KINETIC AND ATP MAINTENANCE STUDIES OF A METABOLICALLY ENGINEERED *Zymomonas mobilis* FERMENTING GLUCOSE AND XYLOSE MIXTURES

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ABSTRACT

The growth (glucose- and xylose-associated processes) and non-growth-mediated (maintenance energy requirements) characteristics of a metabolically engineered strain of *Zymomonas mobilis* capable of fermenting both glucose and xylose to ethanol have been investigated in mixed sugar fermentations. Ideally such a microorganism will also tolerate acetic acid and other inhibitory components typically present in biomass hydrolyzates. We have measured substrates and products concentrations as well as intracellular adenosine-5'-triphosphate (ATP) concentrations across a 2-factor, 3-level factorial design using the methods of analysis, techniques, and calculations described previously (*1*). Batch fermentations were conducted using a 10% (w/v) total sugar concentration (5% glucose/5% xylose mixture) at a temperature of 30 °C at pH 5.0, 5.3, or 6.0 in the presence of varying initial amounts of acetic acid (0, 4, and 8 g/L).

Results show large differences in *Z. mobilis* fermentation kinetics but only modest changes in intracellular ATP levels across the experimental design space. Ethanol process yields varied between 56.6% and 92.3% of theoretical depending upon the test conditions, and illustrate how pH strongly influences the inhibitory effect of acetic acid on fermentation kinetics. For example, maximum specific growth rates varied between a low of 0.06 h^{-1} observed at pH 5 in the presence of 8 g/L acetic acid to a high of 0.20 h^{-1} obtained at pH 6 without any acetic acid present. Reflecting this trend, calculated rates of ATP consumption for growth-mediated-processes ranged from a low of 6.2 g ATP/g DCM-h at pH 5 and 8 g/L acetic acid to a high of 20.9 g ATP/g DCM-h at pH 6 without acetic acid. ATP requirements for maintenance showed an opposite pattern, with the highest maintenance requirement, 12.9 g ATP/g DCM-h, obtained at pH 5 and 8 g/L acetic acid, and the lowest, 0.2 g ATP/g DCM-h, at pH 6 without acetic acid present. Despite the more than sixty-fold difference in maintenance requirements observed across the design space, maximum levels of free intracellular ATP only varied within a narrow range of 1.5 to 3.8 mg ATP/g DCM.

These findings provide quantitative information about how pH and acetic acid concentration affect fermentation kinetics. A mathematical model is being developed to describe how the rate of ATP consumption for maintenance varies as a function of the concentrations of undissociated acetic acid and dissociated acetate ion, which are modulated by pH (hydronium ion concentration), as well as the concentration of ethanol. Preliminary results suggest that in this system the concentration of undissociated acetic acid is the strongest determinant of maintenance requirements.

KEYWORDS

ATP production rates; ATP consumption rates; maintenance; cofermentation; xylose fermentation; multiple substrates; ethanol.

INTRODUCTION

Zymomonas mobilis is of interest as a potential biocatalyst for large-scale ethanol production from lignocellulosic materials due to its high ethanol fermentation yields (*2-5*). Recombinant strains of this gram-negative bacterium are capable of efficiently converting both glucose and xylose to ethanol, and under some conditions can also tolerate acetic acid and other inhibitory components typically present in biomass hydrolyzates. However, the fermentation performance characteristics of *Z. mobilis*, particularly its operable pH range and ability to grow in the presence of ethanol, are influenced by a variety of factors, including sugar type(s) and concentration, acetic acid concentration, and temperature.

A distinctive and significant characteristic of xylose- and glucose-fermenting *Z. mobilis* strains is the uncoupling of ethanol production from cell growth that occurs towards the end of batch mixed sugar fermentations. This uncoupling behavior is often observed after glucose is depleted, whereupon cell growth rate decreases, eventually falling to zero, while the remaining xylose is fermented to ethanol (Figure 1). This phenomenon, in combination with *Z. mobilis* using the low ATP yield Entner-Doudoroff glycolytic pathway (which generates only 1 net mol of intracellular ATP per mol of glucose or xylose consumed), results in relatively more sugars being available for ethanol production compared with other ethanologens. We have speculated that lower ATP accumulation levels may underlie the uncoupling phenomenon, as well as the greater sensitivity to inhibition by acetic acid or ethanol or low pH observed when fermenting xylose. Presumably, the amount of ATP available for growth processes falls late in fermentation as increasing amounts of ATP become required for cell maintenance as sugar concentrations decrease and inhibitory products like ethanol accumulate.

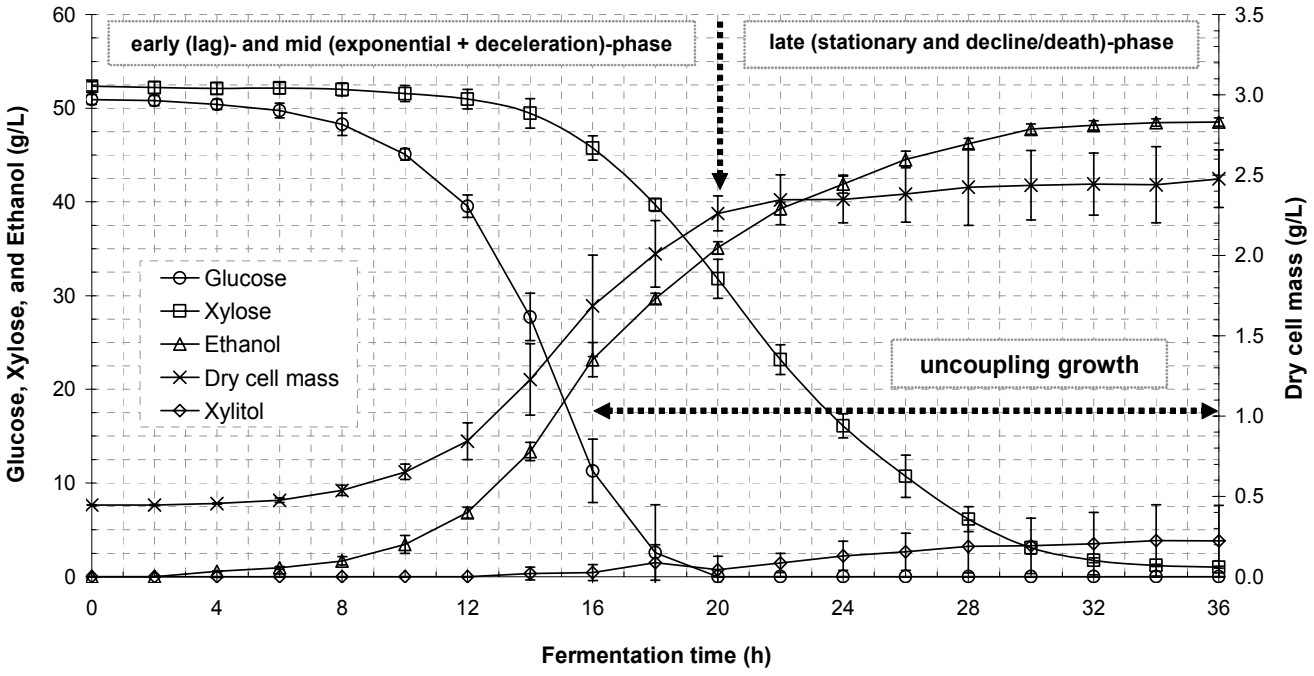

Figure 1. Averaged performance data of *Z. mobilis* cofermenting a 5% glucose/5% xylose sugar mixture at pH 5.

In this study, we measured substrates and products concentrations as well as intracellular ATP concentrations across a 2-factor (acetic acid and pH), 3-level factorial design (Figure 2) using the methods described previously (*1*). In addition to performance data, this work provides quantitative information about how pH and acetic acid concentration affect fermentation kinetics. This is the type of information that is needed to validate a mathematical model describing how the rate of ATP consumption for maintenance varies as a function of the concentrations of undissociated acetic acid and dissociated acetate ion, which are modulated by pH (hydrogen or hydronium ion concentration), as well as ethanol concentration.

MATERIALS AND METHODS

A genomic DNA-integrated glucose- and xylose-fermenting strain of *Z. mobilis* previously developed at NREL was used during this research work (*2, 5*). Ethanol production runs were conducted batchwise in a set of four bench-scale Bioflo 3000 7-L fermentors (New Brunswick Scientific, NJ) using a 4-L working volume. Fermentations were carried out at an initial substrate concentration of 5% (w/v) glucose and 5% (w/v) xylose (i.e., 10% total sugar concentration), under nonaerated conditions, for 24-120 h depending on the operating conditions. The factorial design (Figure 2) was performed to evaluate the effect of pH (5.0, 5.3, and 6.0) and acetic acid concentration (0, 4, and 8 g/L) on intracellular ATP levels and fermentation performance. In all runs, the temperature was kept constant at 30°C, agitation was maintained at 150 rpm, and pH was controlled at its set point using automatic addition of alkali (2 N KOH) or acid (1 N H_2SO_4) solutions. Samples were collected periodically throughout the course of the fermentations, both manually using traditional methods and more frequently using an autosampler system. Samples were analyzed to determine the concentrations of sugars, cell mass, ethanol, byproducts, and intracellular ATP.

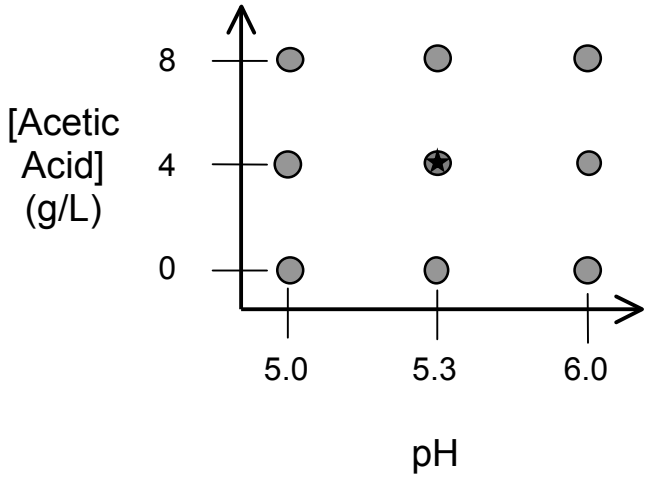

Figure 2. Schematic diagram of the experimental design.

The concentrations of sugars (glucose and xylose), ethanol, and potential byproducts (i.e., xylitol, lactate, glycerol, and acetate) were determined from filtered sample supernatants using High Performance Liquid Chromatography. Cell growth (as optical density at 600 nm) and dry cell mass (DCM) concentrations were measured with a Spectronic 601 spectrophotometer (Milton Roy, NY) and gravimetrically, respectively. Quantification of intracellular ATP levels in *Z. mobilis* cell samples was carried out using a TD-20/20 luminometer (Turner Biosystems, CA). Bioluminescence light intensity is proportional to ATP

concentration in the samples according to the luciferin-firefly luciferase reaction stoichiometry; this methodology was described previously (*1*).

RESULTS AND DISCUSSION

Fermentation performance levels varied significantly across the experimental design space (not shown). For example, ethanol process yields varied between 56.6% and 92.3% of theoretical depending upon test conditions, with the highest ethanol yields (≥ 87%) obtained in fermentations where the initial concentration of acetic acid was low or the pH was high. Fermentations initiated with 8 g/L of acetic acid at pH 5.3 or with 4 or 8 g/L of acetic acid at pH 5.0 were the only ones to result in yields below 87%, achieving yields of 78.2%, 73.1%, and 56.6%, respectively. Similar results were observed for the extent of xylose utilization, which was above 94% for all fermentations except for those run at the three conditions described above, which only used 82%, 64%, and 28%, respectively, of the input xylose. Maximum specific growth rates varied between a low of 0.06 h^{-1} observed at pH 5 in the presence of 8 g/L acetic acid to a high of 0.20 h^{-1} obtained at pH 6 without any acetic acid present. However, the effect of fermentation conditions on ethanol metabolic yields was minimal; metabolic yields were above 86% in all cases.

ATP measurements showed that intracellular ATP accumulated to levels of only 1.5-3.8 mg ATP/g DCM. Therefore, a simplified ATP balance neglecting accumulation and dilution terms was used to calculate the rate of ATP consumption for growth and maintenance, as shown in Equation 1:

$$r_{ATP,\,production} = r_{ATP,\,consumption} = r_{ATP,\,growth} + r_{ATP,\,maintenance} \qquad (1)$$

where the specific rate of ATP production (g ATP/g DCM-h) is equal to the rate of ATP consumption (g ATP/g DCM-h), which is the sum of the rates of ATP consumption for growth-mediated-processes and for maintenance requirements. The specific rate of ATP production was calculated from the sugar uptake rates (g sugar/g DCM-h) measured during the exponential phase of the batch fermentations assuming a stoichiometry of 1 net mol of intracellular ATP produced per mol of glucose or xylose consumed. The specific rate of ATP consumption for growth was estimated as the specific growth rate (μ, h^{-1}) divided by the yield of cell mass on ATP ($Y_{X/ATP}$, g cells/mol ATP), as shown in Equation 2.

$$r_{ATP,\,growth} = \frac{\mu}{Y_{X/ATP}} \qquad (2)$$

The apparent specific growth rate was calculated for each run during its mid exponential phase. A $Y_{X/ATP}$ value of 4.74 g/mol was utilized, which was determined by averaging values for all runs. This averaged $Y_{X/ATP}$ value is consistent with the range of 4.62-9.90 g/mol recently reported for a pair of recombinant *Z. mobilis* strains fermenting glucose and xylose mixtures (*4*). The rate of ATP consumption for maintenance was calculated as the difference between the specific rate of ATP production and the estimated specific rate of ATP consumption for growth-mediated processes by rearranging Equation 1.

$$r_{ATP,\,maintenance} = r_{ATP,\,production} - r_{ATP,\,growth} \qquad (3)$$

Calculated rates of ATP consumption for growth ranged from a low of 6.2 g ATP/g DCM-h at pH 5 and 8 g/L acetic acid to a high of 20.9 g ATP/g DCM-h at pH 6 without acetic acid. ATP requirements for maintenance showed an opposite pattern, with the highest maintenance energy requirement, 12.9 g ATP/g DCM-h, obtained at pH 5 and 8 g/L acetic acid, and the lowest, 0.2 g ATP/g DCM-h, at pH 6 without acetic acid present.

The results obtained for the specific ATP production and consumption rates were statistically analyzed using Design-Expert (version 6.0.1) and MATLAB (version 6.5.1) software packages. Response Surface Methodology was used to model the specific ATP production and consumption rates as a function of acetic acid and pH.

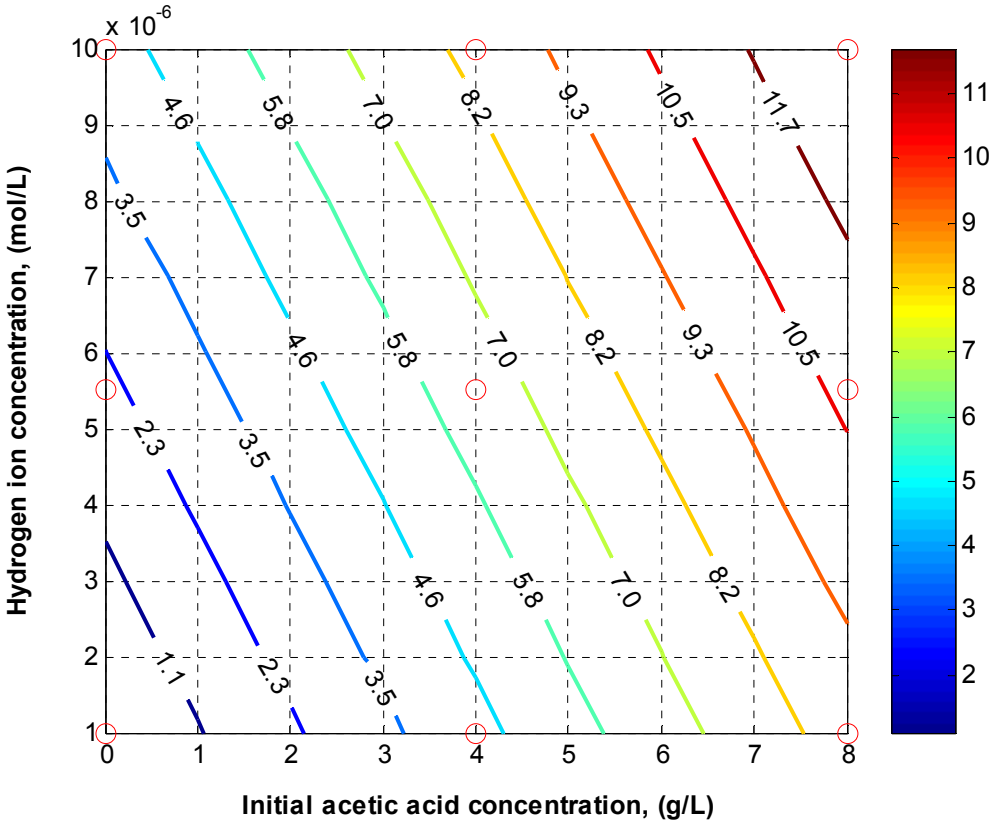

Figure 3. Modeled variation in specific rate of ATP consumption for maintenance as a function of acetic acid and hydrogen ion concentrations ($[H^+] = 10^{-pH}$). Circles show where experimental runs were performed.

Figure 3 shows a contour plot describing how maintenance requirements varied across the design space. This plot shows that *Z. mobilis* maintenance energy requirements are more sensitive to changes in acetic acid concentration than to changes in pH. However, as the diagonal contours show, there is a pronounced interaction effect, with pH strongly influencing the inhibitory effect of acetic acid. As maintenance energy requirements increase as hydrogen ion (hydronium ion) and acetic acid concentrations increase, ATP maintenance requirements increase and the rate of ATP consumption for growth decreases (in an opposite pattern to that shown in Figure 3), which results in poorer *Z. mobilis* fermentation performance.

These findings provide quantitative information about how pH and acetic acid concentration affect ATP consumption rates. This information is being used to develop a mathematical model describing how the rate of ATP consumption for maintenance varies as a function of the concentrations of undissociated acetic acid and dissociated acetate ion, which are modulated by pH. The concentration of undissociated acetic acid depends upon the total acetic acid concentration and the pH (hydronium ion concentration) according to the Henderson-Hasselbalch equation (*6*). Cell membranes are permeable and susceptible to the undissociated form of acetic acid (*7*), and presumably the amount of ATP available for cell growth late in a batch fermentation falls as increasing amounts are required for cell maintenance as sugar concentrations decrease, ethanol concentrations increase, and nutrients are consumed.

Preliminary modeling results on this system suggest that the concentration of undissociated acetic acid exerts the strongest effect on maintenance requirements. The implications of our findings to date will be discussed.

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