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Response Surface Methodology for Understanding Glucose and Xylose Utilization by *Clostridium beijerinckii* NCIMB 8052

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We applied response surface methodology to understand the effect and extend of carbon catabolite repression (CCR) on growth of *Clostridium beijerinckii* NCIMB 8052 using xylose and glucose as representative lignocellulosic sugars. We performed batch growth experiments based on the central composite design with different concentrations of glucose and xylose, and estimated the respective growth rates as the response. Fitting the quadratic model with interaction coefficient to experimental data gave a good quality of fit (R-squared=0.939). We found that glucose is the most significant factor affecting the growth rate. Interaction between glucose and xylose is another highly significant factor. Response surface illustrated that increasing or decreasing both sugar concentrations at the same time results in a decreasing growth rate, and increasing either sugar concentration while decreasing the other sugar increases the growth rate. It is an important finding as it suggests that CCR can be not only from glucose on xylose but also from xylose on glucose. A transcriptional study will be necessary to understand the repression mechanism and to improve the utilization of sugars in mixed form, thus lignocellulosic fermentation processes.

1. Introduction

Lignocellulosic biomass is a promising feedstock, since it is the most abundant renewable biomass resource on the planet. Moreover, it prevents the direct fuel-versus-food competition due to use of edible plants such as corn and sugar cane for biofuel production. Cellulose, hemicellulose, and lignin are the main constituents of lignocellulosic biomass. Hemicellulose contains a large fraction of pentose sugars such as xylose as well as hexose sugars such as glucose. Therefore, lignocellulosic biomass hydrolysis yields both hexose and pentose sugars (Zaldivar et al., 2001). Furthermore, the composition of biomass depends on the plant species, and is subject to change depending on age and growth conditions. As reported by Jørgensen et al., (2007), typical dry weight of lignocellulosic biomass consists of 34.2-46.4 % glucose, 4.9-24.9 % xylose, 1.1-2.9 % arabinose, 0.3-12 % mannose, and 11.9-29.4 % lignin. Therefore, fermentation performance of microorganisms such as product yield depends on their capacity for simultaneous utilization of these mixed sugars in the hydrolysate. However, the cells' efficiency at utilizing different sugars in mixed form tends to decrease due to 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 is an ongoing research on metabolic engineering (Lee et al., 2016) to develop strains capable of simultaneously utilizing both hexose and pentose sugars as substrates. However, co-utilization of mixed sugars does not guarantee that CCR is inactive (Zhang et al., 2016). Therefore, limited quantitative knowledge about the effect and extend of CCR on mixed sugar utilization remains as a bottleneck, and importance of studying the growth on mixed sugars to elucidate the synergic effects stressed by earlier studies (Ferone et al., 2016). We aim to target this bottleneck in this study by using response surface methodology (RSM). RSM is a statistical tool, which includes an experimental design with a minimum number of experiments and builds a model that can predict the interaction and correlation between a set of independent variables and responses (Hanharan and Hu, 2006). RSM has been widely applied for optimization of fermentation processes aiming to maximize the product yield and titer (Ramli et al., 2017; Kana et al., 2012; Samsudin et al., 2017). Our objective is to apply response surface methodology to understand the

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utilization of xylose and glucose as representative lignocellulosic sugars by *Clostridium beijerinckii* NCIMB 8052.

2. Materials and Methods

2.1 Microorganism and medium

Wild type *Clostridium beijerinckii* NCIMB 8052 was used in this study due to its ability to utilize both sugars (Zhang et al., 2012). We applied a two-stage pre-growth strategy based on our previous work, which enables the culture to co-utilize glucose and xylose (Birgen et al., 2018). First, a frozen work ampoule (1 ml) was grown for 14 hours on reinforced Clostridial medium (CM0149, Oxoid). Secondly, we inoculated a fresh medium flask with the culture grown on CM0149 (5% v/v). 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 xylose containing medium, we started designed batch growth experiments by inoculating medium flasks with the culture grown on xylose medium (4% v/v). Growth medium contained glucose and xylose amounts defined by the design of experiments, and the rest of the components were the same as in the xylose pre-growth medium.

2.2 Batch growth experiments

We conducted 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. There was no pH control applied. We took 2 ml samples every 2 hours from the start of the experiment. Experiments 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. Samples exceeding 0.4 OD were diluted with water so that the Beer-Lambert Law applies.

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 sufficient that the growth rate is independent of nutrient concentration. Therefore, μ_{max} equals to specific growth rate, μ (h^{-1}), which is shown in Eq(1).

$$\frac{dX}{dt} = \mu X \quad (1)$$

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

2.5 Response surface methodology

We used a circumscribed central composite (CCC) design for 2 factors, glucose and xylose concentrations due to its ability provide good accuracy of estimation over the entire design space using built-in Matlab function ccdesign. CCC design resulted in 16 experimental runs with 8 of them in the centre point to reduce the effects of correlations between the factors. We determined the minimum and maximum values as 1 g/l and 4 g/l for both factors and then obtained real values for each experiment according to the coded levels of the respective CCC design. Table 1 shows levels and codes of CCC design.

Table 1: Real and coded values of circumscribed central composite design for 2 factors.

Factor	Symbol	Code level				
		-1.4142	-1	0	1	1.4142
Glucose concentration (g/l)	X_1	1.0	1.43934	2.5	3.56066	4
Xylose concentration (g/l)	X_2	1.0	1.43934	2.5	3.56066	4

We fitted the model with the data given in Eq(2) by using Matlab function fitlm for linear regression analysis applying least squares method.

$$Y = A_0 + \sum A_i X_i + \sum A_{ii} X_i^2 + \sum A_{ij} X_i X_j \quad (2)$$

Y is the response, A_0 is the constant coefficient, A_i is the linear coefficient, A_{ii} is the quadratic coefficient and A_{ij} is the interaction coefficient. X_i and X_j are factor level values.
We used coefficient of determination (R-squared) for assessing the quality of the fit, and pValue using the F-test for significance of regression model coefficients.

3. Results

3.1 Model fitting

We performed 16 batch growth experiments designed according to CCC design. For each experiment, we estimated the growth rate as described in Section 2.4. Table 2 shows resulting response values for each experiment together with the factor levels, sugar concentrations, defined by CCC design.

Table 2: Experimental values of circumscribed central composite design and responses.

Experiment No.	Glucose concentration (g/l)	Xylose concentration (g/l)	Growth rate (h^{-1})
1	1.439339828	0.7095	0.7095
2	1.439339828	1.076	1.076
3	3.560660172	1.439339828	0.8514
4	3.560660172	3.560660172	0.6811
5	1	2.5	0.6153
6	4	2.5	0.7045
7	2.5	1	0.9313
8	2.5	4	0.8396
9	2.5	2.5	0.8877
10	2.5	2.5	0.7343
11	2.5	2.5	1.058
12	2.5	2.5	0.838
13	2.5	2.5	0.8426
14	2.5	2.5	0.7039
15	2.5	2.5	0.7544
16	2.5	2.5	0.8755

Fit results provided insight into the quality of the fit. The R-squared value was 0.939, which means that the model can explain 93.9% of the variability in the response variable, growth rate, and 6.1% of the variability cannot be represented by this model. The root mean squared error (RMSE) value, which is the standard deviation of the error distribution, was 0.0696. The pValue for the F-test on the model was 8.83e-06 indicating that the model was significant. Moreover, residuals from the least squares are important for assessing the accuracy of the model. Therefore, normal probability distribution of residuals and residuals versus fitted values plots are shown in Figure 1 a) and b), respectively.

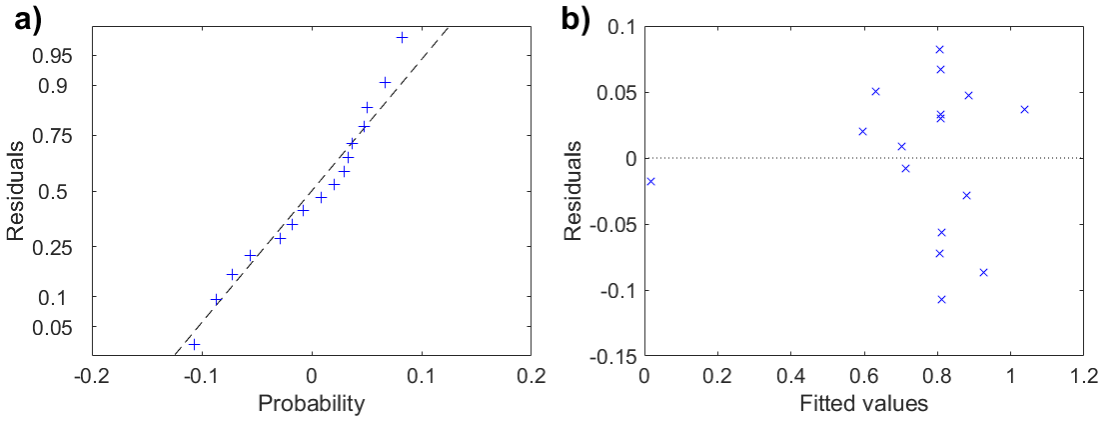

Figure 1: Normal probability distribution of residuals a) and residuals versus fitted values b).

The plots in Figure 1 confirmed that the normality assumption was satisfied since the probability of the residuals fell on a straight line.
We fitted the experimental data given in Table 2 to the model presented in Section 2.5. Model fitting resulted in Eq(3) with the actual variables (glucose and xylose concentrations) and the predicted response (growth rate).

$$\text{Growth rate} = 0.017828 + 0.60737*\text{Glucose} + 0.048654*\text{Xylose} - 0.058592*\text{Glucose}^2 + 0.049852*\text{Xylose}^2 - 0.12668*\text{Glucose}*\text{Xylose} \quad (3)$$

After the model fitting was done and the quality of the fit was found satisfactory, we plotted the response surface by using the model in Eq(3), the resulting response surface a) and its contour plot b) can be seen in Figure 2.

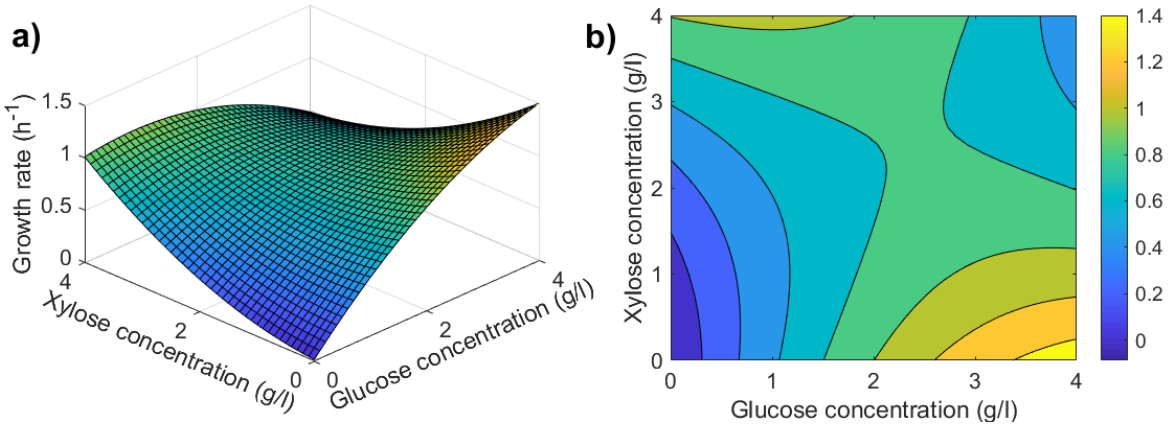

Figure 2: Response surface a) and contour plot b) of glucose and xylose concentrations versus growth rates.

Figure 2 shows that a minimum appeared when both variables are zero, since cells cannot grow without any carbon source. When glucose was the only sugar present, growth rate increased as its concentration increased, and a maximum growth rate value was obtained at the highest glucose concentration. Even though the growth rate was lower for only xylose containing experiments than for only glucose containing ones, the growth rate was still proportional with the xylose concentration. Contour plot b) shows that the stationary point is a saddle point. From this point near the centre of the experimental design, increasing or decreasing both factors at the same time results in a decreasing response. However, from the stationary point, increasing either factor when decreasing the other one results in an increasing response.

3.2 Effects of factors

Fit results provided the model coefficient estimates and their statistical values: Standard error of the coefficients (SE), t-statistic for each coefficient to test the null hypothesis that the corresponding coefficient is zero against the alternative, which is different from zero, given the other predictors in the model (tStat), p-value for the F statistic of the hypotheses test that the corresponding coefficient is equal to zero or not (pValue). Table 3 shows the statistical values of the model coefficients.

Table 3: Model coefficients and corresponding statistical values.

Source	SE	tStat	pValue	Significance
Intercept	0.067230	0.26518	0.79626	
X_1	0.097101	6.255	9.4434e-05	**
X_2	0.090592	0.53707	0.60296	
X_1^2	0.022422	-2.6131	0.025901	*
X_2^2	0.019307	2.5821	0.027318	*
X_1X_2	0.022318	-5.6758	0.00020501	**

* Significant ($p<0.05$)
** Highly significant ($p<0.01$)

Glucose (X_1) was the most significant variable among all and the only highly significant linear variable affecting the growth rate. Second highly significant variable was the interaction between the variables (X_1X_2). Both quadratic variables (X_1^2 and X_2^2) were significant, while the linear variable of xylose (X_2) was not significant.
Figure 3 shows the normalized coefficients versus the response values (growth rate) to illustrate the effect of each model coefficient on the response.

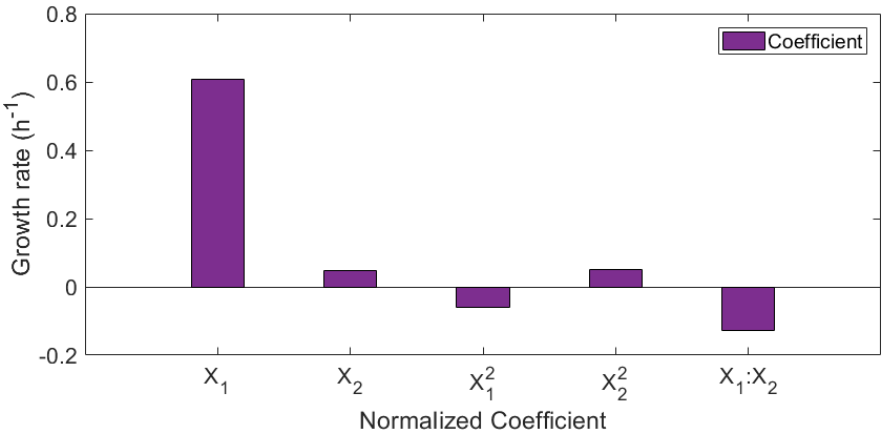

Figure 3: Normalized values of model coefficients versus growth rate (h^{-1}).

We examined the effects of the model coefficients using Figure 3. Linear glucose (X_1) was the most significant variable with the greatest impact on the response. The growth rate increased as glucose concentration increased. The second most significant variable was the interaction between the variables. The interaction term had a negative impact on the growth rate. The quadratic variable of glucose (X_1^2) had a negative effect on the response, while quadratic variable of xylose (X_2^2) was increasing the growth rate.

4. Discussion

In this study, we applied response surface methodology to understand the effect and extend of CCR on growth of *Clostridium beijerinckii* NCIMB 8052 using xylose and glucose as representative lignocellulosic sugars. We performed 16 batch growth experiments on different mixtures of glucose and xylose as the factors determined by the central composite design and estimated the respective growth rates as the response. Experimental data fitting to the quadratic model gave a good quality of fit with an R-squared value of 0.939. Therefore, our model is able to explain 93.9% of the variability in growth rate. Furthermore, we analyzed the residuals, and distribution of the residuals showed a normal distribution with matched variance. In addition, pValue of the model showed high significance (pValue=8.83e-06 <0.01) confirming the validity of the model.
We plotted the response surface and contour plots for better examination of response. The highest growth rate was achieved when glucose was the only sugar and its concentration was the highest within the experimental design space. Therefore, the effect of glucose concentration on the growth rate is greater than xylose as it is the preferred carbon source. The response surface illustrated that the stationary point was a saddle point. From this point, which is near the center of the experimental design, increasing or decreasing both sugar concentrations at the same time results in a decreasing growth rate. However, from the stationary point, increasing either sugar concentration while decreasing the other one results in an increasing response. This trend demonstrates the importance of interaction between the sugars. Therefore, CCR is still active and both sugars are most likely to be repressing utilization of each other, thus decreasing the growth rate. This is an interesting finding as glucose was found to repress the growth of xylose in earlier studies (Ren et al., 2010); while our findings suggest that, the repression effect can apply to both sugars. This might also be due to our two-stage pre-growth strategy, where the culture was grown on a medium containing only xylose as the sugar to activate the xylose pathway and achieve co-utilization of sugars.
We determined the significance level of the model coefficients by using pValue. Glucose (pValue=9.4434e-05<0.05) was the most significant variable among all and the only highly significant linear variable affecting the growth rate. This is in agreement with our examination of the response surface plot. Second highly significant variable was the interaction between the variables (pValue=0.00020501<0.05), which explains the behaviour around the saddle point that we observed in the contour plot of the response surface. Both

quadratic variables (pValue=0.025901<0.01 for glucose and pValue=0.027318<0.01 for xylose) were significant, while the linear variable of xylose was not significant. Information about the significance of the model parameters by using pValue was confirmed by the plot showing the effect of the coefficients on the response, growth rate.

5. Conclusions

To our knowledge, this is the first application of response surface methodology for understanding the effect and extend of CCR on the utilization of glucose and xylose. We obtained a quadratic model with interaction coefficient. We found that glucose is the most significant factor affecting the growth rate. Interaction between glucose and xylose is another highly significant factor, which reduces the growth rate. The response surface illustrated that increasing or decreasing both sugar concentrations at the same time results in a decreasing growth rate, while increasing either sugar concentration and decreasing the other one increases the growth rate. It is an important finding as this suggests that the CCR mechanism can be active not only from glucose on xylose utilization, but also vice versa, from xylose on glucose utilization. A transcriptional study is necessary to understand the repression mechanisms and to improve the utilization of sugars in mixed form, thus lignocellulosic fermentation processes.

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