch fermentation by HMS174AcGFP1 at 10g kg^{-1} initial glucose concentration.

4. Conclusions

A mathematical unstructured model describing the recombinant protein production (GFP) in *E. coli* HMS174AcGFP1 using acetate inhibition kinetics was developed for batch fermentations. The model considers the inhibition of recombinant cultures by acetate inhibition. Using this modelling approach a satisfactory prediction was achieved (5-10 g Kg^{-1}), with the parameter obtained by experimental data fitting. Within the range of the model, the Jerusalimsky and Cockshott approaches should be favoured.

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