

The Effect of Initial Cell Concentration on Xylose Fermentation by *Pichia stipitis*

FRANK K. AGBOGBO,^{*,1} GUILLERMO COWARD-KELLY,¹ MADS
TORRY-SMITH,¹ KEVIN WENGER,¹ AND THOMAS W. JEFFRIES²

¹Novozymes North America Inc., 77 Perry Chapel Church Road,
Franklinton, NC 27525, E-mail: fkag@novozymes.com; and

²Institute from Microbial and Biochemical Technology, Forest products
Laboratory, One Gifford Pinchot Dr., Madison, WI 53705

Abstract

Xylose was fermented using *Pichia stipitis* CBS 6054 at different initial cell concentrations. A high initial cell concentration increased the rate of xylose utilization, ethanol formation, and the ethanol yield. The highest ethanol concentration of 41.0 g/L and a yield of 0.38 g/g was obtained using an initial cell concentration of 6.5 g/L. Even though more xylitol was produced when the initial cell concentrations were high, cell density had no effect on the final ethanol yield. A two-parameter mathematical model was used to predict the cell population dynamics at the different initial cell concentrations. The model parameters, a and b correlate with the initial cell concentrations used with an R^2 of 0.99.

Index Entries: Cell concentration; fermentation; model; *Pichia stipitis*; xylose.

Introduction

Cost-effective production of fuel ethanol from lignocellulosic biomass requires fermentation of the hemicellulose fraction, which contains xylose as the major sugar component in agricultural residues (1). Hemicellulose includes 24–35% of the dry biomass of some lignocellulosic materials, such as corn fiber, corn cob, corn stover, and sugar cane bagasse (1). Native strains of *Pichia stipitis* have the ability to ferment xylose, glucose, and cellobiose (2,3).

Low levels of oxygen (1.5–5 mmol/L·h) are necessary for the conversion of xylose to ethanol in order to maintain cell viability and NADH balance (4–7). A high level of oxygen leads to ethanol reassimilation and biomass production, and therefore, low yields (8–10). Under anaerobic conditions, cell growth and ethanol production are severely restricted (11). The cell growth of *P. stipitis* is inhibited at low ethanol concentrations (approx 34.0 g/L) (12–13) and the highest ethanol tolerance reported for

*Author to whom all correspondence and reprint requests should be addressed.

growth is 64.0 g/L (11). Previous studies have shown that the fermentation rate can be increased by addition of 10.0 mg/L zinc (14) and that it can be decreased by forced cycling of pH (15). The initial cell concentrations used in *P. stipitis* fermentations varies from 0.01 to 6 g/L (14,16). However, how the initial cell concentration affects fermentation rate, final ethanol concentration, and yield has not been documented. The purpose of this work is to look at the effect of the initial cell concentration on the rate of fermentation, final ethanol concentrations, and yield using synthetic media containing xylose.

Materials and Methods

Microorganism and Media

P. stipitis CBS 6054 stock cultures were maintained on 20% glycerol at -15°C . Stock culture (100 μL) of *P. stipitis* CBS 6054 was cultured on yeast extract, peptone, xylose agar plates at 30°C for 3 d. The plates were prepared from 10.0 g/L yeast extract, 20.0 g/L peptone, 20.0 g/L xylose, and 20.0 g/L agar. Colonies from the plates were grown overnight in a filter-sterilized fermentation medium containing 1.7 g/L yeast nitrogen base (without amino acid and ammonium sulfate), 2.27 g/L urea, 6.56 g/L peptone, and 20.0 g/L xylose. The cells were centrifuged at 3000 rpm for 5 min and the supernatant was discarded. The cells were resuspended in distilled water media to have a cell concentration of 55.0 g/L.

Synthetic Media Preparation

Xylose solution (140.0 g/L) was prepared and filter-sterilized. Nutrient solution (50X the concentration used) was prepared by dissolving 1.7 g of yeast nitrogen base, 2.27 g of urea, and 6.56 g of peptone in 20 mL of water.

Fermentation

Fermentations were performed in sterile 125-mL Erlenmeyer flasks (with 0.2 μm vent cap) using an air-shaker incubator at 30°C and 100 rpm. The initial pH was 6.3. Each Erlenmeyer flask contained 50 mL of xylose solution, 1 mL of nutrient solution, and 7 mL of inoculum, which had been diluted to the appropriate cell concentration. Initial cell concentrations of 1.8, 4.3, and 6.5 g/L were investigated in this study. All experiments were performed in triplicate.

Analytical Methods

Fermentation was monitored for 8 d by taking 1 mL of sample for xylose, ethanol, and xylitol analyses. The concentration of xylose, ethanol, and xylitol was determined using an Agilent high-performance liquid

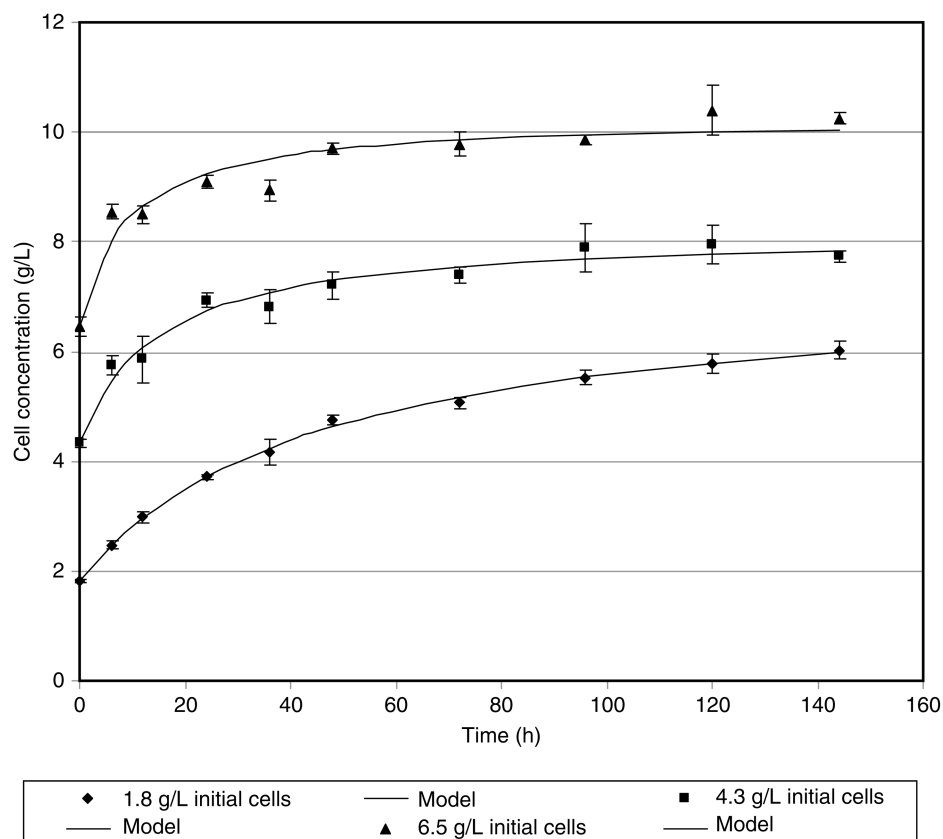


Fig. 1. Cell concentrations during fermentation. Error bars are ± 1 standard deviation.

chromatography System with an analytical BIO-RAD Aminex HPX-87H column and a BIO-RAD Cation H refill guard column. Cell concentration was determined from optical density measurement of the cells using Cary 3C Ultraviolet-visible spectrophotometer at 600 nm. An optical density of 1 is equivalent to 0.23 g of dry cells/L.

Results and Discussion

Cell Growth and Mathematical Model

The cell concentrations of *P. stipitis* during fermentation on xylose are shown in Fig. 1. From Fig. 1, an initial cell concentration of 1.8, 4.3, and 6.5 g/L increased to 6.0, 7.7, and 10.3 g/L, respectively, after 144 h of fermentation. The average specific cell growth rates decrease at high initial cell concentrations. Previous attempts to model *P. stipitis* fermentation used the Monod model (11,17–19). The Monod model describes substrate-limited growth in which environmental conditions are only related to the substrate concentration. However, the fermentation performed in this work is not limited solely by the amount of substrate supplied initially.

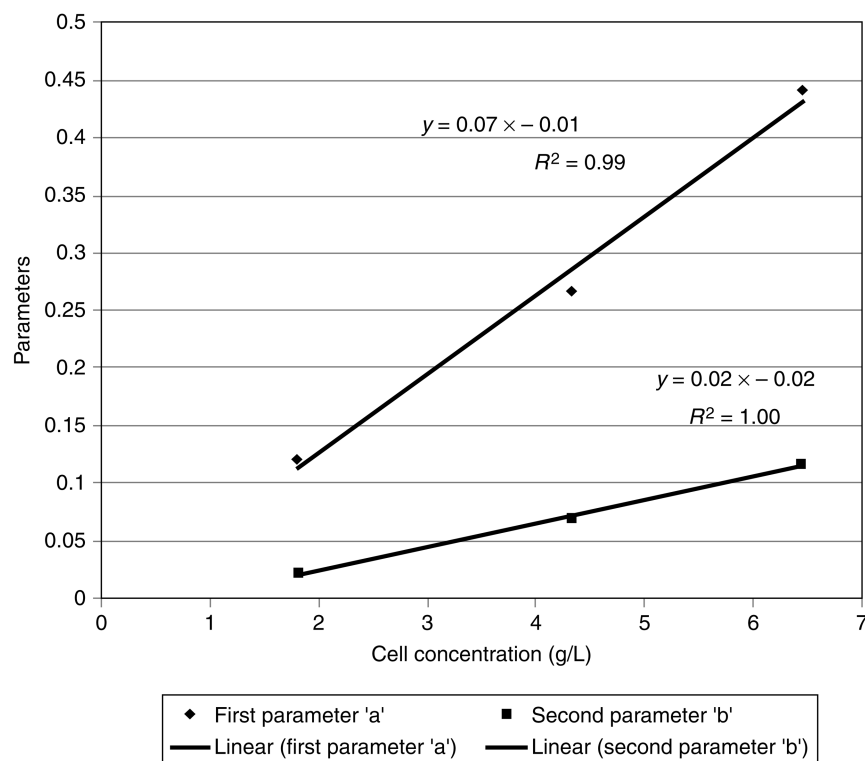


Fig. 2. Variation of model parameters during fermentation on xylose.

Therefore, a two-parameter empirical mathematical model was used to fit the cell concentrations during the fermentation. The different initial cell concentrations were fit to Eq. 1, using least-square analysis of the error between experimental and predicted values.

$$X = X_0 + \frac{at}{1+bt} \quad (1)$$

where X is the cell concentration during fermentation (g/L), X_0 is the cell concentration initially (g/L), t is the time (h), a is a parameter with units (g/L · h), and b is a parameter with units (1/h).

The parameters a and b were determined at the different initial cell concentrations used in the fermentation. The variation of these parameters with the initial cell concentration is shown in Fig. 2. From Fig. 2, a linear correlation fits the parameters, a and b , with a correlation coefficient of 0.99 and 1, respectively. Therefore, Eq. 1 predicts the population dynamics with parameters that correlate very well with the initial cell concentrations used in the fermentation. From the graphs in Fig. 2, it is possible to determine the parameters a and b , in the range 1.8–6.5 g/L and then determine the cell concentrations during the fermentation. Although these studies were done in shake flasks at a shaking rate of 100 rpm and sugar concentration of

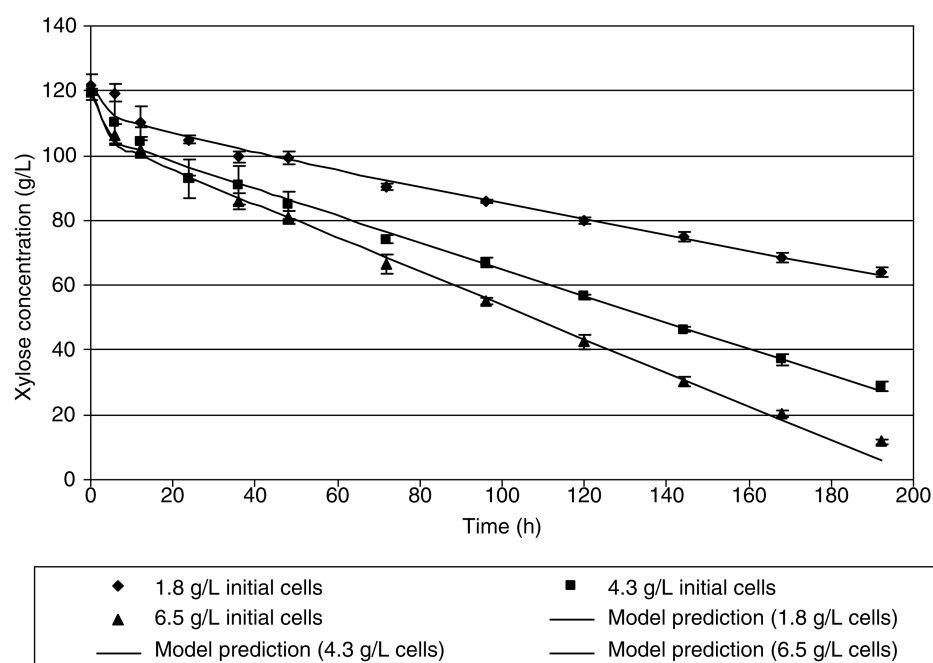


Fig. 3. Xylose concentration at different initial cell concentrations. Error bars are ± 1 standard deviation.

120 g/L, a working population dynamics model for this system could be applied to other systems.

Substrate Consumption

The xylose concentrations at the different initial cell concentrations are shown in Fig. 3. From the graph, the amounts of xylose consumed after 192 h of fermentation were 58.0, 92.0, and 108.0 g/L at initial cell concentrations of 1.8, 4.3, and 6.5 g/L, respectively. At high initial cell concentrations, the amount of substrate consumed was high. As the amount of substrate consumed depends on the initial cell concentration used, inoculating with the right initial cell concentration is vital for complete substrate consumption with *P. stipitis*. In a recent study (20), using xylose at a concentration of 60.0 g/L and an initial cell concentration of 2.0 g/L, approx 60.0 g/L of the substrate was consumed in 120 h compared with 192 h in this study. The decrease in the ethanol yield and the rate of substrate utilization in this work compared with our previous study (20) is similar to observations made by Du Preez et al (21).

The rate of substrate consumption is modeled using the Leudeking-Piret kinetics (22) as shown in Eq. 2.

$$\frac{dS}{dt} = -\alpha X - \beta \frac{dX}{dt} \quad (2)$$

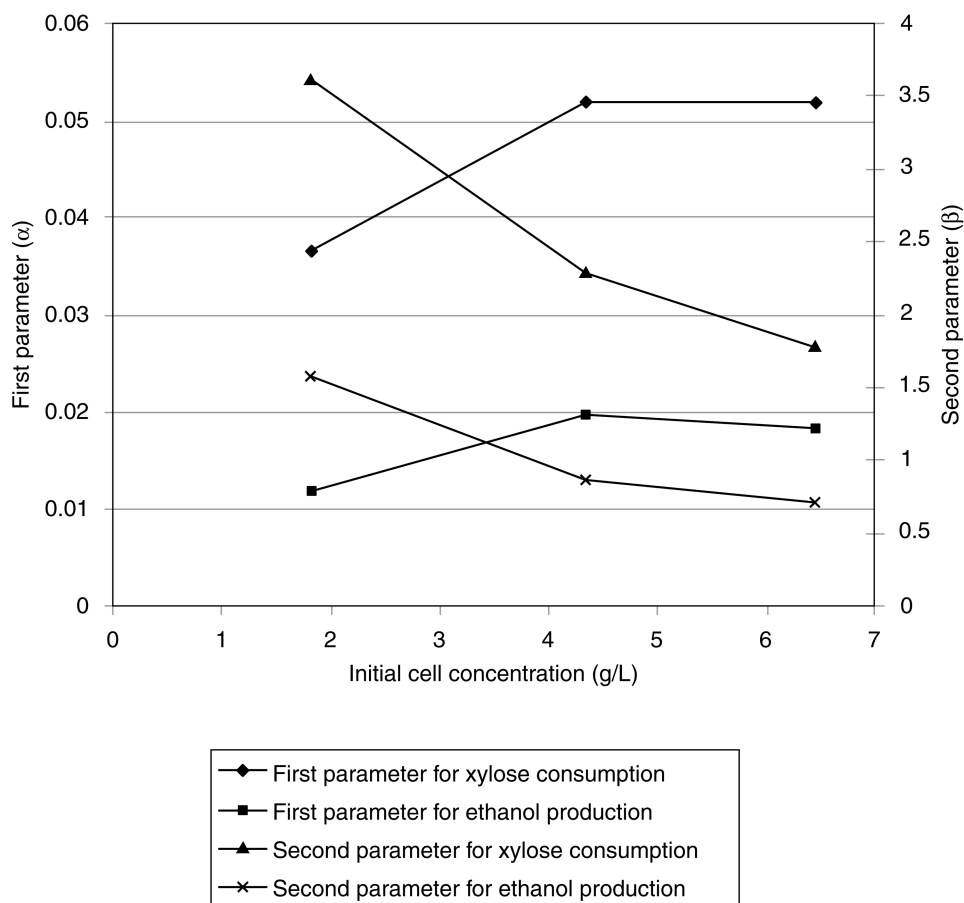


Fig. 4. Variation of model parameters with initial cell concentrations.

where α is a parameter associated with cell maintenance (1/h) and β is a parameter associated with growth (g substrate/g cells). Substituting Eq. 1 and its differential in Eq. 2 and integrating, we obtain Eq. 3.

$$S = S_0 - \alpha \left[X_0 t + \frac{at}{b} - \frac{a}{b^2} \ln(1+bt) \right] - \beta \left[X_0 + \frac{at}{1+bt} \right] \quad (3)$$

where S is the substrate concentration at time t (g/L) and S_0 is the initial substrate concentration (g/L). Equation 3 was fitted to experimental data using least square minimization of the error between experimental and predicted values. The experimental and predicted substrate concentrations at the different initial cell concentrations are shown in Fig. 3. The model parameters (α and β), at the different initial cell concentrations are shown in Fig. 4. From Fig. 4, the first parameter (α), which is related to the maintenance coefficient, increased when the initial cell concentration increased from 1.8 to 4.3 g/L. However, the parameter remained essentially constant when initial cell concentrations increased from 4.3 to 6.5 g/L. The first

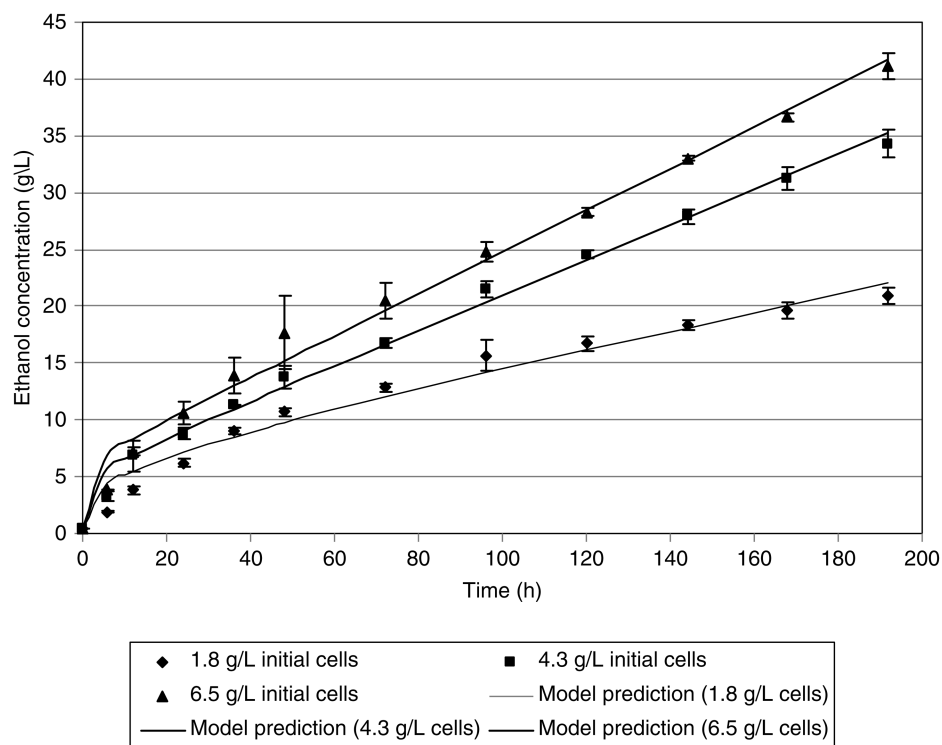


Fig. 5. Ethanol concentration at different initial cell concentrations. Error bars are ± 1 standard deviation.

parameter is the major contributor to the rate of substrate consumption after the initial growth period ($t > 48$ h). The results indicate that the initial cell concentration needs to be more than some threshold value in order to get substantial substrate consumption. The second parameter β , which is the growth associated parameter, decreased as the initial cell concentrations increased (see Fig. 4). As the cells grow more when the initial cell concentration is low, this parameter is high when the initial cell concentrations are low. Although β is high at low initial cell concentrations, it has no significant effect on the initial fermentation rate.

Ethanol Production

Figure 5 shows the ethanol concentration at the different initial cell concentrations. The final ethanol concentrations after 192 h of fermentation were 20.9, 34.3, and 41.1 g/L at the initial cell concentrations of 1.8, 4.3, and 6.5 g/L, respectively. The final ethanol concentration was high when the initial cell concentration was high because there were more cells to convert the xylose into ethanol. The amount of ethanol produced in the fermentation depends on the amount of cells used in the initial inoculum. Xylitol, a byproduct of the fermentation, was at concentrations of 2.2, 6.9, and 10.4 g/L at the initial cell concentrations of 1.8, 4.3, and 6.5 g/L,

Table 1
Summary of Fermentation Results After 192 h of Fermentation

Substrate	Xylose		
Substrate concentration (g/L)		120.3 ± 1.4	
Initial cell concentration (g/L)	1.82 ± 0.04	4.33 ± 0.08	6.45 ± 0.16
Maximum ethanol concentration (g/L)	20.9 ± 0.72	34.3 ± 1.22	41.09 ± 1.12
Ethanol yield (g/g)	0.363 ± 0.019	0.381 ± 0.017	0.378 ± 0.006
Xylitol yield (g/g)	0.038 ± 0.003	0.077 ± 0.007	0.096 ± 0.002
Substrate consumption rate (g/L·h)	0.301 ± 0.021	0.470 ± 0.006	0.565 ± 0.007
Ethanol production rate (g/L·h)	0.109 ± 0.004	0.179 ± 0.006	0.214 ± 0.006
Ethanol selectivity (g/g)	0.906 ± 0.002	0.832 ± 0.013	0.798 ± 0.006

Errors are ±1 standard deviation.

respectively, after 192 h of fermentation. The xylitol concentrations and fraction of the total product converted to xylitol, increased with an increase in the initial cell concentration.

A summary of the fermentation results are shown in Table 1. The ethanol yield was 0.36, 0.38, and 0.38 g/g at the initial cell concentrations of 1.8, 4.3, and 6.5 g/L, respectively. The ethanol yield was slightly higher at initial cell concentrations of 4.3 and 6.5 g/L because the substrate was used for ethanol production rather than for cell biomass production. There was no significant difference between the yield at initial cell concentrations of 4.3 and 6.5 g/L, although the fraction of the substrate converted to xylitol was higher at initial cell concentration of 6.5 g/L. This might be owing to the neutralizing effect of the two factors, an increased yield owing to less cell biomass production, and a decreased yield owing to xylitol production. The selectivity for ethanol was high at low initial cell concentrations and decreased as the initial cell concentrations increased.

The rate of ethanol production modeled using the Leudeking–Piret kinetics for product formation as shown in Eq. 4.

$$\frac{dP}{dt} = -\alpha X + \beta \frac{dX}{dt} \quad (4)$$

where α is a parameter associated with cell maintenance (1/h) and β is a parameter associated with growth. Substituting Eq. 1 and its differential in Eq. 4 and integrating, we obtain Eq. 5.

$$P = P_0 + \alpha \left[X_0 t + \frac{at}{b} - \frac{a}{b^2} \ln(1+bt) \right] + \beta \left[X_0 + \frac{at}{1+bt} \right] \quad (5)$$

where P is the ethanol concentration at time t (g/L) and P_0 is the initial ethanol concentration (g/L). Equation 5 was fit to experimental data using least square minimization of the error between experimental and predicted values. The experimental and predicted substrate concentrations at the different initial cell concentrations are shown in Fig. 5. The model parameters at the different initial cell concentrations are shown in Fig. 4.

From Fig. 5, the observations made for the substrate consumption are similar to that made for ethanol production. The first parameter (α), which is related to the maintenance coefficient, is the major contributor to the rate of ethanol production after the initial growth period. The results from this study indicate that the initial cell concentration needs to be more than some threshold in order to get substantial product formation rate. The second parameter (β), decreased as the initial cell concentrations increased (see Fig. 5). The parameter β , is a growth associated parameter and has a significant effect on the initial ethanol production rate.

Conclusions

This work shows that the initial cell concentration of *P. stipitis* affects the fermentation rate, ethanol concentrations, and yield. At low initial cell concentrations, cell growth was higher compared with fermentations with high initial cell concentrations. The rate of xylose consumption and ethanol production was high when the initial cell concentrations were high. Ethanol yield was high when the initial cell concentration was high because the cells used the substrate for ethanol production rather than for cell growth. However, the ethanol selectivity was low at higher initial cell concentrations.

The cell growth curve is a function of the initial cell concentration used in the fermentation. The form of the empirical mathematical model used in this work describes the population dynamics with just two parameters, which correlate very well with the initial cell concentration used. This cell concentration model was used with the Leudeking-Piret kinetics to predict substrate consumption and product formation during the fermentation. From the results, the substrate consumption and ethanol production rate are both functions of the initial cell concentration used. In the use of *P. stipitis* for biomass fermentation, an adequate amount of cells is necessary for complete substrate utilization and adequate ethanol yield.

Acknowledgment

This investigation was supported by the Abengoa-DOE project. We like to thank David Milam for helping with the high-performance liquid chromatography analysis.

References

1. Saha, B. C. (2003), *J. Ind. Microbiol. Biotechnol.* **30**, 279–291.
2. Toivola, A., Yarrow, D., van den Bosch, E., van Dijken, J. P., and Scheffers, W. A. (1984), *Appl. Environ. Microbiol.* 1221–1223.
3. Du Preez, J. C., Bosch, M., and Prior, B. A. (1986), *Appl. Microbiol. Biotechnol.* **23(3–4)**, 228–233.
4. Chandakant, P. and Bisaria, V. S. (1998), *Crit. Rev. Biotechnol.* **18(4)**, 295–331.
5. Moniruzzaman, M. (1995), *World J. Microbiol. Biotechnol.* **11**, 646–648.
6. Grootjen, D. R. J., van der Lans, R. G. J. M., and Luyben, K. Ch. A. M. (1990), *Enzyme Microb. Technol.* **12**, 20–23.
7. Grootjen, D. R. J., van der Lans, R. G. J. M., and Luyben, K. Ch. A. M. (1991), *Enzyme Microb. Technol.* **13**, 648–654.
8. Passoth, V., Zimmermann, M., and Klinner, U. (2003), *Appl. Biochem. Biotechnol.* **57/58**, 201–212.
9. du Preez, J. C. (1994), *Enzyme Microb. Technol.* **16**, 944–956.
10. Gorgens, J. F., Passoth, V., van Zyl, W. H., Knoetze, J. H., and Hahn-Hagerdal, B. (2005), *FEMS Yeast Res.* **5**, 677–683.
11. Slininger, P. J., Branstrator, L. E., Bothast, R. J., Okos, M. R., and Ladisch, M. R. (1991), *Biotechnol. Bioeng.* **37**, 973–980.
12. Meyrial, V., Delgenes, J. P., Romieu, C., Moletta, R., and Gounot, A. M. (1995), *Enzyme Microb. Technol.* **17**, 535–540.
13. Delgenes, J. P., Moletta, R., and Navarro, J. M. (1988), *J. Ferment. Technol.* **66(4)**, 417–422.
14. Sreenath, H. K. and Jeffries, T. W. (2000), *Bioresour. Technol.* **72**, 253–260.
15. Ryding, P. -O., Niklasson, C., and Liden, G. (1993), *Can. J. Chem. Eng.* **71**, 911–915.
16. Sanchez, S., Bravo, V., Castro, E., Moya, A. J., and Camacho, F. (2002), *J. Chem. Technol. Biotechnol.* **77**, 641–648.
17. Dominguez, H., Nunez, M. J., Chamy, R., and Lema, J. M. (1993), *Biotechnol. Bioeng.* **41**, 1129–1132.
18. Kompala, D. S., Ramkrishna, D., and Tsao, G. T. (1984), *Biotechnol. Bioeng.* **26**, 1272–1281.
19. Dhurjati, P., Ramkrishna, D., Flickinger, M. C., and Tsao, G. T. (1985), *Biotechnol. Bioeng.* **27**, 1–9.
20. Agbogbo, F. K., Coward-Kelly, G., Torry-Smith, M., and Wenger, K. S. (2006), *Process Biochem.* (Accepted).
21. du Preez, J. C., Bosch, M., and Prior, B. A. (1986), *Enzyme Microb. Technol.* **8**, 360–364.
22. Leudeking, R. and Piret, E. L. (1959), *J. Microbiol. Technol. Eng.* **1(4)**, 393–412.