

Kinetic Study of Butanol Production from Mixtures of Glucose and Xylose and Investigation of Different Pre-growth Strategies

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Abstract

This study proposes a dynamic model that describes key characteristics of fermentative butanol production from glucose and xylose mixtures. The model has 12 parameters and incorporates noncompetitive inhibitory interaction between sugars as well as inhibitions due to high substrate and butanol concentrations. Different pre-growth strategies to achieve co-utilization of sugars were explored together with their effects on fermentation kinetics. Mixed sugar fermentation by the cultures pre-grown on a mixture of glucose and xylose showed a higher endurance to inhibition, a 2-fold increase in butanol production and a 1.5-fold increase in total sugar consumption compared to cultures pre-grown on xylose only. The average squared correlation coefficients (r^2) between experimental observations and model predictions were 0.917 and 0.926 for fermentations done by the cultures pre-grown on xylose only, and pre-grown on a mixture of glucose and xylose, respectively. Sensitivity analysis on the model parameters revealed that the growth parameters were the most critical. The proposed model can serve as a basis for modeling of microbial butanol production from lignocellulosic biomass and be applied to other substrates and microorganisms.

Keywords: Butanol; *Clostridia*; lignocellulosic biomass; mixed sugars; dynamic model.

1. Introduction

Biofuels have gained global interest due to environmental concerns, volatility of oil price and supply, and legal restrictions limiting the use of nonrenewable energy sources [1]. n-Butanol (in the further run referred to as butanol) is a promising biofuel alternative based on several advantages compared to the more established biofuels ethanol and methanol: a longer carbon chain length, higher volatility, polarity, combustion value, octane rating and lower corrosive effects and lesser ignition problems due to lower heat of vaporization [2]. Furthermore, diesel engines can run on pure butanol or butanol and diesel blends without any modifications [3].

Sustainable and economical fermentative butanol production faces several challenges such as high substrate cost, solvent toxicity, low cell density and by-product formation. Fermentation substrate has the greatest share in the total costs, accounting for 66% of all [4]. To tackle this problem, many feedstock alternatives have been studied [5] and lignocellulosic biomass is among them because, it is the most abundant renewable biomass resource, and it circumvents the direct fuel-versus-food competition compared to e.g. corn and sugar cane in biofuel production. Hydrolysis of lignocellulosic biomass yields a mixture of pentoses and hexoses, which are fermented to butanol and by-products by the microorganisms. Full exploitation of all sugars bound in lignocellulosic biomass is necessary to decrease the substrate costs. However, the cells' efficiency at using different sugars in mixed form tend to decrease due to the phenomenon *Carbon Catabolite Repression* (CCR). CCR can reduce or prevent the utilization of pentoses such as xylose when a preferred carbon source such as glucose is present [6].

Developing strains capable of co-utilizing hexose and pentose for butanol production is an active research topic in metabolic engineering [6, 7]. Even though Lee et al. (2016) [5] stated metabolic engineering is required for simultaneous utilization of sugars, researchers have developed pre-growth strategies achieving co-utilization without any strain manipulation [8-14]. The suggested pre-growth methods comprised of subjecting a culture to a less favorable carbon source, mostly sole xylose for early activation of its utilization pathway. When a mixture of a sugars, usually glucose and xylose, was then added to the fermentation medium, the culture pre-grown on xylose could simultaneously utilize them. This strategy has successfully been applied for lignocellulosic hydrolysate fermentations and significantly enhanced the fermentation performance in terms of product yield and substrate utilization. However, only two of the above mentioned studies have reported time course data of mixed sugar fermentations for cultures pre-grown on different sugars to illustrate the effect of pre-growth in a systematic manner [8, 11]. Therefore, we investigated the improvement in mixed sugars fermentations with respect to the suggested pre-growth strategies as the literature is lacking analysis and thorough discussion on this topic.

First attempts to model fermentative butanol production date back to 1984 employing carbon balance to determine product yields [15]. Even though a wide variety of model structures has been proposed since then [16, 17], there is still not a consensus about the most appropriate model to use for process design, control and optimization. Moreover, the majority of models is only valid under certain process conditions and regimes [18]. Dynamic models developed by Shinto et al. (2007, 2008) to describe fermentation of glucose and xylose for butanol production

have been central to the recent modeling attempts [19, 20]. For example, Raganati et al. (2015) applied their models for a wider range of pentose and hexose sugars [21]. In a more recent study, Diaz and Willis (2018) extended the model to include CCR for fermentation of glucose and xylose [22] and it was developed simultaneously with our initial model [23]. To our knowledge, these two are currently the only mixed sugar fermentation models developed for butanol production. Despite their rich information content, the complexity level in the models of Shinto et al. (2007, 2008) is high and they require larger experimental datasets for estimation of larger number of parameters necessary to construct the models. On the contrary, traditional unstructured models are simpler and easier to interpret, thus they are still actively being used for describing butanol production by fermentation [24].

The main objective of this study is therefore twofold: i) to establish a dynamic model for fermentative butanol production from mixtures of glucose and xylose, and ii) to investigate the effects of pre-growth strategies on the fermentation kinetics.

2. Theory

2.1. Modeling approach

In this study, we propose a dynamic model based on unstructured mathematical models that have typically been used to estimate the state of fermentative butanol production [25]. The model describes the cell mass (X) growth, uptake of glucose (SG) and xylose (SX) and butanol (B) production. We choose the proper forms of the kinetic equations such that they can describe the key characteristics of the process while avoiding the overparameterization of the model. Following assumptions are made by employing the fermentation biochemistry knowledge to establish the proposed model:

- Glucose and xylose are the only limiting substrates.
- There is no nitrogen limitation.
- Growth inhibition sources are i) high substrate concentration, ii) butanol accumulation and iii) interaction between sugars.
- High substrate inhibition effects are combined for glucose and xylose, and it is in noncompetitive form [26].
- Butanol inhibition is noncompetitive described by parabolic function [26, 27].
- Inhibition effects of substrates on each other is significant and noncompetitive [28].
- Substrate assimilation is only for butanol and cell mass production.
- Substrate consumption for maintenance is negligible.
- Luedeking-Piret model with a growth-associated part describes the butanol production.

We developed the model with the light of the assumptions above using the data of Fond et al. (1986) [11], and our experimental data separately for cultures pre-grown on xylose, and pre-grown on xylose and glucose. We checked the validity and accuracy using two more datasets for each model. Then, we identified the critical parameters by sensitivity analysis.

2.2. Dynamic model for the microbial butanol production from mixtures of glucose and xylose

In our previous study, the Monod equation was modified to describe the growth on mixtures of glucose and xylose together with noncompetitive inhibition between them [28]. Cell mass growth on glucose, μ_{SG} , and xylose, μ_{SX} , are shown in Equation 1 and Equation 2, respectively.

$$\mu_{SG} = \frac{\mu_{\max G} * SG}{(K_{sG} + SG) * \left(1 + \frac{SX}{K_{sX}}\right)} \quad (1)$$

$$\mu_{SX} = \frac{\mu_{\max X} * SX}{(K_{sX} + SX) * \left(1 + \frac{SG}{K_{sG}}\right)} \quad (2)$$

where $\mu_{\max G}$ and $\mu_{\max X}$ are maximum specific growth rates on glucose and xylose, and K_{sG} and K_{sX} are substrate affinity constants for glucose and xylose, respectively. We extended the growth model with substrate and butanol inhibition terms in this study as shown in Equation 3.

$$\mu_g = (\mu_{SG} + \mu_{SX}) * \left(\frac{K_I}{K_I + SG + SX}\right) * \left(1 - \frac{B}{B_{\max}}\right)^{i_B} \quad (3)$$

where μ_g is the specific growth rate of cell mass, K_I is the substrate inhibition constant, B_{\max} is the concentration of butanol at which cell mass growth stops, and i_B is the butanol inhibition constant to cell mass growth. Net growth rate of cell mass, μ_{net} , is the difference between the specific growth rate and specific death rate, k_d , therefore, the cell mass change over time shown in Equation 4 and Equation 5, respectively.

$$\mu_{net} = \mu_g - k_d \quad (4)$$

$$\frac{dX}{dt} = \mu_{net} * X \quad (5)$$

Glucose and xylose uptakes are given in terms of the amounts utilized for cell mass growth and butanol formation, which can be seen in Equation 6 and Equation 7, respectively.

$$\frac{dSG}{dt} = -\mu_{SG} * \left(\frac{1}{Y_{X/SG}} + \frac{1}{Y_{B/SG}}\right) * X \quad (6)$$

where $Y_{X/SG}$ is the cell yield on glucose and $Y_{B/SG}$ is the butanol yield on glucose.

$$\frac{dSX}{dt} = -\mu_{SX} * \left(\frac{1}{Y_{X/SX}} + \frac{1}{Y_{B/SX}}\right) * X \quad (7)$$

where $Y_{X/SX}$ is the cell yield on xylose and $Y_{B/SX}$ is the butanol yield on xylose. Equation 8 shows the butanol formation.

$$\frac{dB}{dt} = (\mu_{SG} * Y_{B/XG} + \mu_{SX} * Y_{B/XX}) * X \quad (8)$$

where $Y_{B/XG}$ is the butanol yield on cell mass utilizing glucose and $Y_{B/XX}$ is the yield on cell mass utilizing xylose. The relationships between the cell mass yields, butanol yields on the substrates and on the cell mass are shown in Equation 9 and Equation 10.

$$Y_{B/XG} = \frac{Y_{B/SG}}{Y_{X/SG}} \quad (9)$$

$$Y_{B/XX} = \frac{Y_{B/SX}}{Y_{X/SX}} \quad (10)$$

The empirical coefficients shown in Equations 1-10 are called as parameters in this study due to their dependency on the species, substrate and environmental conditions.

2.3. Parameter estimation

The proposed model includes 12 parameters listed in Section 2.2 are unknown a priori; therefore, they are estimated from experimental data. In this work, the observed concentrations of cell mass, glucose, xylose, and butanol are used:

$$y = [X][SG][SX][B]^T$$

for estimation of parameters

$$\Theta = [Y_{X/SG} \ Y_{X/SX} \ Y_{B/SG} \ Y_{B/SX} \ k_d \ B_{\max} \ i_B \ K_I \ \mu_{\max G} \ K_{sG} \ K_{sX} \ \mu_{\max X}]^T$$

The parameter estimation poses a nonlinear least-squares optimization problem

$$\hat{\Theta} = \underset{\Theta}{\operatorname{argmin}} \sum_{i=1}^{N_m} \sum_{j=1}^{N_v} (y_{ij}^{\text{obs}} - y_{ij}^{\text{pred}})^2 \quad (11)$$

where $\hat{\Theta}$ denotes the estimated values of the parameters Θ ; and y^{obs} and y^{pred} denote the observed and predicted concentrations of the components. Number of components, N_m , is 4. Number of observations, N_v , is 14. The subscript ij denotes the j^{th} value of the i^{th} component.

The objective of the parameter estimation problem in Equation 11 is to determine the parameters, Θ , by minimizing the squared difference between the observed and predicted concentrations of the components in y . The constrained nonlinear optimization problem is solved using `fmincon` in MATLAB 2017b Optimization Toolbox based on the interior point algorithm together with `ode45` solver (Runge-Kutta 4th order method) for the mass balance equations.

The parameter bounds considered in the optimization problem are given in Table 1. The bounds for yield coefficients were determined using stoichiometric relations between the components [16, 26]. The rest of the parameter bounds was taken from literature [24, 29]. The optimization problem was initialized with the initial points, which were the middle points between the upper and lower bounds of the parameters.

Table 1 Model parameters, their bounds and initial points used for initialization.

Parameter	Unit	Lower bound	Upper bound	Initial point	Source
$Y_{X/SG}$	g/g	0	0.689	0.345	Stoichiometry
$Y_{X/SX}$	g/g	0	0.689	0.345	Stoichiometry
$Y_{B/SG}$	g/g	0	0.412	0.206	Stoichiometry
$Y_{B/SX}$	g/g	0	0.494	0.247	Stoichiometry

k_d	h^{-1}	0	0.500	0.250	[24]
B_{max}	g/l	10	20	15	[24]
i_B	-	0	10	5	[24]
K_I	g/l	125	250	187	[24]
μ_{maxG}	h^{-1}	0	2.000	1.000	[28]
K_{sG}	g/l	0	10	5	[28]
K_{sX}	g/l	0	10	5	[28]
μ_{maxX}	h^{-1}	0	2.000	1.000	[28]

2.4. Index of model accuracy

We obtained the model after parameter estimation, and performed model simulations to check its validity for other experimental data. We calculated sum of squared error (SSE) between the observed and predicted values of the components shown in Equation 12.

$$SSE = \sum_{t=0}^{t_{end}} (y_i^{obs}(t) - y_i^{pred}(t))^2, \quad \forall t=[0, t_{end}], \quad i=X, SG, SX, B \quad (12)$$

where $y_i^{obs}(t)$ and $y_i^{pred}(t)$ denote the observed and predicted concentrations of the components at time t , the subscript i denotes value of the i^{th} measured component.

We calculated sum of squared total (SST), which is a quantification of the observations', $y_i^{obs}(t)$ variation around their mean, \bar{y}_i^{obs} shown in Equation 13.

$$SST = \sum_{t=0}^{t_{end}} (y_i^{obs}(t) - \bar{y}_i^{obs})^2, \quad \forall t=[0, t_{end}], \quad i=X, SG, SX, B \quad (13)$$

By using SSE and SST, we calculated average squared correlation coefficients (r^2) for each component with the formula given in Equation 14.

$$r^2 = 1 - \frac{SSE}{SST} \quad (14)$$

r^2 values were used as the index of model accuracy.

2.5. Sensitivity analysis of model parameters

We conducted the sensitivity analysis by 10% perturbations in each of 12 parameters. The sensitivity can be measured by comparing the final concentrations of components with perturbed and unperturbed parameters. One parameter is perturbed at a time while keeping the rest of the model parameters the same as their original estimates, and model simulations are done with the new parameter set which involves one perturbed and 11 unperturbed parameters. As a result, end point deviations (ED) of cell mass, glucose, xylose and butanol are calculated with respect to their reference values obtained with the parameter set consisting only of the unperturbed parameters. ED values were calculated as in Equation 15.

$$ED_i^P(\%) = \frac{y_i(\Theta \pm \Delta\Theta, t_{end}) - y_i^{ref}(\Theta, t_{end})}{y_i^{ref}(\Theta, t_{end})}, \quad i=X, SG, SX, B \quad (15)$$

where $y_i(\Theta, t_{\text{end}})$ and $y_i(\Theta \pm \Delta\Theta, t_{\text{end}})$ represent the predicted concentration of i^{th} component at time t_{end} associated with unperturbed parameter Θ and perturbed parameter $\Theta \pm \Delta\Theta$, respectively. $y_i^{\text{ref}}(\Theta, t_{\text{end}})$ is the end concentration of the i^{th} component in the reference.

3. Materials and methods

3.1. Microorganism and culture conditions

Wild type *Clostridium beijerinckii* NCIMB 8052 was used in this study, since it can utilize both glucose and xylose [30]. We applied a two-stage pre-growth strategy based on our previous work, which enables the culture to co-utilize glucose and xylose [8]. First step was the growth of a frozen work ampoule (1 ml) for 14 hours on 50 ml of reinforced clostridial medium (CM0149, Oxoid) under static conditions at 37 °C. In the second step, the culture was grown for 6 hours on a medium containing 2.5 g/l Na-acetate, 5 g/l yeast extract, 2 g/l $(\text{NH}_4)_2\text{SO}_4$, 0.01 g/L NaCl, 0.75 g/l KH_2PO_4 , 1.5 g/l K_2HPO_4 , 0.2 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g/l $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.01 g/l $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g/l p-aminobenzoic acid, 0.01 g/l biotin, 0.1 g/l thiamine, and 5 g/l xylose as the sole sugar to obtain xylose pre-grown culture or 5 g/l glucose and xylose with a ratio of 1:1 for glucose and xylose pre-grown culture. In fed-batch fermentations, the same medium was used as in the second pre-growth step with different total sugar concentrations. As part of a literature review study, we collected data from 175 lignocellulosic biomass fermentations documented in publications the past 30 years, and the results showed that the total amount of glucose and xylose used was in the range of 18 and 47 g/l. Therefore, we selected minimum and maximum concentrations as 15 and 45 g/l and did parameter estimation with 30 g/l as their mean value.

3.2. Fed-batch fermentations

We conducted fed-batch growth experiments in 120 ml serum flasks with 50 ml working volume in an incubator with temperature controlled at 37°C under static and anaerobic conditions. We used an inoculum size of 4% v/v. There was no pH control applied. We took 1 ml samples at sampling times of 0, 4, 8, 12, 16, 24, 26, 28, 30, 32, 36, 40 and 48 hours with respect to the start of the experiment. The feeding frequency was selected according to average sugar consumption rates that we obtained in our earlier experiments, adjusted with respect to the sugar concentrations (15, 30, 45 g/l). Sugar feeding was done with a concentrated, 232.5 g/l sugar solution containing equal amounts of glucose and xylose. The feeding was done such that the volumes removed during sampling and the volumes added during feeding were equal and the same for all the experiments. The cultures with 15 g/l total sugars (X15 and GX15, validation datasets) were fed with the sugar solution every 8 hours, the ones with 30 g/l (X30 and GX30, parameter estimation datasets) were fed every 16 hours, and the ones with 45 g/l (X45 and GX45, validation datasets) were fed every 24 hours. Experiments terminated after 48 hours.

3.3. Analytical methods

Optical density (OD) was used as a measure for cell mass concentration, measured at 660 nm with a UV-vis spectrophotometer UV-1700 (Shimadzu) with water as the reference. Samples exceeding 0.4 OD were diluted with water so that the Beer-Lambert Law is applied. The OD readings were converted to cell mass concentrations according to the correlation obtained in

our previous study [8]. The samples for determining concentrations of glucose, xylose and butanol were filtrated (Millipore filter, 0.2 μm) before analysis using a HPLC system (Shimadzu Model 9) equipped with UV (210 nm) and RI detector and an Aminex HPX-87H column (Biorad). Samples were eluted with 5 mM H_2SO_4 , flow rate 0.6 ml/min at 45°C. Quantification was performed using standards for each component.

4. Results and discussion

4.1. Effect of pre-growth strategy on fermentation kinetics

We estimated the model parameters for 4 datasets: glucose and xylose fermentations by i) xylose pre-grown (FondX), and ii) glucose and xylose pre-grown cultures (FondGX) from the work of Fond et al. (1986) [11], and our experimental data of glucose and xylose fermentations by iii) xylose pre-grown (X30), and iv) glucose and xylose pre-grown cultures (GX30) with 30 g/l total sugar containing equal amounts of glucose and xylose. Parameter estimates for all 4 datasets given in Table 2 were interpreted to illustrate the effects of different pre-growth strategies on the fermentation kinetics.

Table 2 Parameter estimation results.

Parameter	Xylose pre-grown		Glucose and xylose pre-grown	
	X30 (This study)	FondX [11]	GX30 (This study)	FondGX [11]
$Y_{X/SG}$	0.199	0.097	0.523	0.157
$Y_{X/SX}$	0.292	0.260	0.058	0.223
$Y_{B/SG}$	0.265	0.250	0.196	0.319
$Y_{B/SX}$	0.057	0.389	0.232	0.399
k_d	0.055	0.033	0.076	0.026
B_{\max}	15.632	15.796	15.658	17.243
i_B	1.125	2.138	0.616	1.695
K_I	143.629	186.199	171.492	187.667
$\mu_{\max G}$	0.982	1.444	0.730	1.204
K_{sG}	1.842	5.634	1.293	4.637
K_{sX}	2.066	6.974	4.469	5.884
$\mu_{\max X}$	0.487	1.292	0.615	1.025

Cell mass yields on glucose ($Y_{X/SG}$) were 0.199 and 0.523 g/g, and cell mass yields on xylose ($Y_{X/SX}$) were 0.292 and 0.058 g/g for xylose pre-grown culture (X30), and glucose and xylose pre-grown culture (GX30), respectively. Therefore, a greater proportion of glucose was converted to cell mass in GX30 than in X30, and vice versa for xylose. This difference in GX30 and X30 cultures may relate to different expression levels of sugar utilization enzymes. To elaborate, glucose utilization enzymes were likely to be relatively more prominent than xylose utilization enzymes in GX30 since it is the preferred carbon source [6]. Similarly, xylose assimilation enzymes were readily available in X30 when the glucose and xylose mixture was added and could utilize xylose at a higher efficiency for cell mass production. Analogously, FondGX had higher $Y_{X/SG}$ and lower $Y_{X/SX}$ than FondX showing the same trend. Maximum specific growth rate of cell mass on glucose, $\mu_{\max G}$ and substrate affinity constant for glucose,

K_{sG} was lower for FondGX and GX30 indicating that the cells pre-grown on glucose and xylose grew slower on glucose and were more attracted to glucose. However, changes in μ_{maxX} and K_{sX} exhibited opposite trends for our cultures, X30 and GX30, and the results of Fond et al. (1986) [31], FondX and FondGX. This difference in the parameters of xylose growth model can be the result of *C. beijerinckii* (used in our experiments) having more sets of xylose metabolic pathway genes than *C. acetobutylicum* (used in Fond et al. (1986)) [31]. Thus, different sugar utilization mechanisms may have caused variance in the xylose growth parameters.

Both butanol yield on glucose, $Y_{B/SG}$ and butanol yield on xylose, $Y_{B/SX}$ were greater for glucose and xylose pre-grown culture, FondGX than xylose pre-grown culture, FondX. The same increase was observed for $Y_{B/SX}$ in GX30 compared to X30. Therefore, glucose and xylose pre-grown cultures produced more butanol. The highest $Y_{B/SG}$ was 0.319 g/g and the highest $Y_{B/SX}$ was 0.399 g/g representing the 77.4% of the maximum theoretical butanol yield on glucose, 0.412 g/g, and 80.7% of the maximum theoretical butanol yield on xylose, 0.494 g/g, respectively. The concentrations of butanol at which cell mass growth stops, B_{max} were 15.632, 15.658, 15.796 and 17.243 g/l for X30, GX30, FondX and FondGX, respectively. Even though B_{max} values obtained for our datasets, X30 and GX30 were similar, this might indicate that glucose and xylose pre-grown cultures were more tolerant to butanol toxicity. Similarly, butanol inhibition constants to cell mass growth, i_B were 1.125, 0.616, 2.138, 1.695 for X30, GX30, FondX and FondGX, respectively indicating the extent of butanol inhibition is greater for xylose pre-grown cultures. The toxic butanol concentration values and butanol inhibition constants are in good agreement with both previously reported experimental observations [27] and estimations in modeling studies [24]. Further experimental evidence confirms that the inhibitory effects of butanol on *C. acetobutylicum* were more pronounced in xylose-grown cells than in glucose-grown cells, and glucose and xylose permease were inhibited when butanol concentration reached 12 and 8 g/l, respectively [14].

Substrate inhibition constant, K_I values were 143.629, 171.492, 186.199, and 187.667 g/l for X30, GX30, FondX and FondGX, respectively. Thus, the substrate inhibition on the growth was greater for the xylose pre-grown cultures. K_I estimates coincide with the literature information where it was stated that the cell growth was inhibited strongly when the total substrate concentration was 200 g/l and stopped entirely when it was 250 g/l for a mixture of sugars containing mostly glucose [32]. These results principally agree with the results of Raganati et al. (2015), in which they found that the cultures fed with glucose possessed the highest metabolic activity and lowest tendency to sporulate compared to the cultures with pentose sugars [21]. Even though this might be an indication of a shorter lifespan for xylose pre-grown cells, further investigation is necessary.

4.2. Comparison of model predictions and experimental observations

Simulations were performed by employing the model with the parameter estimates given in Table 2. The model predictions and the observed values for the FondX and FondGX datasets are shown in Figure 1.

Figure 1 Comparison of model predictions and experimental observations with the data from Fond et al. (1986) for a) xylose pre-grown culture, FondX, and b) glucose and xylose pre-grown culture, FondGX [11].

Figure 1.a shows the predicted and observed values of cell mass, glucose, xylose and butanol concentrations for fermentation of a sugar mixture of 53 g/l xylose and 25 g/l glucose by xylose pre-grown culture, FondX. Exponential growth phase during which the cell mass increased rapidly was observed until 10 hours with respect to the start of the experiment, and followed by a steady cell mass growth until 40 hours. Glucose utilization started immediately and lasted for 25 hours. Only after complete depletion of glucose, xylose utilization became apparent and lasted until the end of the fermentation with a decreased rate from 64 hours when butanol concentration reached 14 g/l. Butanol production was slow initially, became faster between 16 and 54 hours, and almost stopped around 64 hours. The residual xylose concentration was 11 g/l, and butanol was 14.5 g/l in the end of the fermentation. Figure 1.b shows the predicted and observed values for fermentation of a sugar mixture of 36 g/l xylose and 39 g/l glucose by glucose and xylose pre-grown culture, FondGX. Exponential growth lasted longer in this fermentation, continued for 22 hours, and then steady growth occurred until 47 hours and cell mass concentration decreased until the end of the experiment. Initially, glucose was consumed rapidly, and xylose utilization was very slow. After 22 hours, both sugars were co-utilized almost at the same rate. Glucose was completely depleted at 47 hours, while xylose consumption continued. Xylose consumption rate decreased when butanol concentration was 16 g/l. The residual xylose and butanol concentrations were 4.3 and 16.6 g/l, respectively. Higher butanol concentration and lower residual xylose in FondGX than in FondX are line with the estimated parameters as well as the butanol concentrations when the sugar utilization rates dropped. Therefore, the model can describe the fermentation kinetics for both pre-growth strategies. A more detailed overview of the model accuracy in terms of average squared correlation coefficients (r^2) between the predicted and observed values is given in Table 3.

The model predictions and our experimental observations for X30 and GX30, parameter estimation experiments, and X15, GX15, X45, and GX45, validation experiments are shown in Figure 2.

Figure 2 Comparison of model predictions and our experimental observations for xylose pre-grown cultures a) X15, b) X30, c) X45, and glucose and xylose pre-grown cultures d) GX15, e) GX30, f) GX45.

Figures 2.a, 2.b and 2.c show the predicted and observed values of cell mass (volume corrected), accumulated consumption of glucose, xylose and accumulated butanol concentrations for fed-batch fermentations by xylose pre-grown cultures, X15, X30 and X45. All the cultures showed the same growth pattern; cell mass concentrations increased exponentially as soon as the experiments started followed by a steady growth phase, followed by a decay phase during which the apparent cell mass concentration decreased [26]. In all the experiments, xylose consumption rates were slightly higher and utilizations were simultaneous with glucose. Consumption rates of both glucose and xylose decreased as the fermentations

proceeded. Butanol concentrations showed the same trend as the cell mass confirming the growth-associated production as shown in Equation 8.

Figures 2.d, 2.e and 2.f show the model predictions and experimental observations of cell mass (volume corrected), accumulated consumption of glucose, xylose and accumulated butanol concentrations for fed-batch fermentations by glucose and xylose pre-grown cultures, GX15, GX30 and GX45. Cell mass concentrations showed the same growth pattern as in the fermentations with xylose pre-grown cultures explained above. In all experiments, sugar utilizations were simultaneous and almost at the same rate unlike the xylose pre-grown culture experiments in which the xylose consumption rates were higher than those for glucose. Sugar consumption rates became slower as the fermentations continued. Similarly, butanol and cell mass productions were closely linked.

In accordance with the results from Fond et al. (1986) [31], glucose and xylose pre-grown cultures in our experiments showed better tolerance to inhibitions as well as 2-fold increase in butanol production and 1.5-fold increase in sugar utilizations. For all our experiments, cell mass growth rate, total amounts of sugar utilizations and butanol production increased with decreasing total sugar concentration. The reason is the inhibition due to high substrate concentration and noncompetitive inhibition between sugars. Model predictions and observations were in good agreement as derivable from the r^2 values shown in Table 3.

Table 3 Average squared correlation coefficients (r^2) between predicted and observed values.

Dataset	Cell mass	Glucose	Xylose	Butanol	Average
X15	0.122	0.944	0.851	0.903	0.705
X30	0.842	0.975	0.973	0.914	0.926
X45	0.552	0.921	0.894	0.524	0.723
GX15	0.754	0.935	0.873	0.925	0.872
GX30	0.934	0.982	0.991	0.778	0.917
GX45	0.752	0.094	0.248	0.221	0.329
FondX	0.595	0.998	0.350	0.591	0.633
FondGX	0.657	0.997	0.623	0.826	0.776
Average	0.651	0.856	0.725	0.710	0.735

Average squared correlation coefficients (r^2) for the parameter estimation datasets were 0.926, 0.917, 0.633, and 0.776 for X30, GX30, FondX and FondGX, respectively. Our results are in the range of r^2 values calculated in similar studies. The r^2 values between model predictions and experimental observations found in the works of Shinto et al. were 0.909 and 0.970; in results of Raganati et al. were 0.894 and 0.890 for fermentations of 65.7 mM (10 g/l) xylose and 70.6 mM (12.7 g/l) glucose, and 60 g/l glucose and 60 g/l xylose, respectively [19-21]. It is important to note that the fermentations in these studies were single sugar fermentations. To our knowledge, the only model proposed for mixed sugar fermentation was by Diaz and Willis (2018), and r^2 was 0.955 for fermentation of 32 g/l xylose and 31 g/l glucose [22]. The higher

average squared correlation coefficients can be due to use of a more detailed model considering more metabolites and a variety of different datasets used in parameter estimation.

The r^2 values were further assessed to check the model accuracy with our validation datasets. Model predictability was satisfactory for X15, X45 and GX15, while poor for GX45. The lowest average r^2 between predicted and observed values for cell mass concentration was 0.571. This eventually caused a lower overall model accuracy. Predictability of cell mass was lowest in the literature values as well. Therefore, further efforts need to focus on enhancing the model predictability for cell mass concentration.

4.3. Sensitivity analysis on model parameters

We identified the critical parameters by performing a sensitivity analysis. The reference trajectory was the concentration profiles from model simulations under the given initial concentrations and model parameters estimated for X30 and GX30. Figure 3 shows the sensitivity analysis for the cell mass, glucose, xylose and butanol in terms of end point deviations (%) in concentrations with 10% perturbations in the parameters.

Figure 3 Sensitivity analysis on model parameters for a) and e) cell mass, b) and f) glucose, c) and g) xylose, and d) and h) butanol concentrations.

End point deviations (%) were significantly larger in cell mass, glucose and xylose concentrations for GX30 than X30, while in the same range for butanol. Figures 3.a and 3.b show that +10% variations of all growth parameters resulted in a greater end point cell mass concentration due to increased net growth rate. While positive influence of $\mu_{\max G}$ and $\mu_{\max X}$ on growth is apparent, the same impact of K_{sG} and K_{sX} can be explained by decreased competitive inhibition between the sugars when $SG > K_{sG}$ and $SX > K_{sX}$ as shown in Equations 1 and 2. The greatest end point deviation in cell mass was 49.68% in GX30 with respect to a +10% increase in $\mu_{\max G}$. Another critical parameter was specific death rate of cell mass, k_d and its -10% perturbation caused 11.28 and 18.48% end point deviations in cell mass concentrations in X30 and G30, respectively as a result of increased net growth rate, μ_{net} .

Figures 3.b and 3.f illustrate that glucose concentrations were most sensitive to the perturbations in growth parameters due to its correlation with cell mass concentration as given in Equation 6. Therefore, any perturbation of any parameter causing an increase in cell mass concentration results in a decrease in glucose concentration. Moreover, +10% variation in $Y_{B/SG}$ yielded 3.84 and 16.68% end point deviations for X30 and GX30, respectively, as a result of greater growth inhibition due to increased butanol level. Figures 3.c and 3.g show that the critical parameters were the same for xylose as for glucose. The greatest end point deviations in xylose concentrations were 12.66% for X30 and 52.8% for G30 resulting from -10% variations in $\mu_{\max X}$ and $\mu_{\max G}$, respectively.

Growth parameters were the most critical for butanol since its production is dependent on growth and cell mass concentrations as given in Equation 8. Figures 3.d and 3.h show that +10% variation in the most critical parameter, $\mu_{\max G}$ resulted in 21.17 and 38.95% end point deviations, while +10% variation of the second most important parameter, K_{sX} caused 16.88 and 21.27% end point deviations for X30 and GX30, respectively. Sensitivity analysis

identified critical parameters, which can be re-estimated with more experimental data to improve the model accuracy.

5. Conclusions

A dynamic model structure describing key characteristics of fermentative butanol production from glucose and xylose mixtures was proposed. Literature data [11] and our experimental results of fermentations with cultures pre-grown on xylose as the sole sugar, and cultures pre-grown on a mixture of glucose and xylose were used for estimation of the parameters in the proposed model structure. Parameter estimates for both literature and our experimental data revealed that pre-growth has a profound impact on the kinetics, and parameter values coincide with observations done in similar studies. Sugar utilization and butanol production were higher in fermentations by cultures pre-grown on glucose and xylose. Sugar utilizations and butanol productions decreased with increasing initial sugar concentrations, which is consistent with the results of our exploratory data analysis performed by using data of 175 fermentations with lignocellulosic hydrolysates and mixed sugars (manuscript submitted). We validated both models developed for both pre-growth strategies with two more experimental datasets for each, and average squared correlation coefficients (r^2) between predicted and observed values were satisfactory. Growth parameters were critical for all components as identified by sensitivity analysis. The main contributions of this study can be stated as below:

1. This is the first study, which investigates the effect of different pre-growth strategies on kinetics of mixed sugar fermentations.
2. The proposed model is the first attempt to incorporate the noncompetitive inhibition between sugars together with high substrate and butanol inhibitions.
3. This paper provides insight into contributions of each sugar to cell mass growth and butanol formation in terms of yield parameters.

The suggested model can serve as a basis to describe fermentations of lignocellulosic biomass. The cell mass growth part of the model can be extended with the inhibitions due to the presence of inhibitory components in real biomass-derived substrates, which would reflect on sugar utilization and thus butanol production. Therefore, the extension of the suggested model would improve its applicability to industrial fermentation processes.

Authors contributions

CB performed the experiments and wrote the manuscript. OTB, HAP, AW contributed with drafting and finalizing the manuscript.

Acknowledgements

This work was supported by the Research Council of Norway, grant no. 246821/E20 (EcoLodge). The authors thank Kathinka Lystad and Kari Hjelen for guidance during HPLC analysis, Sidsel Markussen and Randi Aune for sharing their knowledge and expertise in fermentation, and Bernd Wittgens for discussions about the process and modeling.

Nomenclature

B = concentration of butanol (g/l)

B_{acc} = accumulated concentration of produced butanol (g/l)

B_{max} = concentration of butanol at which cell mass growth stops (g/l)

i_B = butanol inhibition constant to cell mass growth

k_d = specific death rate of cell mass (h^{-1})

K_I = substrate inhibition constant (g/l)

K_{sG} = substrate affinity constant for glucose (g/l)

K_{sX} = substrate affinity constant for xylose (g/l)

SG = concentration of glucose (g/l)

SG_{acc} = accumulated concentration of consumed glucose (g/l)

SX = concentration of xylose (g/l)

SX_{acc} = accumulated concentration of consumed xylose (g/l)

X = concentration of cell mass (g/l)

$Y_{B/SG}$ = butanol yield on glucose (g/g)

$Y_{B/SX}$ = butanol yield on xylose (g/g)

$Y_{B/XG}$ = butanol yield on cell mass utilizing glucose (g/g)

$Y_{B/XX}$ = butanol yield on cell mass utilizing xylose (g/g)

$Y_{X/SG}$ = cell mass yield on glucose (g/g)

$Y_{X/SX}$ = cell mass yield on xylose (g/g)

Greek letter

μ_g = specific growth rate of cell mass (h^{-1})

μ_{maxG} = maximum specific growth rate of cell mass on glucose (h^{-1})

μ_{maxX} = maximum specific growth rate of cell mass on xylose (h^{-1})

μ_{net} = net growth rate of cell mass (h^{-1})

μ_{SG} = specific growth rate of cell mass on glucose (h^{-1})

μ_{SX} = specific growth rate of cell mass on xylose (h^{-1})

References

- [1] A. Ranjan, V.S. Moholkar, Biobutanol: science, engineering, and economics, International Journal of Energy Research, 36 (2012) 277-323.
- [2] L.J. Visioli, H. Enzweiler, R.C. Kuhn, M. Schwaab, M.A. Mazutti, Recent advances on biobutanol production, Sustainable Chemical Processes, 2 (2014) 15.

- [3] J. Campos-Fernández, J.M. Arnal, J. Gómez, M.P. Dorado, A comparison of performance of higher alcohols/diesel fuel blends in a diesel engine, *Applied Energy*, 95 (2012) 267-275.
- [4] Y. Jiang, J. Liu, W. Jiang, Y. Yang, S. Yang, Current status and prospects of industrial bio-production of n-butanol in China, *Biotechnology Advances*, 33 (2015) 1493-1501.
- [5] S.-H. Lee, E.J. Yun, J. Kim, S.J. Lee, Y. Um, K.H. Kim, Biomass, strain engineering, and fermentation processes for butanol production by solventogenic clostridia, *Applied Microbiology and Biotechnology*, 100 (2016) 8255-8271.
- [6] C. Ren, Y. Gu, S. Hu, Y. Wu, P. Wang, Y. Yang, C. Yang, S. Yang, W. Jiang, Identification and inactivation of pleiotropic regulator CcpA to eliminate glucose repression of xylose utilization in *Clostridium acetobutylicum*, *Metabolic Engineering*, 12 (2010) 446-454.
- [7] F. Xin, Y. Wu, J. He, Simultaneous fermentation of glucose and xylose to butanol by *Clostridium* species strain BOH3, *Applied and Environmental Microbiology*, (2014) AEM. 00337-00314.
- [8] C. Birgen, S. Markussen, A. Wentzel, H.A. Preisig, The Effect of Feeding Strategy on Butanol Production by *Clostridium beijerinckii* NCIMB 8052 using Glucose and Xylose, *Chemical Engineering Transactions*, 65 (2018) 283-288.
- [9] A. Boonsombuti, K. Komolpis, A. Luengnaruemitchai, S. Wongkasemjit, Enhancement of ABE fermentation through regulation of ammonium acetate and D-xylose uptake from acid-pretreated corncobs, *Annals of Microbiology*, 64 (2014) 431-439.
- [10] A. El Kanouni, I. Zerdani, S. Zaafa, M. Znassni, M. Loutfi, M. Boudouma, The improvement of glucose/xylose fermentation by *Clostridium acetobutylicum* using calcium carbonate, *World Journal of Microbiology and Biotechnology*, 14 (1998) 431-435.
- [11] O. Fond, J.M. Engasser, G. Matta-El-Amouri, H. Petitdemange, The acetone butanol fermentation on glucose and xylose. I. Regulation and kinetics in batch cultures, *Biotechnology and Bioengineering*, 28 (1986) 160-166.
- [12] B.L. Magalhães, M.C.B. Grassi, G.A. Pereira, M. Brocchi, Improved n-butanol production from lignocellulosic hydrolysate by *Clostridium* strain screening and culture-medium optimization, *Biomass and Bioenergy*, 108 (2018) 157-166.
- [13] T. Noguchi, Y. Tashiro, T. Yoshida, J. Zheng, K. Sakai, K. Sonomoto, Efficient butanol production without carbon catabolite repression from mixed sugars with *Clostridium saccharoperbutylacetonicum* N1-4, *Journal of Bioscience and Bioengineering*, 116 (2013) 716-721.
- [14] K. Ounine, H. Petitdemange, G. Raval, R. Gay, Regulation and butanol inhibition of D-xylose and D-glucose uptake in *Clostridium acetobutylicum*, *Applied and Environmental Microbiology*, 49 (1985) 874-878.
- [15] E.T. Papoutsakis, Equations and calculations for fermentations of butyric acid bacteria, *Biotechnology and Bioengineering*, 67 (1984) 813-826.
- [16] R. Mayank, A. Ranjan, V.S. Moholkar, Mathematical models of ABE fermentation: review and analysis, *Critical Reviews in Biotechnology*, 33 (2013) 419-447.
- [17] T. Millat, K. Winzer, Mathematical modelling of clostridial acetone-butanol-ethanol fermentation, *Applied Microbiology and Biotechnology*, 101 (2017) 2251-2271.
- [18] H.I. Velázquez-Sánchez, R. Aguilar-López, Novel kinetic model for the simulation analysis of the butanol productivity of *Clostridium acetobutylicum* ATCC 824 under different reactor configurations, *Chinese Journal of Chemical Engineering*, 26 (2018) 812-821.

- [19] H. Shinto, Y. Tashiro, M. Yamashita, G. Kobayashi, T. Sekiguchi, T. Hanai, Y. Kuriya, M. Okamoto, K. Sonomoto, Kinetic modeling and sensitivity analysis of acetone–butanol–ethanol production, *Journal of Biotechnology*, 131 (2007) 45-56.
- [20] H. Shinto, Y. Tashiro, G. Kobayashi, T. Sekiguchi, T. Hanai, Y. Kuriya, M. Okamoto, K. Sonomoto, Kinetic study of substrate dependency for higher butanol production in acetone–butanol–ethanol fermentation, *Process Biochemistry*, 43 (2008) 1452-1461.
- [21] F. Raganati, A. Procentese, G. Olivieri, P. Götz, P. Salatino, A. Marzocchella, Kinetic study of butanol production from various sugars by *Clostridium acetobutylicum* using a dynamic model, *Biochemical Engineering Journal*, 99 (2015) 156-166.
- [22] V.H.G. Díaz, M.J. Willis, Kinetic modelling and simulation of batch, continuous and cell-recycling fermentations for acetone-butanol-ethanol production using *Clostridium saccharoperbutylacetonicum* N1-4, *Biochemical Engineering Journal*, 137 (2018) 30-39.
- [23] C. Birgen, H.A. Preisig, Dynamic Modeling of Butanol Production from Lignocellulosic Sugars, *Computer Aided Chemical Engineering*, Elsevier2018, pp. 1547-1552.
- [24] M.-H. Eom, B. Kim, H. Jang, S.-H. Lee, W. Kim, Y.-A. Shin, J.H. Lee, Dynamic modeling of a fermentation process with ex situ butanol recovery (ESBR) for continuous biobutanol production, *Energy and Fuels*, 29 (2015) 7254-7265.
- [25] A. Srivastava, B. Volesky, Updated model of the batch acetone-butanol fermentation, *Biotechnology Letters*, 12 (1990) 693-698.
- [26] M.L. Shuler, F. Kargi, *Bioprocess Engineering: Basic Concepts*. 2nd, Upper Saddle2002.
- [27] X. Yang, G.T. Tsao, Mathematical modeling of inhibition kinetics in acetone-butanol fermentation by *Clostridium acetobutylicum*, *Biotechnology Progress*, 10 (1994) 532-538.
- [28] C. Birgen, S. Markussen, A. Wentzel, H. Preisig, B. Wittgens, Modeling the growth of *clostridium beijerinckii* ncimb 8052 on lignocellulosic sugars, *Chemical Engineering Transactions*, 65 (2018) 289-294.
- [29] E.A. Buehler, A. Mesbah, Kinetic study of acetone-butanol-ethanol fermentation in continuous culture, *PloS One*, 11 (2016) e0158243.
- [30] L. Zhang, S.A. Leyn, Y. Gu, W. Jiang, D.A. Rodionov, C. Yang, Ribulokinase and transcriptional regulation of arabinose metabolism in *Clostridium acetobutylicum*, *Journal of Bacteriology*, (2011) JB. 06241-06211.
- [31] J. Nölling, G. Breton, M.V. Omelchenko, K.S. Makarova, Q. Zeng, R. Gibson, H.M. Lee, J. Dubois, D. Qiu, J. Hitti, Genome sequence and comparative analysis of the solvent-producing bacterium *Clostridium acetobutylicum*, *Journal of Bacteriology*, 183 (2001) 4823-4838.
- [32] N. Qureshi, B.C. Saha, M.A. Cotta, Butanol production from wheat straw hydrolysate using *Clostridium beijerinckii*, *Bioprocess and Biosystems Engineering*, 30 (2007) 419-427.