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Modeling the Growth of *Clostridium beijerinckii* NCIMB 8052 on Lignocellulosic Sugars

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To our knowledge, this is the first growth model of *Clostridium beijerinckii* NCIMB 8052 on glucose and xylose as representative lignocellulosic sugars, which considers the synergistic effects of sugars on the growth rate. We fitted models with different types of interactions between the substrates to the growth rate data obtained with varying sugar concentrations. Noncompetitive binary substrate growth model gave the best fit with the smallest mean standard errors (MSE), and sum of squares error (SSE), 0.0778 and 0.0071, respectively. Confidence intervals for the parameter estimates showed that the substrate affinity constant for xylose, K_{sX} (g/l) had the largest uncertainty, while the maximum specific growth rate on xylose, μ_{maxX} (h^{-1}) had the smallest. The correlation matrix showed that the model parameters were highly correlated. Carbon catabolite repression (CCR) effect on the growth rate was of the noncompetitive type. Validation with other sugar concentration values is necessary to evaluate the prediction capability of the proposed model. A transcriptional study will be beneficial to understand global gene regulation mechanisms as guidance for improving the efficiency of lignocellulosic fermentation processes.

1. Introduction

Lignocellulosic biomass is a promising feedstock, since it is the most abundant renewable biomass resource on the planet. Its hydrolysis yields both hexoses and pentoses, and the composition of the biomass depends on the plant species, which also can vary depending on age and growth conditions. Jørgensen et al., (2007) reported a typical dry weight composition of lignocellulosic biomass shown in Table 1.

Table 1: Typical dry weight of lignocellulosic biomass (Jørgensen et al., 2007).

	Glucose	Xylose	Arabinose	Mannose	Lignin
Dry weight (%)	32.2 - 46.4	4.9 - 24.9	1.1 - 2.9	0.3 - 12	11.9 - 29.4

Fermentation performance of microorganisms depends on their capacity to co-utilize these sugars in the hydrolysate. However, the cells' efficiency in utilizing different sugars in mixed form tends to decrease due to mechanisms of carbon catabolite repression (CCR). CCR reduces or prevents the utilization of pentose sugars in the presence of a preferred carbon source (Ren et al., 2010). There are ongoing efforts of metabolic engineering (Lee et al., 2016) of strains which can simultaneously utilize both hexose and pentose sugars. However, co-utilization of sugars does not guarantee that CCR is inactive (Zhang et al., 2016). Thus, limited knowledge about the effect and extent of CCR on growth is still a bottleneck. Our earlier study aimed to deal with this bottleneck, and showed that the interaction between sugars was significant and repressive for growth (Birgen et al., 2018a). However, the type of interaction remains unknown, and successful design of lignocellulosic fermentation processes requires the kinetic model for accurately predicting the cell mass growth. Therefore, our objective was to develop a growth model for *Clostridium beijerinckii* NCIMB 8052 on glucose and xylose as representative lignocellulosic sugars.

2. Materials and Methods

2.1 Microorganism and medium

Wild type *Clostridium beijerinckii* NCIMB 8052 was used in this study as it can utilize both glucose and xylose (Zhang et al., 2012). We employed a two-stage pre-culture strategy based on our previous work (Birgen et al., 2018b), which enables the culture to co-utilize glucose and xylose. First, the cells stored at -80 ^{o}C (1 ml, 20% glycerol) were cultivated for 14 hours on reinforced Clostridial medium (CM0149, Oxoid). Then, inoculum from the first stage of the pre-growth was cultivated in the second fresh medium (5% v/v). The second growth medium contained 5 g/l xylose, 2.5 g/l Na-acetate, 5 g/l yeast extract, 2 g/l $(NH_4)_2SO_4$, 0.01 g/L NaCl, 0.75 g/l KH_2PO_4, 1.5 g/l K_2HPO_4, 0.2 g/l $MgSO_4.7H_2O$, 0.01 g/l $MnSO_4.H_2O$, 0.01 g/l $FeSO_4.7H_2O$, 0.01 g/l p-aminobenzoic acid, 0.01 g/l biotin and 0.1 g/l thiamine. After 6 hours of growth on the second medium, we started batch growth experiments by inoculating medium flasks with the culture grown on the second medium (4% v/v). Media for the batch growth experiments contained both glucose and xylose, and the rest of the components were the same as in the second growth medium.

2.2 Batch growth experiments

We performed batch growth experiments in 120 ml serum flasks with 50 ml working volume in an incubator with temperature controlled at 37^{o}C under static and anaerobic conditions. We used an inoculum size of 4% v/v. No pH control was applied. We took 2 ml samples every 2 hours from the start of the experiment. Experiments were terminated after 6 hours when the exponential growth phase ended.

2.3 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. We diluted the samples exceeding 0.4 OD with water so that the Beer-Lambert Law applied.

2.4 Estimation of growth rate

We estimated the maximum specific growth rate, μ_{max} (h^{-1}) during the exponential growth phase where the nutrient concentration is large enough that the growth rate is independent of nutrient concentration. Therefore, μ_{max} is equal to the growth rate, μ (h^{-1}), which is shown in Eq(1).

$$\frac{dX}{dt} = \mu X \tag{1}$$

where X is the cell mass concentration (g/l) and t is time (h). The growth rate was determined during the exponential growth phase by estimating the slope of the OD (660 nm) versus time (h) plot.

2.5 Binary substrate growth models

We chose the binary substrate growth models with the interaction term, since our previous findings showed that the synergistic effect between the sugars has a significant influence on the growth rate (Birgen et al., 2018a). Moreover, we chose the growth models such that they can describe the key characteristics of the growth while avoiding its overparameterization. Thus, we employed noncompetitive, competitive, uncompetitive, interactive and noninteractive binary substrate growth models (Segel, 1975; Bell, 1980; Megee et al., 1972; Yoon et al., 1977). The models were based on the Monod equation, and the classification of the interactions for these models was based on the enzyme kinetics (Okpokwasili and Nweke, 2006). Table 2 shows the models we used, where S_G is glucose concentration, S_X is xylose concentration, μ_{maxG} is the maximum specific growth rate on glucose, μ_{maxX} is the maximum specific growth rate on xylose, K_{sG} is the substrate affinity constant for glucose and K_{sX} is the substrate affinity constant for xylose. These are empirical coefficients, and due to their dependency on the species, substrate and environmental conditions, we call them parameters in this study. We fitted the models given in Table 2 by using the Matlab function nlinfit for nonlinear regression, which uses the Levenberg-Marquardt nonlinear least squares algorithm. The parameter estimates minimize the least squares equation, Eq(2), where $f(x_i,b)$ is the nonlinear function, x_i are the predictors for i^{th} observation, i = 1,...,N, and b are the parameters.

$$\sum_{i=1}^{N} [y_i - f(x_i,b)]^2 \tag{2}$$

We used nlparci to obtain 95% confidence intervals for the model parameters, and the CovB output argument of nlinfit to get the covariance matrix, which we then converted to a correlation matrix by using corrcov function. We used the R argument of the nlinfit function to get raw residuals data to visualize the difference between the simulated and observed values of the growth rate.

3. Results

3.1 Model fitting

We used the growth rate data obtained in our previous work (Birgen et al., 2018a) for model fitting. The dataset of the observed values consisted of 16 data points, which was the result of a central composite design of experiments to understand the effect of glucose and xylose concentrations on the growth rate and the synergistic effects between them. Glucose and xylose concentrations, S_G and S_X are predictor variables and the growth rate, μ is the response variable. Table 2 shows fit results in terms of degrees of freedom of error (DFE), mean standard errors (MSE), and sum of squares error (SSE) for the chosen binary substrate growth models.

Table 2: Fit results for binary substrate growth models.

Model	Model type	DFE	SSE	MSE	Reference
$\mu=\frac{\mu_{maxG}*S_G}{(K_{sG}+S_G)*\left(1+\frac{S_X}{K_{sX}}\right)}+\frac{\mu_{maxX}*S_X}{(K_{sX}+S_X)*\left(1+\frac{S_G}{K_{sG}}\right)}$	Noncompetitive	11	0.0778	0.0071	Segel, 1975
$\mu=\frac{\mu_{maxG}*S_G}{K_{sG}+S_G*\left(1+\frac{S_X}{K_{sX}}\right)}+\frac{\mu_{maxX}*S_X}{K_{sX}+S_X*\left(1+\frac{S_G}{K_{sG}}\right)}$	Uncompetitive	12	0.1516	0.0126	Segel, 1975
$\mu=\frac{\mu_{max}*S_G*S_X}{(K_{sG}+S_G)*(K_{sX}+S_X)}$	Interactive	13	0.1867	0.0144	Megee et al., 1972
$\mu=\frac{\mu_{maxG}*S_G}{K_{sG}+S_G}+\frac{\mu_{maxX}*S_X}{K_{sX}+S_X}$	Noninteractive	12	0.1847	0.0154	Bell, 1980
$\mu=\frac{\mu_{maxG}*S_G}{K_{sG}+S_G+S_X*\left(\frac{K_{sG}}{K_{sX}}\right)}+\frac{\mu_{maxX}*S_X}{K_{sX}+S_X+S_G*\left(\frac{K_{sX}}{K_{sG}}\right)}$	Competitive	12	0.1928	0.0161	Yoon et al., 1977

Fit results showed that the noncompetitive binary substrate growth model delivered the smallest SSE and MSE values, 0.0778 and 0.0071, respectively. Eq(3) shows the resulting model with the estimated parameters. We found the quality of the fit satisfactory.

$$\mu(S_G,S_X)=\frac{1.435*S_G}{(1.236+S_G)*\left(1+\frac{S_X}{4.601}\right)}+\frac{1.508*S_X}{(4.601+S_X)*\left(1+\frac{S_G}{1.236}\right)} \quad (3)$$

The model simulations together with the observed values, the resulting plot a) and its residuals plot b) are presented in Figure 1.

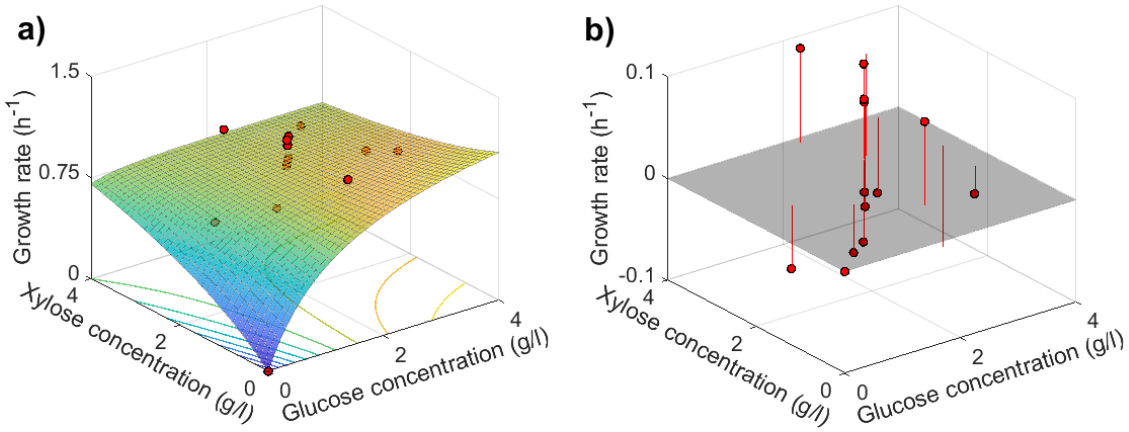

Figure 1: Simulation of the noncompetitive binary substrate growth model a) and residuals plot of glucose and xylose concentrations versus growth rate b).

Figure 1 a) shows that a minimum response, the growth rate, appeared when both glucose and xylose concentrations were zero, since cells cannot grow without any carbon source. When glucose was the sole sugar, the growth rate increased as its concentration increased, and a maximum growth rate value was obtained at its highest concentration. Even though the growth rate was lower for xylose than for glucose, the growth rate was still proportional with the xylose concentration. The projection of the simulation surface plot a) shows that increasing or decreasing both sugar concentrations at the same time results in a decreasing growth rate. However, increasing either of the sugars when decreasing the other one results in an increasing growth rate. The residuals plot b) shows the raw residual values, which are the difference between the simulated and observed values of the growth rate.

3.2 Effects of model parameters

We chose initial values for parameter estimation as 0.001 for all the parameters, and did not specify the parameter bounds. Table 3 shows the parameter estimates and their 95% confidence intervals for the noncompetitive binary substrate growth model. The maximum specific growth rate on glucose, μ_{maxG} (h^{-1}) was greater than the maximum specific growth rate on xylose, μ_{maxX} (h^{-1}). On the other hand, the substrate affinity constant for xylose, K_{sX} (g/l) was larger than the substrate affinity constant for glucose, K_{sG} (g/l).

Table 3: Parameters of the noncompetitive binary substrate growth model.

Parameter	Estimate	95% Confidence interval
μ_{maxG}	1.4354	-2.2643 – 5.1351
K_{sG}	1.2360	-7.1363 – 9.6082
K_{sX}	4.5996	-23.9499 – 33.1491
μ_{maxX}	1.5078	-1.8106 – 4.8262

μ_{maxX} had the smallest confidence interval, thus the uncertainty associated with this parameter was the smallest among others, while K_{sX} had the largest confidence interval. 95% confidence intervals of the parameters in ascending order were μ_{maxX}, μ_{maxG}, K_{sG} and K_{sX}.
We computed the correlation matrix for the noncompetitive binary substrate growth model. Table 4 shows the resulting matrix.

Table 4: Correlation matrix of noncompetitive binary substrate growth model.

	μ_{maxG}	K_{sG}	K_{sX}	μ_{maxX}
μ_{maxG}	1.0000	0.9918	-0.9945	-0.9728
K_{sG}	0.9918	1.0000	-0.9860	-0.9455
K_{sX}	-0.9945	-0.9860	1.0000	0.9542
μ_{maxX}	-0.9728	-0.9455	0.9542	1.0000

The correlation coefficient ranges from −1 to +1. The greater the absolute value of the correlation coefficient, the stronger the relationship between those parameters. Furthermore, the sign of the correlation coefficient indicates the direction of the relationship. If both parameters tend to increase or decrease simultaneously, the coefficient is positive. Therefore, the highest correlation was found between μ_{maxG} and K_{sX}, -0.9954, and one parameter decreased as the other one increased. We observed the weakest correlation between μ_{maxX} and K_{sG}, -0.9455. The relationships between maximum specific growth rates, μ_{maxG} and μ_{maxX}, and substrate affinity constants, K_{sG} and K_{sX}, were negative. On the other hand, the relationship between model parameters for single sugars, μ_{maxG} and K_{sG}, and μ_{maxX} and K_{sX}, were positive.
We did model simulations to illustrate the effect of the model parameters on the growth rate. We varied the parameters over their respective confidence intervals, and plotted them against the resulting growth rate values. We kept the sugar concentrations constant at their centre value over the experimental concentration space, S_G and S_X = 2.5 g/l. Figure 2 shows the resulting plots for all 4 model parameters.

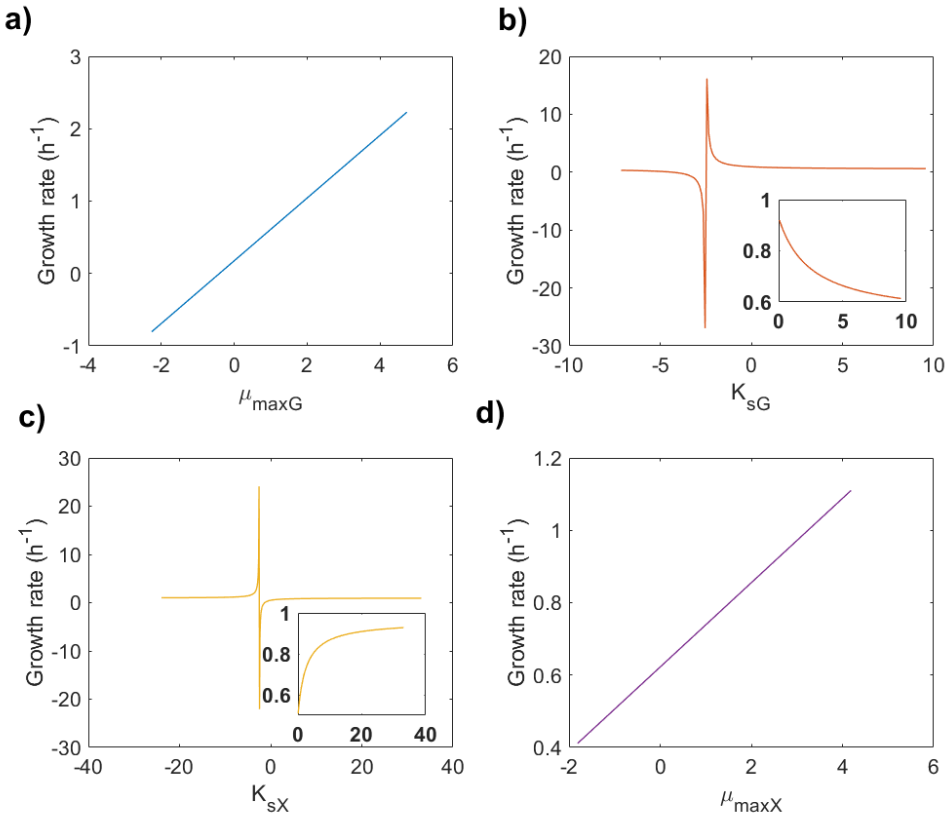

Figure 2: Effects of parameters of the noncompetitive binary substrate growth model on the growth rate (h^{-1}).

The maximum specific growth rate on glucose, μ_{maxG}, increased the growth rate, μ more than maximum specific growth rate on xylose, μ_{maxX}, since the slope of μ_{maxG} vs. μ plot was greater, 0.41, than slope of μ_{maxX} vs. μ plot,, 0.23, shown in Figure 2 a) and d), respectively. We constructed the substrate affinity constant vs. growth rate plots together with their magnified plots for their values of K_{sX} and K_{sG} > 0. Figure 2 b) shows the K_{sG} vs. μ plot in the shape of an exponential decay curve, therefore μ increased exponentially with decreasing K_{sG}. On the other hand, the K_{sX} vs. μ plot was in the shape of an exponential growth curve as shown in Figure 2 c), thus μ increased exponentially, while K_{sG} was increasing.

4. Discussion

In this study, we developed a kinetic model of *C.beijerinckii* NCIMB 8052 growth on xylose and glucose. We fitted different growth models to the growth rate data obtained for various concentrations of glucose and xylose. We chose binary substrate growth models, which include the synergistic effects between sugars. Evaluation of model performance by using error values is a commonly applied practice (Cecilia et al., 2013). Among other models, noncompetitive binary substrate growth model gave the best fit with the smallest SSE and MSE values, 0.0778 and 0.0071, respectively. In addition, we checked the distribution of the residuals as a measure of the model quality, and the probability of the residuals fell on a straight line (data not shown) indicating that the normality assumption was satisfied.

Parameter estimation showed that μ_{maxX} was greater than μ_{maxG}. Therefore, the rate of growth during the exponential growth phase was higher on xylose than on glucose. The reason might be our two-stage pre-culture development strategy, where the culture was grown on a medium containing only xylose as the only sugar to activate the xylose utilization pathway. On the other hand, K_{sX} was larger than K_{sG}, meaning that the cells were more attracted to glucose, which is the preferred carbon source over xylose due to CCR (Ren et al., 2010). Therefore, our observation is in line with previous findings. The confidence interval for a parameter is an indicator of its uncertainty. Therefore, the larger the confidence interval the larger the uncertainty associated with that parameter. Our results showed that K_{sX} had the largest, and μ_{maxX} had the smallest uncertainty. We computed the correlation matrix, and it showed that the model parameters were highly correlated. The correlation coefficients between μ_{maxG} and μ_{maxX}, K_{sG} and K_{sX}, μ_{maxG} and K_{sX} and, μ_{maxX} and

K_{sG} were negative. Thus, increasing K_{sX} would result in a higher growth rate on xylose, and a lower growth rate on glucose due to the CCR effect of xylose on glucose, and vice a versa. Therefore, our results suggest that repression effects apply to both sugars. We did model simulations to examine the effects of model parameters on the growth rate. Even though μ_{maxG} was smaller than μ_{maxX}, it had a greater influence on the growth rate. There is a relation between the sugar concentration and the substrate affinity constant (Shuler et al., 2017). When $S_X < K_{sX}$, the term for repression effect of xylose on glucose utilization ($1+S_X/K_{sX}$) decreases with increasing K_{sX}, and vice a versa. Therefore, a change in K_{sG} and K_{sX} resulted in different effects on the growth rate, Figure 2 b) and c), since S_G=2.5 g/l > K_{sG}=1.2360 g/l, and S_X=2.5 g/l < K_{sX}=4.5996 g/l.

5. Conclusions

To our knowledge, this is the first attempt to model the growth of *C.beijerinckii* NCIMB 8052 on glucose and xylose, which considers the synergistic effects of sugars. We fitted growth models with different types of interactions to the growth rate data obtained with varying sugar concentrations. A noncompetitive binary substrate growth model gave the best fit. Both sugars had CCR effects on the growth rate, and interaction was of the noncompetitive type. Validation with other sugar concentration values will be necessary to evaluate the prediction capability of the proposed model. A transcriptional study will be beneficial to understand global gene regulation mechanisms as guidance for improving the efficiency of lignocellulosic fermentation processes.

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