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Kinetic Models for Growth and Product Formation on Multiple Substrates

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Abstract Hydrolyzates from lignocellulosic biomass contain a mixture of simple sugars; the predominant ones being glucose, cellobiose and xylose. The fermentation of such mixtures to ethanol or other chemicals requires an understanding of how each of these substrates is utilized. *Candida lusitaniae* can efficiently produce ethanol from both glucose and cellobiose and is an attractive organism for ethanol production. Experiments were performed to obtain kinetic data for ethanol production from glucose, cellobiose and xylose. Various combinations were tested in order to determine kinetic behavior with multiple carbon sources. Glucose was shown to repress the utilization of cellobiose and xylose. However, cellobiose and xylose were simultaneously utilized after glucose depletion. Maximum volumetric ethanol production rates were 0.56, 0.33, and 0.003 g/L·h from glucose, cellobiose and xylose, respectively. A kinetic model based on cAMP mediated catabolite repression was developed. This model adequately described the growth and ethanol production from a mixture of sugars in a batch culture.

Keywords: kinetic model, ethanol fermentation, multiple substrates

INTRODUCTION

Low-cost fermentation substrates frequently contain mixtures of carbon sources including hexoses, pentoses, and disaccharides. For example, hydrolyzates from lignocellulosic biomass generally contain a mixture of glucose, xylose, and cellobiose, along with small amounts of other sugars [1,2]. Although numerous microorganisms have been evaluated for the conversion of such substrate mixtures into ethanol, a single species which gives high yields on all these sugars has not yet been found. Therefore, it is often beneficial to use a combination of organisms, either together or sequentially, to efficiently utilize the major substrate components rather than rely on a pure culture to complete the fermentation [3,4].

In developing processes for the production of ethanol from biomass hydrolyzates, the major substrate components which must be considered are glucose, xylose and cellobiose. Although numerous organisms that ferment glucose to ethanol are available, some are more efficient than others. In addition, only a few of these organisms can also ferment cellobiose and xylose. Of the organisms which have been evaluated for fermentation of cellobiose, *Candida lusitaniae* ferments glucose at the same rate as *Saccharomyces cerevisiae* and gives a high ethanol yield from cellobiose [5]. The utilization of a cellobiose-fermenting yeast in simultaneous saccharification and fermentation (SSF) of cellulose for ethanol production appeared advantageous, because of the ability of the microorganism to ferment both cellobiose and glucose [6,7]. Therefore, *C. lusitaniae* appears to be a strong candidate for biomass hydrolyzate fermentation.

To design an efficient ethanol fermentation process reliable kinetic models are required for the growth and product formation of organisms. Frequently, yeasts growing on multiple carbon sources exhibit diauxic growth, where one carbon source represses the utilization of the others [8,9]. This diauxic growth usually is attributed to catabolite repression by the preferential substrate. If catabolite repression does not occur, other types of controls may lead to the preferential use of one substrate.

In many cases where multiple substrates are present, one will cause the catabolite repression of the others. There have been many theoretical models developed to describe catabolite repression [10-13]. Although each of these is based on a different mechanism for catabolite repression, each model includes a term denoting the fraction of promoter genes open to transcription. In most cases, this term (denoted Q) is of the following form:

$$Q = \frac{1 + AS_1^n}{1 + BS_1^n} \tag{1}$$

where A and B are constants, S_1 is the concentration of the substrate causing catabolite repression, and n is an integer representing the number of binding sites required for the specific mechanism assumed. For systems in which complete repression of the second substrate (S_2)

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occurs in the presence of high levels of the first (S_1), the value of A must be zero.

As pointed out by Slaff and Humphrey [11], the growth rate on the substrate subject to catabolite repression, S_2, should be proportional to the fraction, Q:

$$\mu_{m2R} = \mu_{m2} \cdot Q \quad (2)$$

where μ_{m2R} is the maximum growth rate on S_2 in the presence of S_1 and μ_{m2} is the maximum growth rate on pure S_2.

When mixtures of two or more carbon sourses are available, as in the case of biomass hydrolyzate, it is likely that catabolite repression will be caused by only one of the substrates, with the other substrates producing general inhibitory effects on one another. Thus, the overall growth rate is the sum of the growth rates on each individual substrate with catabolite repression and inhibition effects taken into account. Allowing S_1 to be the substrate causing catabolite repression and assuming the other substrates interact according to the general model of Yoon *et al.* [14], the growth rate for N substrates can be modeled as

$$\mu = \sum_{i=1}^{N} \mu_i = \sum_{i=1}^{N} \left[\frac{\mu_{mi} S_i}{K_i + \sum_{j=1}^{N} a_{ij} S_j} \right] \left[\frac{1}{1 + B_i S_1^n} \right] \quad (3)$$

where $a_{ij}=1$ and a_{ij} represents the inhibitory effect of S_j on S_i. If there are no effects of S_j on S_i, then $a_{ij}=0$. For any substrate, S_2, subject to catabolite repression by S_1, $a_{12}=a_{21}=0$. For any substrate, S_3, not subject to catabolite repression, $B_3=0$ but a_{13} and a_{31} are not necessarily zero.

In this paper, we utilize this model to describe the kinetics of growth and product formation by *C. lusitaniae* on mixed sugars. Experiments were carried out to obtain kinetic data for the production of ethanol from glucose, cellobiose and xylose. Various combinations of the three sugars were also tested to determine the kinetic behavior with multiple carbon sources.

MATERIALS AND METHODS

Microorganism

C. lusitaniae NRRL Y-5394 was used in this study. Selection was based on the ability of *C. lusitaniae* to produce ethanol from cellobiose but not xylose. The culture was obtained from the Nothern Regional Center Culture Collection, U. S. Department of Agriculture (Peoria, IL, USA). It was maintained on YM agar slants (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 10 g/L glucose and 20 g/L agar).

Growth Medium

A mineral salts medium supplemented with yeast extract was used for all kinetic studies. The composition of the medium (per L) was as follows: $(NH_4)_2SO_4$, 5 g; KH_2PO_4, 5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.05 g; yeast extract, 5 g; $FeSO_4 \cdot 7H_2O$, 2.5 mg; $MnSO_4 \cdot H_2O$, 0.6 mg; $ZnSO_4 \cdot 7H_2O$, 1.76 mg; $CoCl_2 \cdot 6H_2O$, 1.0 mg; carbon source, 10.0 g. The carbon source was dissolved in distilled water and autoclaved separately from other ingredients to prevent undesirable reactions. The pH of each solution was adjusted to 4.5 with HCl prior to autoclaving.

Experimental Conditions

Batch fermentations were conducted at 30°C using BioFlo II systems (New Brunswick Sci. Co., Edison, NJ, USA) containing 1 L medium. The pH was controlled at 4.5 by the addition of 1 N NaOH. Semi-aerobic conditions were maintained by an air flowing at 0.2 VVM (volume air/volume liquid/min) into the headspace with 100 rpm agitation.

Inocula were aerobically grown in shake flasks using two transfers to get a consistent final cell mass concentration of about 6 g/L. For each run, the fermentor was inoculated with 3% inoculum to give an initial cell mass of about 0.2 g/L. Samples were periodically withdrawn from the cultures for subsequent analysis. The optical density was immediately measured and the culture broth was frozen until needed for further analysis.

Growth, Ethanol and Carbohydrates Analysis

Cell growth was determined by measuring the optical density (OD) at 600 nm and then compared to a known calibration curve. To provide standardized OD measurements for different fermentations, cells were harvested by centrifugation at 5,000×g for 10 min, and then resuspended in distilled water for the OD measurement. Ethanol concentrations of fermentation broths were determined by gas chromatography using a 6 ft Porapak-Q column (Supelco, Bellefonte, PA, USA). Peaks were detected with a flame ionization detector. The oven temperature was maintained at 190°C.

Sugar concentrations were determined by HPLC (Waters Associates, Milford, MA, USA) using a Bio-Rad HPX-87P cation exchange column. The column was maintained at 85°C. Water was used as the mobile phase at a flowrate of 0.6 mL/min. Peaks were detected using a differential refractometer.

RESULTS AND DISCUSSION

Fermentation of Single Sugars

Fermentations of single sugars (glucose, cellobiose, and xylose) were conducted under semi-aerobic conditions. Maximum specific growth rates (μ_m), saturation coefficients (K), cell yields (Y) and product yields (Yp) on each substrate are summarized in Table 1. *C. lusitaniae* utilized glucose (9.74 g/L) and cellobiose (9.93 g/L) rapidly, within 8 h and 13 h, respectively. Xylose was assimilated

Table 1. Kinetic parameters for growth and ethanol production from batch culture of *C. lusitaniae* on single substrates

Parameters	Glucose	Cellobiose	Xylose
$\mu_m(h^{-1})$	0.38	0.16	0.03
Max. ethanol conc. (g/L)	4.50	4.92	0.38
Time for max. ethanol (h)	8.00	15.00	132.00
Percent theoretical yield	92.00	86.00	16.00
K (g/L)	0.30	0.20	0.70
Y (g cell/g substrate)	0.19	0.15	0.30
Y_p (g ethanol/g substrate)	0.47	0.49	0.08
$Y_{p/x}$ (g ethanol/g cell)	2.53	3.34	0.25
Q_p (g ethanol/L·h)	0.56	0.33	0.003
q_p (g ethanol/g cell·h)	0.96	0.54	0.01
q_s (g substrate/g cell·h)	2.04	1.11	0.20

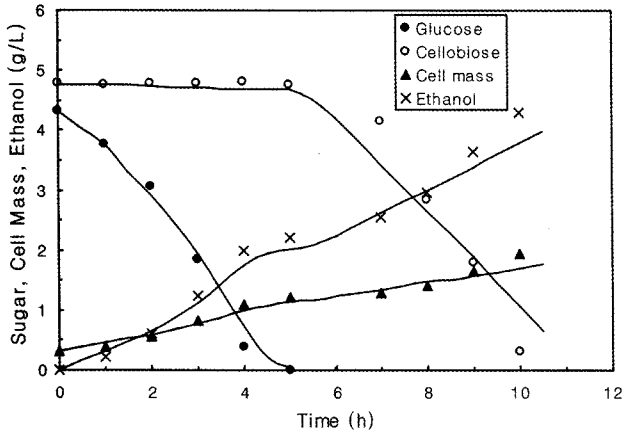

Fig. 1. Comparison of model predictions (solid lines) and experimental data for batch culture of *C. lusitaniae* on a glucose/cellobiose mixture.

at a far lower rate, with less than 30% consumed after 60 h. This slow utilization of xylose could be attributed to oxygen limitations under the semi-aerobic condition.

Ethanol was produced on all three substrates. However, the yield from xylose was very small. Ethanol concentrations were maximal after 8 h with glucose and 15 h with cellobiose; ethanol production from both substrates was growth associated. During the course of fermentation, ethanol yields were 0.47 g ethanol/g glucose and 0.49 g ethanol/g cellobiose, which represents 92 and 86% of theoretical, respectively. The maximum volumetric ethanol production rates were 0.56, 0.33, and 0.003 g/L·h from glucose, cellobiose and xylose, respectively.

Fermentation of Multiple Sugars

Fig. 1 shows the growth, ethanol production and substrates utilization by *C. lusitaniae* grown on a mixture of glucose (4.32 g/L) and cellobiose (4.8 g/L). The growth pattern shows diauxic behavior as expected, with glucose

Table 2. Effects of glucose concentration on the growth rate of *C. lusitaniae* on cellobiose

Substrates (g/L)	Specific growth rate (h^{-1})		Diauxic lag (h)
	Glucose	Cellobiose	
Glucose (9.74)	0.38	–	–
Cellobiose (9.93)	–	0.16	–
Glu/Cell (1/5)	0.22	0.16	0.50
Glu/Cell (3/5)	0.33	0.13	1.50
Glu/Cell (4.32/4.78)	0.34	0.09	2.50

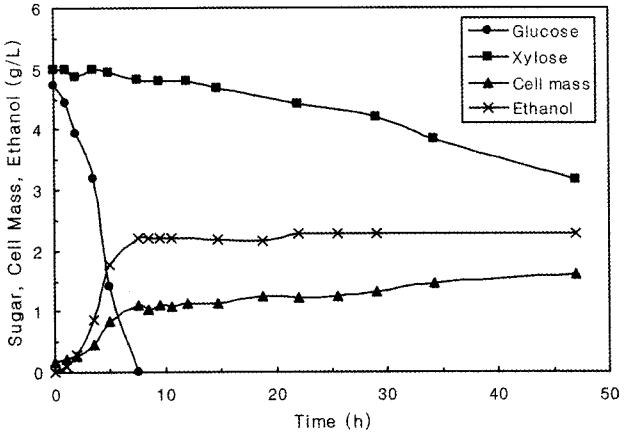

Fig. 2. Batch growth of *C. lusitaniae* on a glucose/xylose mixture.

repressing the utilization of cellobiose. The two phases of cell growth and ethanol production were separated by a lag phase of approximately 2 h to derepress the enzyme involved in the catabolism of cellobiose. Maximum ethanol concentration was the same as the amount calculated from the yield for each single substrate fermentation.

Specific growth rate data from trials using various ratios of glucose to cellobiose are provided in Table 2. The initial concentration of glucose in the mixture had a marked effect on the growth rate during the cellobiose utilization phase. As the initial glucose concentration was increased, the growth rate for the cellobiose phase decreased. In addition, diauxic lag time between phases increased. This reduced growth rate on the second substrate is referred to as permanent repression [11]. As the glucose concentration drops below some critical value, the derepression of enzymes occurs. However, the rate of enzyme formation remains lower than for a system which has not been repressed. The presence of cellobiose had no significant effect on the growth rate on glucose. However, when the glucose concentration was 1 g/L, the specific growth rate during the glucose phase was also lower, indicating a relatively high value for the substrate utilization constant (K).

A typical diauxic pattern is also seen for the growth of *C. lusitaniae* on a mixture of glucose (4.74 g/L) and xylose (5.0 g/L) (Fig. 2). The specific growth rate for the

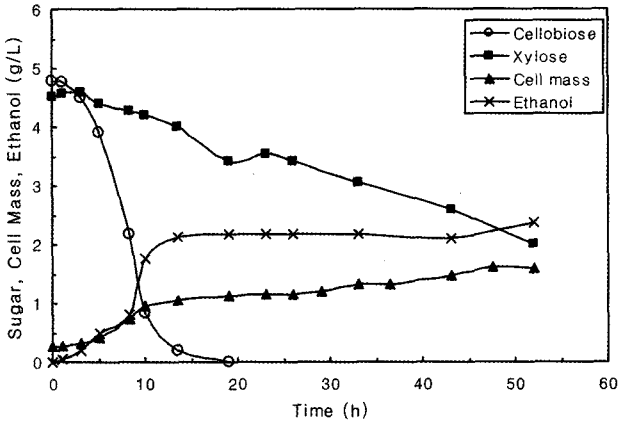

Fig. 3. Batch growth of *C. lusitaniae* on a cellobiose/xylose mixture.

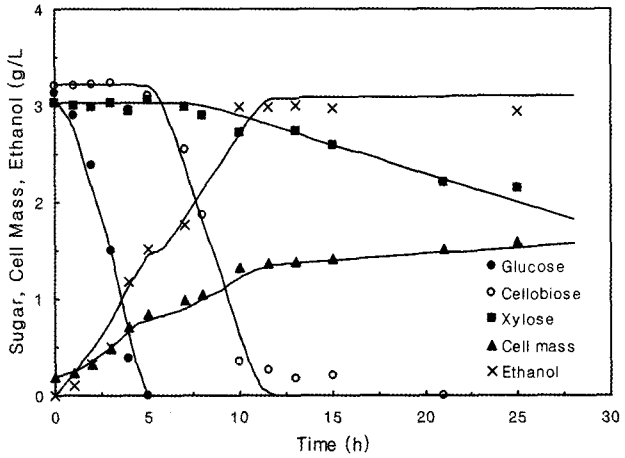

Fig. 4. Comparison of model predictions (solid lines) and experimental data for batch culture of *C. lusitaniae* on a glucose/cellobiose/xylose mixture.

xylose phase was lower than for pure xylose (0.01 vs. 0.03 h^{-1}), while that for the glucose phase was unchanged (0.36 h^{-1}). Ethanol was produced mainly from glucose, with a small contribution from xylose. It was consumed along with xylose after 50 h. The lag between the phases appeared to be insignificant.

C. lusitaniae grown on a mixture of cellobiose (4.77 g/L) and xylose (4.52 g/L) did not show diauxic growth (Fig. 3). Cellobiose and xylose were utilized simultaneously; however, cellobiose was consumed much more rapidly than xylose. The initial lag time in the xylose utilization (3 h) was likely caused by growth of the inoculum without xylose present. Ethanol production and consumption followed the same trend as the glucose/xylose mixture. The ethanol yield on the cellobiose/xylose mixture was the same as the sum of that on the single substrates. Although not shown in Fig. 3, after about 50 h, ethanol was simultaneously consumed with xylose.

Growth, substrate utilization and ethanol production on mixture of glucose (3.13 g/L), cellobiose (3.21 g/L) and xylose (3.03 g/L) are shown in Fig. 4. A triauxic growth pattern with distinct lag phases is seen in cell growth; however, ethanol production showed only two phases and ethanol was consumed simultaneously with xylose after cellobiose depletion. Glucose was used as the sole carbon source during the first phase. Cellobiose was utilized after glucose depletion. Several hours after the glucose was depleted, xylose began to be utilized along with cellobiose. Maximum ethanol production from the three sugar system was slightly lower than that calculated from the yield for each substrate fermented individually (3.0 versus 3.1 g/L). Table 3 summarizes specific growth rates for single and multiple substrate runs.

Table 3. Specific growth rates for growth of *C. lusitaniae* on various substrates

Substrates (g/L)	Specific growth rate (h^{-1})		
	Glucose	Cellobiose	Xylose
Glucose (9.74)	0.38	–	–
Cellobiose (9.93)	–	0.16	–
Xylose (10.0)	–	–	0.03
Glu/Cell (4.32/4.78)	0.34	0.09	–
Glu/Xyl (4.74/5.0)	0.36	–	0.01
Cell/Xyl (4.77/4.52)	–	0.15	0.01
Glu/Cell/Xyl (3.13/3.21/3.03)	0.38	0.10	0.01

Kinetic Model

The results from multiple substrate fermentations indicate that glucose results in the catabolite repression of cellobiose and xylose utilization but that neither cellobiose nor xylose have any effect on the utilization of each other. Allowing glucose, cellobiose and xylose to be represented by S_1, S_2, and S_3, repectively, specific growth rates on each of the substrates in a three substrate system are as follows:

$$\mu_1 = \frac{\mu_{m1} S_1}{K_1 + S_1} \tag{4}$$

$$\mu_2 = \left[\frac{\mu_{m2} S_2}{K_2 + S_2}\right]\left[\frac{1}{1 + B_2 S_1^n}\right] \tag{5}$$

$$\mu_3 = \left[\frac{\mu_{m3} S_3}{K_3 + S_3}\right]\left[\frac{1}{1 + B_3 S_1^n}\right] \tag{6}$$

The overall specific growth rate (Eq. (3)) then becomes:

$$\mu = \left[\frac{\mu_{m1} S_1}{K_1 + S_1}\right] + \left[\frac{\mu_{m2} S_2}{K_2 + S_2}\right]\left[\frac{1}{1 + B_2 S_1^n}\right] + \left[\frac{\mu_{m3} S_3}{K_3 + S_3}\right]\left[\frac{1}{1 + B_3 S_1^n}\right] \tag{7}$$

Table 4. Kinetic parameters used for model simulations of pure culture of *C. lusitaniae*

Parameters	Glu/Cell	Glu/Cell/Xyl
μ_{m1}	0.34	0.38
μ_{m2}	0.10	0.10
μ_{m3}	–	0.01
K_1	0.30	0.30
K_2	0.20	0.20
K_3	–	0.70
Y_1	0.19	0.19
Y_2	0.15	0.15
Y_3	–	0.30
Y_{p1}	0.47	0.47
Y_{p2}	0.49	0.49
Y_{p3}	–	0.08
n	1.00	1.00
B_2	50.00	50.00
B_3	–	50.00

These expression may be used in mass balances to give the following set of differential equations:

$$\frac{dX}{dt} = D(X_0 - X) + \mu X \tag{8}$$

$$\frac{dS_1}{dt} = D(S_{10} - S_1) - \frac{1}{Y_1}\mu_1 X \tag{9}$$

$$\frac{dS_2}{dt} = D(S_{20} - S_2) - \frac{1}{Y_2}\mu_2 X \tag{10}$$

$$\frac{dS_3}{dt} = D(S_{30} - S_3) - \frac{1}{Y_3}\mu_3 X \tag{11}$$

$$\frac{dP}{dt} = D(P_0 - P) + Y_{P1}\mu_1 X + Y_{P2}\mu_2 X + Y_{P3}\mu_3 X \tag{12}$$

where D is the dilution rate (h^{-1}), Y_i is the yield of cell mass (g cells/g substrate) and Y_{Pi} is the yield of ethanol (g ethanol/g cells).

Batch fermentation of glucose/cellobiose mixture was simulated by setting D=0 and solving the resulting set of non-linear differential equations using a 4th order Runge-Kutta procedure. The kinetic parameters used for the simulation are summarized in Table 4. Values of K_i, Y_i, and Y_{Pi} were taken from the single substrate results (Table 1). Since n is the stoichiometric coefficient for the combining of cAMP with the catabolite gene activator protein, it must have an integer value. Values for n and B in the catabolite repression model were obtained by selecting an integer value of n and varying B to get the best fit of experimental data for the glucose/cellobiose batch fermentation. Because of the permanent repression effect, the value of μ_{m2} was taken as 0.1 h^{-1} which was obtained from the glucose/cellobiose experimental data.

The results of the simulation for a two sugar system are shown in Fig. 1 (solid lines) to compare with experimental data for the growth of *C. lusitaniae* on a mixture of glucose and cellobiose. There was a slight underestimation for both cell mass and ethanol concentrations, especially during the lag between growth phases. This discrepancy might be due to estimation errors in K or other parameters. In Fig. 4, simulation curves for the glucose/cellobiose/xylose mixture are compared with experimental data. As with the glucose/cellobiose simulation, the cell concentration was slightly underestimated.

CONCLUSION

The yeast *C. lusitaniae*, which can ferment cellobiose as well as glucose, has been evaluated as being one member of a model system for mixed culture fermentations. Pure culture experiments were performed to obtain kinetic data for growth and ethanol production from multiple carbon sources.

C. lusitaniae can produce ethanol in high yields from glucose and cellobiose and has a slight capability to ferment xylose. Data from batch culture of mixed sugars indicate that glucose causes the catabolite repression of cellobiose and xylose utilization. However, cellobiose and xylose are utilized simultaneously. Also, glucose causes a permanent repression of the cellobiose utilization by reducing growth rates during the cellobiose phase of glucose/cellobiose fermentation.

Ethanol yields from mixtures of sugar were generally added for each of the substrates, indicating that the yield from one substrate is not affected by the presence of other substrates. The growth model for multiple substrates was developed based on a cAMP mediated catabolite repression mechanism. This model adequately describes the growth and ethanol production by *C. lusitaniae* for batch fermentation of multiple sugars.

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