Stoichiometry and Kinetics of Xylose Fermentation by *Pichia stipitis*


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INTRODUCTION AND THEORETICAL CONSIDERATIONS

Conclusions of previous investigations have led us to focus on *Pichia stipitis* as a yeast with high potential for producing ethanol from xylose-rich, wood-processing wastes. Given 150 g/L xylose in complex medium, strain Y-7124 functions optimally at 25–26°C and pH 4–7 to accumulate 56 g/L ethanol with negligible xylitol by-production. In a past report, we cited the need for an optimal bioreactor system; toward this end, we put oxygen uptake, growth, and death kinetics into mathematical form. The present report builds on our previous work as the pathways and stoichiometry of xylose metabolism are examined and models of xylose uptake and ethanol production are identified.

Yeasts begin xylose metabolism with the sequence, xylose → xylitol → xylulose → xylulose 5-phosphate. In balanced form, this sequence is:

\[ \begin{align*}
6 \text{C}_5\text{H}_{10}\text{O}_5 + 6f \text{NADPH}, \text{H}^+ &+ 6(1 - f) \text{NADH}, \text{H}^+ \\
&\rightarrow 6 \text{C}_5\text{H}_{12}\text{O}_5 + 6f \text{NADP}^+ + 6(1 - f) \text{NAD}^+ \quad (1) \\
6 \text{C}_5\text{H}_{12}\text{O}_5 + 6 \text{NAD}^+ &\rightarrow 6 \text{C}_5\text{H}_{10}\text{O}_5^+ + 6 \text{NADH}, \text{H}^+ \quad (2) \\
6 \text{C}_5\text{H}_{10}\text{O}_5^+ + 6 \text{ATP} &\rightarrow 6 \text{C}_5\text{H}_{11}\text{O}_3\text{PO}_3 + 6 \text{ADP}. \quad (3)
\end{align*} \]

Here, \( \text{C}_5\text{H}_{10}\text{O}_5 \) designates xylulose, an isomer of xylose. Verduyn *et al.* have shown that *Pichia stipitis* has a xylose reductase with dual cofactor specificity. The parameter \( f \) designates the fraction of reductase activity supported by NADPH, and the difference \( 1 - f \) designates the fraction supported by NADH. Our stoichiometry model

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presumes that metabolism of xylulose 5-phosphate can continue via any of four processes: assimilation, pentose phosphate oxidation, respiration, and ethanolic fermentation.

The following equation describes xylose assimilation for cell synthesis and maintenance:

\[
883 \text{C}_5\text{H}_{10}\text{O}_5 + 280 \text{NH}_2\text{CONH}_2 + (912 + 883f') \text{NADPH,H}^+ \\
+ (1322 + 883f') \text{NAD}^+ + 3219.2 [\text{ATP} + \text{H}_2\text{O}] \\
\rightarrow 1000 \text{C}_4\text{H}_7\text{O}_5\text{N}_0\text{S}_6\text{O}_{2.20} + 695.5 \text{CO}_2 + (912 + 883f') \text{NADP}^+ \\
+ (1322 + 883f') \text{NADH,H}^+ + 1105 \text{H}_2\text{O} + 3219.2 [\text{ADP} + \text{P}_i].
\] (4)

In building this equation, we assumed that the empirical formula for Y-7124 biomass was \(\text{C}_4\text{H}_7\text{O}_5\text{N}_0\text{S}_6\text{O}_{2.20}\) (98 g/mole) based on elemental analysis of lyophilized cells (Galbraith Laboratories); that 0.736 glucose + 0.56 NH\(_2\) + 3.07 ATP + 0.9124 \(\text{NADPH,H}^+ + 1.322 \text{NAD}^+\) produces 0.4155 CO\(_2\) + 1 mole yeast plus the complementary cofactors;\(^{10,11}\) that urea hydrolysis obeys the stoichiometry, \(\text{NH}_2\text{CONH}_2 + \text{H}_2\text{O} \rightarrow 2 \text{NH}_3 + \text{CO}_2\); and that xylose and glucose are related by

\[
6 \text{C}_5\text{H}_{10}\text{O}_5 + 6f'\text{NADPH,H}^+ + 6f'\text{NAD}^+ + [\text{ATP} + \text{H}_2\text{O}] \\
\rightarrow 5 \text{C}_6\text{H}_7\text{O}_6 + 6f'\text{NADH,H}^+ + 6f'\text{NADP}^+ + [\text{ADP} + \text{P}_i].
\] (5)

Equation 6 accounts for the conversion of xylulose 5-phosphate to glucose 6-phosphate via the pentose phosphate pathway:

\[
6 \text{C}_5\text{H}_{11}\text{O}_5\text{PO}_3 + \text{H}_2\text{O} \rightarrow 5 \text{C}_6\text{H}_{12}\text{O}_6\text{PO}_3 + \text{P}_i.
\] (6)

Equation 7 compensates for the difference in ATP required to phosphorylate glucose and the carbon-equivalent of xylose:

\[
5 \text{C}_6\text{H}_{12}\text{O}_6\text{PO}_3 + 5 \text{ADP} \rightarrow 5 \text{C}_6\text{H}_{12}\text{O}_6 + 5 \text{ATP}.
\] (7)

Equation 5 is the sum of equations 1–3, 6, and 7. Thus, the stoichiometry for xylose assimilation was derived from current knowledge of pentose and glucose metabolism in yeasts. Note that there is a small (<1%) hydrogen deficit on the left side of equation 4 that stems from the H:O ratio fixed by our empirical cell formula.

Pentose phosphate oxidation, respiration, and ethanolic fermentation can be classified as dissimilatory processes, which supply the cofactors and energy needed for assimilation. Three major enzyme systems participate, including the pentose phosphate (PP), Embden-Meyerhof-Parnas (EMP), and tricarboxylic acid (TCA) pathways.\(^8,12\) Anaerobic cycling of the PP pathway oxidizes xylose to \(\text{CO}_2\) as ATP is consumed and reduced cofactors are produced:

\[
6 \text{C}_5\text{H}_{10}\text{O}_5 + 6f' \text{NAD}^+ + (60 - 6f') \text{NADP}^+ + 6 [\text{ATP} + \text{H}_2\text{O}] \\
+ 30 \text{H}_2\text{O} \rightarrow 30 \text{CO}_2 + (60 - 6f') \text{NADPH,H}^+ \\
+ 6f' \text{NADH,H} + 6 [\text{ADP} + \text{P}_i].
\] (8)

As a primary function, the PP pathway supplies \(\text{NADPH,H}^+\) to assimilation. However, it also produces the intermediates, glyceraldehyde 3-phosphate and fructose 6-phosphate, which can enter the EMP pathway to form pyruvate. The pyruvate
formed either enters the TCA cycle or is fermented to ethanol. Under aerobic conditions, respiration of xylose occurs via the path, PP \rightarrow EMP \rightarrow TCA, which adds up to ATP production with no net change in reducing equivalents:

\[
6 \text{C}_5\text{H}_{10}\text{O}_5 + 6f\text{NADPH},\text{H}^- + 6f\text{NAD}^+ + 30\text{O}_2
+ 180 [\text{ADP} + P_i] \rightarrow 30\text{CO}_2 + 6f\text{NADH},\text{H}^+
+ 6f\text{NADP}^+ + 180 [\text{ATP} + \text{H}_2\text{O}] + 30\text{H}_2\text{O}. \tag{9}
\]

During oxygen limitation, fermentation via the sequence, PP \rightarrow EMP \rightarrow ethanol, is expected to predominate, even though it yields less energy than respiration:

\[
6 \text{C}_5\text{H}_{10}\text{O}_5 + 6f\text{NADPH},\text{H}^- + 6f\text{NAD}^+
+ 10 [\text{ADP} + P_i] \rightarrow 10\text{C}_2\text{H}_5\text{OH} + 10\text{CO}_2
+ 6f\text{NADH},\text{H}^- + 6f\text{NADP}^+ + 10 [\text{ATP} + \text{H}_2\text{O}]. \tag{10}
\]

Optimistically, the fraction of xylose sent through each of the four processes is regulated such that production and consumption of ATP, NADH$_2^-$, and NADPH$_2^-$ are balanced. If this does not occur, metabolism may stall as intermediates accumulate. For example, xylitol accumulation is believed to occur because of imperfect recycling of NADH$_2^-$ and NAD$^+$ between xylose reductase and xylitol dehydrogenase activities. If xylose reductase activity uses NADPH,H$^-$ instead of NADH,H$^-$ (i.e., $f > 0$), an NAD$^+$ shortage is indicated by the two-step reaction (equations 1 and 2) in which xylose is isomerized to xylulose. Cofunctioning of other pathways can lessen the likelihood of this imbalance. For instance, assimilation consumes NADPH,H$^-$ and produces NADH,H$^-$, thereby increasing the relative availability of NADH,H$^-$ for use by the reductase.

If we consider batch culture kinetics, xylose uptake and ethanol production are stoichiometrically related as follows:

\[
dX/dt = (1/Y_{E/X})dE/dt, \tag{11}
\]

where X and E are xylose and ethanol concentrations, respectively, and $Y_{E/X}$ is the yield of ethanol per xylose consumed. Previously, we verified that growth is an obligately aerobic process for strain Y-7124$^T$ and, although growth is expected to stimulate ethanol production, the necessary presence of oxygen brings on respiration as well. Considering that respiration provides more ATP than fermentation, we might expect $Y_{E/X}$ to drop with increasing $\mu$.

Batch culture ethanol concentration increases at a rate proportional to the viable biomass concentration ($b$) by the specific productivity ($p_E$):

\[
dE/dt = p_E b. \tag{12}
\]

Because fermentation is coupled to assimilation through ATP production, we propose that $p_E$ is at least partly growth-associated. Although originally applied to lactate production, Luedeking and Piret's model$^{13}$ expresses this behavior in the following form:

\[
p_E = Y_{E/b} \mu + m_E. \tag{13}
\]
In the context of ethanol production, \( Y_{E/b} \) represents the ethanol yielded per biomass formed, \( \mu \) is the specific growth rate based on viable cells, and \( m_E \) is the specific ethanol productivity that provides maintenance energy to resting cells. The rate \( p_E \) and the parameters \( Y_{E/b} \) and \( m_E \) are expected to vary with ethanol and xylose concentration.\(^{14,15}\)

Experiments were designed to test the application of these concepts to modeling of \( Y_{E/b} \) during oxygen-limited growth and to evaluate the parameters of useful kinetic expressions. Continuous culture yields indicated how well our stoichiometry model (equations 4 and 8–10) accounted for production and consumption of carbon and cofactors. Overall stoichiometry and \( p_E \) were studied as functions of \( \mu \) in cultures operated at various dilution rates, and dependences of \( p_E \) and \( Y_{E/X} \) on \( E \) and \( X \) were obtained from initial batch performances of concentrated cell populations.

**MATERIALS AND METHODS**

Organism and Media

Lyophilized *Pichia stipitis* NRRL Y-7124 (CBS 5773) was acquired from the ARS Culture Collection (Northern Regional Research Center, Peoria, Illinois). Stock cultures maintained on agar slants were used to prepare fermentor inocula adapted to xylose broth.\(^6\) The complex media for slants (YM) and liquid cultures (CCY) have been described in detail.\(^6\) CCY medium contained yeast extract, urea, potassium phosphate buffer, mineral salts, and xylose, and it was supplemented in fermentors with 1 g/L Hodag FD-62 antifoam.

Continuous Culture Evaluation of Ethanol Productivity and Stoichiometry as Functions of Growth

Fermentation rates and stoichiometry were studied in oxygen-limited continuous cultures operated at 25 °C, pH 4.5, and various dilution rates (\( D \)). Aeration and stirring rates were set to provide specific oxygen transfer coefficients (\( K_o a \)), which were evaluated prior to inoculation from semilog plots of oxygen saturation time courses. The configuration of B. Braun Biostat 2ER (2-L) fermentors and our method for evaluating \( K_o a \) have been described.\(^7\) The differential ethanol balance for this system was as follows:

\[
dE/dt = -DE + p_E b.
\]  

Given that the subscript “s” designates concentrations measured at steady state (i.e., \( dE/dt = 0 \)), the specific ethanol productivity \( (p_E) \) was calculated from the equation

\[
p_E = D E_s / b_s.
\]  

In general, specific growth rate \( (\mu) \) is related to \( D \) according to

\[
\mu = D [1 + (\frac{b_{ds}}{b_s})],
\]  

which was derived previously by applying the steady-state criterion to differential balances on viable \( (b) \) and dead \( (b_d) \) biomass concentrations.\(^7\) Note that \( \mu = D \) only if \( b_{ds} = 0 \) or \( b_{ds} < b_s \). We did not find this to be true for our system.
The oxygen to xylose uptake ratio \( \left( \frac{Y_{\text{ox}}}{X} \right) \) and the yields of ethanol \( \left( \frac{Y_{\text{E}}}{X} \right) \), biomass \( \left( \frac{Y_{\text{b}}}{X} \right) \), and carbon dioxide \( \left( \frac{Y_{\text{co}_2}}{X} \right) \) were calculated as follows:

\[
Y_{\text{ox}}/X = K_a [a(C_{\text{ox}} - C_{\text{ox}*})/D(X_t - X_s)], \\
Y_{\text{E}}/X = E_s/(X_t - X_s), \\
Y_{\text{b}}/X = b_{T_s}/(X_t - X_s), \\
Y_{\text{co}_2}/X = Q_g F_{\text{co}_2} d_{\text{co}_2}/[V_1 D(X_t - X_s)].
\]

The parameters \( C_{\text{ox}*} \) and \( C_{\text{ox}}^* \) denote steady-state dissolved oxygen concentration and oxygen solubility, respectively; \( X_t \) represents the feed xylose concentration of 40 g/L; \( Q_g \) is the volumetric rate of gas flow from the fermentor; \( F_{\text{co}_2} \) is the mole fraction of \( \text{CO}_2 \) in the exit gas; \( d_{\text{co}_2} \) is the density of \( \text{CO}_2 \); and \( V_1 \) is the culture liquid volume. The amount of \( \text{CO}_2 \) arising from the PP cycle was the total \( \text{CO}_2 \) minus fermentative, assimilative, and respirative \( \text{CO}_2 \). The yield of PP-cycle-produced \( \text{CO}_2 \) per xylose consumed \( \left( \frac{Y_{\text{co}_2(P/P)}{X}} \right) \) was thus calculated from the available yield data, the stoichiometry of equation 4, and the formula weights (g/mole) of \( \text{CO}_2 \) (44), ethanol (46), biomass (98), and \( \text{O}_2 \) (32):

\[
Y_{\text{co}_2(P/P)}{X} = Y_{\text{CO}_2/X} - Y_{\text{E}/X}(44/46) - Y_{\text{b}/X}(695.5/1000)(44/98) - Y_{\text{ox}/X}(44/32).
\]

Fractions of xylose used in assimilation \( \left( f_a \right) \), PP cycling \( \left( f_p \right) \), respiration \( \left( f_r \right) \), and ethanolic fermentation \( \left( f_e \right) \) were calculated as ratios of the observed yield to that theoretically possible if all xylose was metabolized by a given process:

\[
\begin{align*}
f_a &= (Y_{\text{b}/X})/(0.738 \text{ g biomass per g xylose}), \\
f_p &= (Y_{\text{co}_2(P/P)}{X})/(1.47 \text{ g CO}_2 \text{ per g xylose}), \\
f_r &= (Y_{\text{ox}/X})/(1.066 \text{ g oxygen per g xylose}), \\
f_e &= (Y_{\text{E}/X})/(0.51 \text{ g ethanol per g xylose}).
\end{align*}
\]

Theoretical yields used in the denominators of equations 22–25 were based on the stoichiometry model (equations 4 and 8–10).

If our stoichiometry model is correct in accounting for carbon metabolism by assimilation (equation 4), pentose phosphate oxidation (equation 8), respiration (equation 9), and ethanol fermentation (equation 10), then our yield data should allow us to calculate that

\[
f_a + f_p + f_r + f_e = 1.
\]

Given this is true, the overall equation for a particular fermentation can be constructed by summing the four model equations, which have been scaled to reflect the fraction of xylose used by each path:

\[
\text{overall equation} = \left[ f_a \times \text{(equation 4)} \right]/883 + \left[ f_p \times \text{(equation 8)} \right]/6 + \left[ f_r \times \text{(equation 9)} \right]/6 + \left[ f_e \times \text{(equation 10)} \right]/6.
\]
Division of stoichiometric coefficients by 883 or 6 normalized the equations for reaction of one mole of xylose.

**Maintenance Ethanol Production by Concentrated Cells**

**Cell Cultivation and Harvest**

Cells were grown in a New Brunswick Fermacel CF50 batch fermentor and centrifuged after 48 h, as described earlier. Portions of the harvested cell paste were transferred to fermentors or flasks for resuspension in fresh CCY such that cell concentrations of 5–7 g/L (dry weight) were achieved. These concentrated batch cultures were used to study effects of X and E on maintenance ethanol yield and productivity ($m_E$), that is, $p_E$ when $\mu = 0$.

**Effect of X on Productivity and Yield**

New Brunswick Microferm fermentors with 2-L working volumes were equipped with antifoam control, operated at pH 4.5 and 25 °C, and aerated at $K_Ia = 0.175$ min$^{-1}$. Initial specific ethanol productivities were measured as a function of the xylose concentration provided ($X_0 = 10–150$ g/L). Volumetric productivity ($P_E$) was obtained by linear regression of the early ethanol time course (0 to ~10 h) and $P_E$ was calculated as $P_E/b_{av}$. Because of slow oxygen transfer relative to the large population of viable cells present, $\mu \approx 0$ over this interval and an average value of the biomass concentration ($b_{av}$) was applied in the calculations. $Y_{E/X}$ was calculated as $E/ (X_0 - X)$.

**Effect of E on Productivity and Yield**

Cell suspensions in stirred flask cultures were sparged with CO$_2$ to remove oxygen and to allow equilibration of CO$_2$ and liquid phases at barometric pressure ($P_o$). Once 20 g/L xylose and 3–80 g/L ethanol were added to start the fermentation, CO$_2$ sparging was stopped and flasks were sealed except for connection to an open u-tube Hg manometer. We assumed that CO$_2$ evolution was solely responsible for the pressure increase and calculated the moles of CO$_2$ formed at any time as the difference between the current ($n$) and the initial moles ($n_o$) in gas and liquid phases:

$$n - n_o = \left[ V_{g_o} (P - P_o) / RT \right]$$
$$+ \left[ (V_g - V_{g_o})(P - P_w) / RT \right] + V_l k_H (P - P_o),$$

(28)

where $V_g$ and $P$ represent the volume and absolute pressure of gas-phase CO$_2$ currently in the system, the subscript “o” designates initial conditions, $P_w$ is water vapor pressure (0.031 atm), $T$ is incubator temperature (298 K), $R$ is the ideal gas constant (0.0821 L-atm/K-mole), $V_l$ is the volume of liquid culture, and $k_H$ is the solubility of CO$_2$ in CCY medium (0.0274 moles/L-atm) [Sigma Chemical Company Diagnostic Kit no. 130-A]. Note that $V_g$ depended on manometer mercury position. Assuming equimolar production of ethanol and CO$_2$, we calculated volumetric ethanol productivity as $P_E =$
(the slope of the initial two-hour $n - n_o$ time course)/$V_1$ and the specific productivity as $P_E = P_{Eb}/b_m$. The yield $[Y_{E/X} = (E - E_o)/(X_o - X)]$ was calculated from chromatography analyses of initial and final (6-h) broth samples.

**Analyses**

**Total and Viable Biomass Concentrations**

These were evaluated by light absorbance and staining techniques.

**Dissolved Oxygen Concentration**

This was monitored throughout the course of each fermentation as the product of Ingold electrode response (in terms of the fraction of oxygen saturation) and oxygen solubility ($C_o$). Given air (21% O$_2$) at 25°C and 760 mmHg, $C_o$ (mg/L) was determined as the following function of $X$ (g/L): $C_o = (0.21)(1.08)(34.6 - 0.0644X + 0.000156X^2)$.

**Ethanol, Xylitol, and Xylose Measurements**

Filtered samples were analyzed by gas and liquid chromatography methods. However, samples with $E < 1$ g/L were analyzed on a Hewlett Packard 5890 GC with a 30-m megabore column of 1-micron DB 1701 stationary phase (J&W Scientific). The carrier gas to the column was helium flowing at 6.79 mL/min, split to 1/13. Oven, injector, and flame ionization detector temperatures were 150, 175, and 250°C, respectively.

**Exit Gas CO$_2$**

The mole fraction of CO$_2$ in the gas exiting our continuous fermentors was measured by gas chromatography.

**RESULTS AND DISCUSSION**

**Stoichiometry as a Function of Growth**

**Pathway Usage**

Oxygen-limited continuous cultures were run at various dilution rates to test the effect of growth rate on pathway usage during xylose metabolism. TABLE 1 summarizes these results in terms of the fractions of xylose used by each pathway. The sum of pathway usage fractions was always near 1, indicating that the proposed stoichiometry model (equations 7–10) adequately accounted for xylose utilization. The metabolic state of the yeast varied with specific growth rate. As $\mu$ increased, metabolism shifted from fermentation and the pentose phosphate cycle to assimilation and respiration,
that is, from anaerobic to aerobic processes. In conjunction with this observation, earlier data have shown that specific oxygen uptake (or respiration) rate increases linearly with growth rate.\(^7\)

It is notable that xylitol did not accumulate in any of our continuous cultures although small amounts were observed in batch cultures.\(^6\) This finding was consistent with studies of labeled xylose uptake\(^8\) suggesting that xylose reductase is predominantly NADH-dependent and that \(f = 0\) in our model. A question arises, however, because our \(f_p\) values (TABLE 1) were 3–10 times higher than anticipated from our model, which assumed carbon flow through the PP cycle to be regulated solely by the NADPH, H\(^+\) demanded for assimilation.

**Overall Stoichiometry**

As we pursued this problem, an analysis of the overall stoichiometry for each growth condition (TABLE 2) allowed us to make another interesting observation. This analysis suggested that a net production of 1.42–1.63 moles of ATP and 0.63–1.03 moles of reducing equivalents (NADH, H\(^+\) + NADPH, H\(^+\)) occurred regardless of the value of \(f\). Inaccurate estimations of cofactors required by assimilation (equation 4), which applied to only 10–20\% of xylose metabolism, could not account for the large excesses observed. For example, consumptions of ATP and NADPH, H\(^+\) would have to be raised by factors of 4 and 2–10, respectively, whereas NAD\(^+\) would have to be halved (at \(f = 0\)). Such large adjustments in NADPH, H\(^+\) and NAD\(^+\) would bring our assimilation equation into serious violation of mass and charge conservation principles. The ability of mitochondrial electron transport to relieve an H\(^+\) imbalance in the cytoplasm has been mentioned as a possible explanation for the obligate aerobic growth of xylose-fermenting yeasts.\(^8\) Although such a process might consume the reducing equivalents, it would require twice the oxygen demand observed and further augment the ATP surplus.

### Table 1. Pathway Usage as a Function of Specific Growth Rate (\(K_{i/d} = 0.049\) min\(^{-1}\))

<table>
<thead>
<tr>
<th>Specific Growth Rate (h(^{-1}))</th>
<th>Fraction of Xylose Used by Each Pathway(^a)</th>
<th>(f_s)</th>
<th>(f_p)</th>
<th>(f_r)</th>
<th>(f_e)</th>
<th>sum</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.055</td>
<td></td>
<td>0.093</td>
<td>0.101 (0.009)</td>
<td>0.020</td>
<td>0.83</td>
<td>1.04</td>
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<td>0.144</td>
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<td>0.75</td>
<td>0.98</td>
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<tr>
<td>0.161</td>
<td></td>
<td>0.160</td>
<td>0.059 (0.015)</td>
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<td>0.70</td>
<td>0.95</td>
</tr>
<tr>
<td>0.195</td>
<td></td>
<td>0.161</td>
<td>0.053 (0.015)</td>
<td>0.034</td>
<td>0.71</td>
<td>0.96</td>
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<tr>
<td>0.250</td>
<td></td>
<td>0.220</td>
<td>0.068 (0.021)</td>
<td>0.044</td>
<td>0.66</td>
<td>0.99</td>
</tr>
</tbody>
</table>

\(^a\)The fraction of xylose consumed for xylitol production, \(f_{xOH} = Y_{xOH/X}/1.01\), was 0 for all \(D\). The parameters \(f_s, f_p, f_r,\) and \(f_e\) represent the fractions of xylose consumed by assimilation, pentose phosphate oxidation, respiration, and ethanolic fermentation, respectively.

\(^b\)(...) = value of \(f_c\) calculated from \(f_s\) assuming that \(f = 0\) and that the pentose phosphate cycle supplies all NADPH, H\(^+\) for assimilation.
### TABLE 2. Overall Stoichiometry of Xylose Metabolism as a Function of Specific Growth Rate

<table>
<thead>
<tr>
<th>Reactants</th>
<th>0.055</th>
<th>0.089</th>
<th>0.118</th>
<th>0.161</th>
<th>0.195</th>
<th>0.250</th>
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<td>1.00</td>
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<tr>
<td>urea</td>
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<td>0.05</td>
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<tr>
<td>NAD⁻</td>
<td>0.13 + f</td>
<td>0.20 + f</td>
<td>0.22 + f</td>
<td>0.25 + f</td>
<td>0.25 + f</td>
<td>0.33 + f</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>0.88 – f</td>
<td>0.83 – f</td>
<td>0.45 – f</td>
<td>0.45 – f</td>
<td>0.38 – f</td>
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<tr>
<td>ATP + P₇</td>
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<td>1.57</td>
<td>1.53</td>
<td>1.63</td>
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<td>H₂O</td>
<td>0.28</td>
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<td>1.32</td>
<td>1.23</td>
<td>1.27</td>
<td>1.22</td>
<td>1.24</td>
<td>1.11</td>
</tr>
<tr>
<td>ethanol</td>
<td>1.97</td>
<td>1.96</td>
<td>1.84</td>
<td>1.83</td>
<td>1.82</td>
<td>1.85</td>
</tr>
<tr>
<td>CO₂</td>
<td>0.13 + f</td>
<td>0.20 + f</td>
<td>0.22 + f</td>
<td>0.25 + f</td>
<td>0.25 + f</td>
<td>0.33 + f</td>
</tr>
<tr>
<td>NADH,H⁺</td>
<td>0.88 – f</td>
<td>0.83 – f</td>
<td>0.45 – f</td>
<td>0.45 – f</td>
<td>0.38 – f</td>
<td>0.46 – f</td>
</tr>
<tr>
<td>ATP + H₂O</td>
<td>1.48</td>
<td>1.42</td>
<td>1.57</td>
<td>1.53</td>
<td>1.63</td>
<td>1.56</td>
</tr>
<tr>
<td>H₂O</td>
<td>0</td>
<td>0</td>
<td>0.03</td>
<td>0.06</td>
<td>0.11</td>
<td>0.16</td>
</tr>
</tbody>
</table>

However, this apparent cofactor imbalance was consistent with recent evidence that xylose is transported via a proton symport system. Kilian and Van Uden have shown that xylose transport by *P. stipitis* is accompanied by proton uptake (pH rise) and is likely to be energy-dependent. In this context, averaged data from TABLE 2 suggest that *P. stipitis* generates 1.53 moles of ATP to maintain the chemiosmotic gradient required for cotransport of 0.8 moles of H⁺ and 1 mole of xylose. The fact that $f_p$ values were higher than needed for assimilation alone suggests possible involvement of the pentose phosphate cycle in proton uptake (or acceptance) during transport.

**Ethanol Yield and Its Impact on Xylose Uptake Kinetics**

**Concentration Effects**

Batch cultures provided with high concentrations of nongrowing cells were used to examine the effects of xylose and ethanol concentration on yield. As calculated from initial time courses, the ethanol yield on xylose ($Y_{E/X}$) showed no trend on 10−150 g/L xylose or 3−80 g/L ethanol, but averaged 0.41 (0.054 standard deviation). A similar value was predicted by extrapolating our continuous culture results to $\mu = 0$ as shown later.

**Dependence of Ethanol Yield on Growth**

In agreement with observed shifts in the overall stoichiometry, $Y_{E/X}$ declined gradually with increasing growth rate (FIGURE 1). The empirical relationship that fit
this behavior was the following:

\[ Y_{E/X} = 0.421 - 0.343 \mu. \]  

(29)

Hence, in view of equations 11-13, xylose uptake rate was weakly dependent on \( \mu \) via \( Y_{E/X} \), if not through \( p_E \) as well. This expression predicts that \( Y_{E/X} \) will reach zero at \( \mu = 1.23 \) h\(^{-1}\); however, the highest specific growth rates observed in our experiments were ca. 0.55 h\(^{-1}\), which corresponds to \( Y_{E/X} = 0.23 \) g/g.

**Ethanol Production Kinetics**

**Dependence of Productivity on Growth**

Specific ethanol productivity and growth rate, measured in continuous cultures at steady state, were plotted against one another as shown in FIGURE 2. Regardless of \( K_i a \) setting, the data were compatible with Luedeking and Piret's model at \( Y_{E/b} = 1.73 \) g/g and \( m_E = 0.254 \) g/g/h (equation 13). The fact that this expression was independent of \( K_i a \) is related to our earlier finding that the specific oxygen uptake rate \( (q_o) \) is controlled by \( \mu \). At any particular \( D \), \( q_o \) remains unchanged with shifts in \( K_i a \) as \( b \) shifts proportionately.\(^7\)

The observation that ethanol production is partly growth-associated impacts on our interpretation of the literature in various ways. It provides a kinetic basis for early references to xylose fermentations as "oxygen stimulated". Growth is obligately aerobic and usually oxygen-limited,\(^7\) so \( p_E \), being a function of \( \mu \), is subject to oxygen

![FIGURE 1. Linear dependence of ethanol yield \((Y_{E/X})\) on specific growth rate \((\mu)\). Regression provided a slope \((-0.35 \) h g/g\) and intercept \((0.42 \) g/g\) with a correlation coefficient of 0.80, given variable oxygen transfer \((K_i a)\).](image)
FIGURE 2. Linear dependence of specific ethanol productivity \( p_E \) on growth \( \mu \). The yield provided a slope \( (Y_E/b) \) of 1.73 g/g and an intercept \( (m_E) \) of 0.25 g/g/h with a correlation of 0.95 despite variable oxygen transfer \( (K_{l,a}) \).

limitation kinetics. Our combined expressions for \( p_E \) and \( \mu \) can also account for the curious observation that the slope of \( E \) versus \( t \) (equaling \( p_E\mu \)) is constant in early batch cultures.\(^4\) Rising biomass concentration tends to increase \( dE/dt \), but this effect is offset by declining oxygen concentration, which lowers \( \mu \) and \( p_E \). As indicated by the large value of \( m_E \), ethanol production continues even in the absence of oxygen and growth. In batch cultures, the average value of \( p_E \) is skewed toward \( m_E \) because depletion of oxygen concentration lowers \( \mu \) early on. Therefore, average specific ethanol productivities measured in batch cultures tend to be \( \leq 0.25 \) g/g/h, depending on concentrations of xylose and ethanol.

**Dependence of Maintenance Productivity on X**

As a standard practice, *P. stipitis* was grown batchwise 48 h on 150 g/L xylose and then resuspended at high concentration to measure maintenance ethanol production. Time courses in FIGURE 3 indicate that our practice of growing cells at the extreme xylose concentration was key to eliminating adaptation effects from subsequent measurements of \( m_E \). When resuspended in 150 g/L xylose, cells grown on 20 g/L xylose produced ethanol at one-tenth the rate of those grown on 150 g/L. This finding was consistent with adaptation effects reported by other workers.\(^20\)

Raising xylose to 35–80 g/L stimulated \( m_E \), but higher concentrations inhibited the fermentation (FIGURE 4). Curves through the data are discussed in detail by Slininger\(^25\) and represent best fits of models reviewed by Edwards.\(^15\) Performing nearly
as well as four-parameter equations, the three-parameter empirical models of Aiba et al. and Edwards adequately simulated our data (TABLE 3). However, Edwards' model converged on the most realistic parameter values; to cast it in terms of xylose-dependent maintenance productivity, we gave it the following form:

\[ m_E = m_{E,max} \left[ \exp(-X/K_i) - \exp(-X/K_{i-}) \right]. \]  

(30)

**Dependence of Maintenance Productivity on E**

The effects of xylose on \( m_E \) were compounded by ethanol inhibition. End-product inhibition has received much notoriety and it has been described by numerous mathematical models. As a noncompetitive inhibitor, ethanol affects the maximum specific rate and not saturation kinetics. Consequently, Luong's empirical model has been generally useful for modeling inhibition as follows:\(^{14}\)

\[ m_{E,i}/m_{E,o} = 1 - (E/E'_m)^B. \]  

(31)

In the context of this study, \( m_{E,i} \) and \( m_{E,o} \) are rates in the presence and absence of ethanol as inhibitor, \( E'_m \) is the ethanol concentration that prohibits further production, and \( B \) is an empirical constant. In a previous study, we showed that Luong's model was applicable to the growth of *P. stipitis*. Furthermore, linearization of the data (FIGURE 5) described \( m_E \) as a function of \( E \) when \( E'_m \) and \( B \) were 189 g/L and 0.935, respectively. These parameters were analogous, but not equal to those determined for growth (\( E_a = 64.3 \) g/L, \( A = 1.324 \)).\(^7\) The higher ethanol sensitivity of \( \mu \) (compared with \( p_E \)) was also characteristic of *Pachysolen tannophilus*.\(^8\)
FIGURE 4. Dependence of maintenance productivity ($m_E$) on xylose concentration ($X$). Curves through the data represent best fits of various substrate inhibition models. Parameter values are given in TABLE 3.

CONCLUSIONS

In summary, observed carbon balances were consistent with modeling xylose metabolism as a combination of four process equations: assimilation (equation 4), pentose phosphate oxidation (equation 8), respiration (equation 9), and ethanolic fermentation (equation 10). Cofactor balances indicated excesses of 0.8 moles H⁺ (as NADPH,H⁺ and NADH,H⁺) and 1.53 moles ATP per mole xylose consumed. This condition was consistent with xylose transport by energy-dependent proton symport,

TABLE 3. Optimized Parameters and Prediction Errors Associated with Substrate Inhibition Models

<table>
<thead>
<tr>
<th>Equation</th>
<th>$m_{E,\text{max}}$ (g/g/h)</th>
<th>$K'_i$ (g/L)</th>
<th>$K''_i$ (g/L)</th>
<th>$K$ (g/L)</th>
<th>Average Squared Error ($\pm$g/g/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haldane²¹</td>
<td>0.867</td>
<td>53.4</td>
<td>56.10</td>
<td>—</td>
<td>0.038</td>
</tr>
<tr>
<td>Webb²²</td>
<td>12.300</td>
<td>1350.0</td>
<td>2.09</td>
<td>2.83 x 10⁴</td>
<td>0.027</td>
</tr>
<tr>
<td>Yano et al.²³</td>
<td>3.220</td>
<td>434.0</td>
<td>185.50</td>
<td>4.92</td>
<td>0.021</td>
</tr>
<tr>
<td>Aiba et al.²⁴</td>
<td>5.880</td>
<td>514.0</td>
<td>63.70</td>
<td>—</td>
<td>0.028</td>
</tr>
<tr>
<td>Edwards²⁵</td>
<td>1.430</td>
<td>45.9</td>
<td>72.70</td>
<td>—</td>
<td>0.028</td>
</tr>
</tbody>
</table>
FIGURE 5. Linearization of Luong's model for the dependence of maintenance ethanol productivity ($m_E$) on ethanol concentration ($E$). Regression indicated 0.90 correlation and provided model parameters (equation 31) via the slope ($B = 0.935$) and intercept $[-B \ln(E_m) = -4.903]$.

and high values of $f_p$ (relative to $f_a$) suggested involvement of the pentose phosphate cycle in proton uptake. Although these cofactor excesses remained relatively constant, the overall stoichiometry gradually shifted from fermentation to respiration as specific growth rate increased. Because xylose uptake was proportional to ethanol production (equation 11), these shifts could be accounted for in our kinetic model by a growth-dependent yield coefficient, $Y_{E/X}$ (equation 29). Specific ethanol productivity was also growth-associated as described by Luedeking and Piret's model (equation 13) at $Y_{E/b} = 1.73 \text{ g/g}$. Maintenance productivity ($m_E$) was adequately represented by the combination of Luong's two-parameter model of ethanol inhibition (equation 31) and any of the three-parameter functions of xylose concentration (equation 30).

ACKNOWLEDGMENT

We are grateful to Roy Butterfield for computer optimizations of parameters in substrate inhibition models.

REFERENCES


APPENDIX

Nomenclature

Symbols

\[ B = \text{exponent governing ethanol inhibition of fermentation (dimensionless)} \]
\[ b = \text{viable biomass concentration (g/L)} \]
\[ b_{av} = \text{average viable biomass concentration (g/L)} \]
\[ b_{d} = \text{dead biomass concentration (g/L)} \]
\[ b_{ds} = \text{steady-state dead biomass concentration (g/L)} \]
\[ b_{s} = \text{steady-state viable biomass concentration (g/L)} \]
\[ b_{T} = \text{total biomass concentration (g/L)} \]
\[ b_{T,s} = \text{steady-state total biomass concentration (g/L)} \]
\[ C_{ox} = \text{dissolved oxygen concentration (mg/L)} \]
\[ C_{ox,s} = \text{steady-state dissolved oxygen concentration (mg/L)} \]
\[ C_{ox}^* = \text{oxygen solubility (mg/L)} \]
\[ D = \text{dilution rate (h}^{-1}) \]
\[ d_{CO_2} = \text{density of CO}_2 \text{ (g/L)} \]
\[ E = \text{ethanol concentration (g/L)} \]
\[ E^*_m = \text{maximum ethanol concentration allowing fermentation (g/L)} \]
\[ E_0 = \text{initial ethanol concentration in a batch culture (g/L)} \]
\[ E_s = \text{steady-state ethanol concentration (g/L)} \]
\[ F_{CO_2} = \text{mole fraction of CO}_2 \text{ in the fermentor exit gas (dimensionless)} \]
\( f \) = fraction of xylose reductase activity supported by NADPH, as opposed to NADH (dimensionless)

\( f_a \) = fraction of xylose used in assimilation (dimensionless)

\( f_e \) = fraction of xylose used in ethanol fermentation (dimensionless)

\( f_p \) = fraction of xylose used in pentose phosphate cycle oxidation (dimensionless)

\( f_r \) = fraction of xylose used in respiration (dimensionless)

\( K_{a} \) = lumped oxygen mass transfer coefficient (h\(^{-1}\))

\( K_i \) = parameter governing substrate inhibition of fermentation (g/L)

\( K_s \) = saturation constant governing xylose-limited fermentation (g/L)

\( k_H \) = Henry's law constant (moles/L/atm)

\( m_E \) = specific ethanol productivity for maintenance (g/g/h)

\( m_{E,i} \) = maintenance productivity in the presence of inhibitor (g/g/h)

\( m_{E,\text{max}} \) = maximum specific maintenance productivity (g/g/h)

\( m_{E,o} \) = maintenance productivity in the absence of inhibitor (g/g/h)

\( n \) = total moles of CO\(_2\) in gas and liquid phases

\( n_o \) = initial total moles of CO\(_2\) in gas and liquid phases

\( P \) = absolute pressure (atm)

\( P_E \) = volumetric ethanol productivity (g/L/h)

\( P_o \) = barometric pressure (atm)

\( P_w \) = water vapor pressure (atm)

\( p_E \) = specific ethanol productivity (g/g/h)

\( Q_{ga} \) = volumetric gas flow rate from the fermentor (L/h)

\( q_{ox} \) = specific oxygen uptake rate (mg/g/h)

\( R \) = ideal gas constant (0.0821 L-atm/K-mole)

\( T \) = temperature (K)

\( t \) = time (h)

\( V_g \) = culture headspace gas volume (L)

\( V_{E,0} \) = initial culture headspace gas volume (L)

\( V_L \) = culture liquid volume (L)

\( X \) = xylose concentration (g/L)

\( X_r \) = concentration of xylose in continuous culture feed (g/L)

\( X_s \) = concentration of xylose initially present in a batch culture (g/L)

\( X_s^* \) = steady-state xylose concentration (g/L)

\( Y_{b/X} \) = yield of biomass per xylose consumed (g/g)

\( Y_{CO_2/X} \) = yield of CO\(_2\) per xylose consumed (g/g)

\( Y_{CO_2(p)} \) = yield of pentose phosphate cycle-produced CO\(_2\) per xylose consumed (g/g)

\( Y_{E/b} \) = yield of ethanol per biomass produced (g/g)

\( Y_{E/X} \) = yield of ethanol per xylose consumed (g/g)

\( Y_{OX/X} \) = oxygen to xylose uptake ratio (g/g)

\( \mu \) = specific growth rate (h\(^{-1}\))

Abbreviations

EMP = Embden-Meyerhof-Parnas

PP = pentose phosphate

TCA = tricarboxylic acid