Kinetic Studies and Intracellular ATP Analyses of a Metabolically Engineered *Zymomonas mobilis* Fermenting Glucose and Xylose Mixtures

Juan Carlos Sáez[†], Lorenzo Saliceti Piazza[†], and James D. McMillan*

[†] University of Puerto Rico, Mayagüez Campus, Department of Chemical Engineering, P.O. Box 9046 Mayagüez, Puerto Rico 00681-9046, e-mail: juancarlos12@hotmail.com
* Biotechnology for Fuels and Chemicals Division, National Bioenergy Center, National Renewable Energy Laboratory, 1617 Cole Boulevard, Golden, CO 80401, USA

Abstract

Developing a cost-effective fermentation process for ethanol production from lignocellulosic materials requires a microorganism that is capable of efficiently converting both hexose and pentose sugars to ethanol. In the present study, a metabolically-engineered strain of *Zymomonas mobilis* capable of fermenting both glucose and xylose to ethanol was characterized in batch culture studies. Experiments were carried out at temperatures between 30 and 40 °C over a pH range of 5.0-6.0, and in the presence of varying initial amounts of acetic acid, using 10% w/v total sugar concentration (pure glucose, pure xylose, or glucose/xylose mixtures). The concentrations of the following components were measured: (i) glucose, (ii) xylose, (iii) ethanol, (iv) intracellular adenosine triphosphate (ATP), (v) dry cell mass, (vi) xylitol, and (vii) acetic acid. Specific sugar uptake and product formation rates were determined, as well as the yields of cell mass, ATP and ethanol. In order to improve the consistency and accuracy of the kinetic data, an autosampler system was employed on the fermentors so that more samples could be taken over the course of each run. Results demonstrate that this *Z. mobilis* strain can ferment moderately high concentrations of biomass sugars (up to 100 g/L) to ethanol at yields greater than 85% of theoretical over a range of pH, temperature, and acetic acid conditions. Fermentation pH strongly influences the inhibitory effect of acetic acid on strain performance. The implications of these and other results on applying *Z. mobilis* to convert biomass-derived sugars to ethanol will be discussed.

Objectives

✿ Demonstrate that the luciferase-based ATP measurement method is effective and reliable for the determination of intracellular ATP in *Zymomonas mobilis* cells samples during the fermentation process.

✿ Demonstrate that this *Z. mobilis* strain can ferment moderately high concentrations of biomass sugars (up to 100 g/L) to ethanol at yields greater than 85% of theoretical over a range of pH and acetic acid conditions.

✿ Apply this ATP measurement protocol and results to help validate a previously developed kinetic model that incorporates an ATP balance.

Materials and Methods

✿ **Microorganism**   *rec-Z. mobilis* cells =>
✿ **Fermentation**
  - anaerobic
  - at different temperatures (30-37°C)
  - at different pH (5.0-6.0)
  - varied acetic acid concentrations (0-8 g/L)
✿ **Analytical methods**
  - sugars, ethanol, and byproducts concentrations (HPLC)
  - cell growth by optical density @ 600 nm (spectrophotometer)
  - dry cell mass concentration (gravimetrically)
  - ATP measurement [µg/mg cell dry weight] (luminometer)

Fermentation System
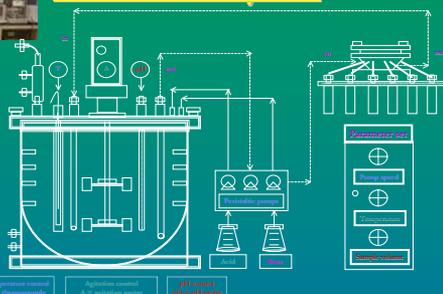
Intracellular ATP measurements

✿ A luminometer combined with ATP luciferin-firefly luciferase reagent, appears to provide a simple and sensitive method for quantifying the amount of ATP in *Z. mobilis* cultures samples.

✿ Method: ATP is extracted from cells using a releasing reagent. Light is emitted when firefly luciferase catalyzes the oxidation of luciferin in the presence of ATP molecules.

✿ The light reaching the luminometer's photomultiplier tube is proportional to the amount of ATP in the sample and, correspondingly, to the number of cells from which it was extracted.

Extraction and Calibration Curve

✿ The extraction of intracellular ATP using a commercially available ATP-releasing reagent (Turner Designs, CA) with phosphatase inhibitor (recommended to avoid major ATP degradation by ATP-converting enzymes) resulted in the efficient release of ATP from within the cells to the outside medium.
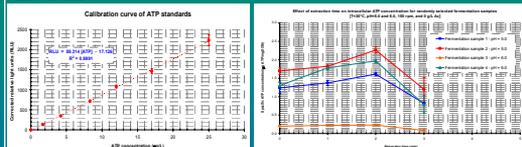
Specific intracellular ATP: Effect of acetic acid
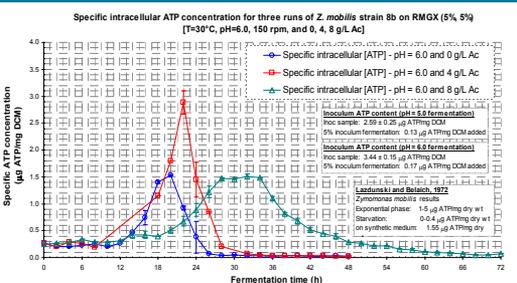
Specific intracellular ATP: Ac effect on growth
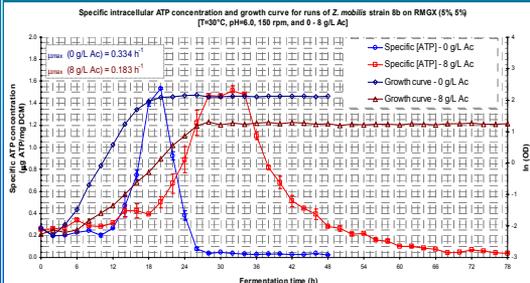
Specific intracellular ATP: Effect of pH
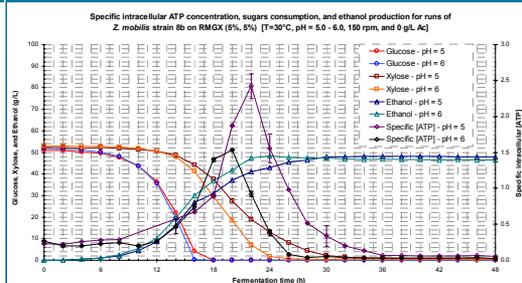
Kinetic parameters: T=30°C, pH=5, and 0 g/L Ac

[a] Kinetic	Glucose/Xylose concentration (g/L) – (T=30°C, pH=5.0, and 0 g/L Ac)				
Parameters	100/0 ([b]n=2)	75/25 (n=1)	50/50 (n=4)	25/75 (n=1)	0/100 (n=2)
µmax (h⁻¹)	0.345 ± 0.001	0.345	0.322 ± 0.016	0.299	0.093 ± 0.001
$r_{max,S}$ (g/[g·h])	11.90 ± 0.15	14.44	12.17 ± 1.46	18.12	3.05 ± 0.02
$r_{max,E}$ (g/[g·h])	6.03 ± 0.46	6.96	6.21 ± 1.04	4.23	1.30 ± 0.01
$r_{max,T}$ (g/[g·h])	N/A	0	0	0.006	0.024 ± 0.001
Y_S (g/g)	0.021 ± 0.001	0.024	0.021 ± 0.002	0.017	0.011 ± 0.001
$Y_{E/S}$ (g/g)	0.48 ± 0.01	0.48	0.48 ± 0.01	0.48	0.47 ± 0.00
Y'_S (g/g)	0.029 ± 0.001	0.024	0.027 ± 0.003	0.038	0.031 ± 0.007
$Y_{E/C}$ (g/g)	17.46 ± 1.26	20.17	19.36 ± 3.69	14.15	13.96 ± 2.21
$Y'_{T/C}$ (g/g)	N/A	0	0	0.02	0.26 ± 0.04
Q_E (g/[L·h])	2.74 ± 0.03	2.04	1.01 ± 0.01	1.02	0.40 ± 0.00
Process yield (%)	93.9 ± 2.6	93.0	92.0 ± 0.62	93.4	91.0 ± 0.6

[a] As defined by Joachimsthal et al. (1999); [b] n means the number of runs

Kinetic data: T=30°C, pH=5.0, and 0 g/L Ac
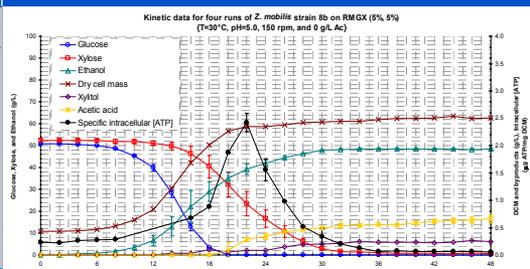
Kinetic parameters: T=30°C, pH=6, and 0–8 g/L Ac

[a] Kinetic	Glucose/Xylose concentration (g/L) – (T=30°C, pH=6.0, and 0 - 8 g/L Ac)				
Parameters	75/25 ([b]n=1)	25/75 (n=1)	50/50 - 0 Ac (n=2)	50/50 - 4 Ac (n=1)	50/50 - 8 Ac (n=1)
µmax (h⁻¹)	0.355	0.302	0.317 ± 0.024	0.272	0.183
$r_{max,S}$ (g/[g·h])	16.90	6.71	7.65 ± 0.71	18.13	11.44
$r_{max,E}$ (g/[g·h])	8.80	3.89	3.91 ± 0.50	8.56	5.29
$r_{max,T}$ (g/[g·h])	0	0.005	0	0	0.021
Y_S (g/g)	0.029	0.026	0.026 ± 0.001	0.016	0.010
$Y_{E/S}$ (g/g)	0.48	0.47	0.46 ± 0.01	0.47	0.45
Y'_S (g/g)	0.021	0.045	0.042 ± 0.001	0.015	0.016
$Y_{E/C}$ (g/g)	24.80	12.88	12.28 ± 0.65	31.46	28.93
$Y'_{T/C}$ (g/g)	0	0.018	0	0	0.115
Q_E (g/[L·h])	2.06	0.99	0.98 ± 0.01	0.99	0.59
Process yield (%)	94.2	91.8	89.2 ± 1.4	91.0	87.0

[a] As defined by Joachimsthal et al. (1999); [b] n means the number of runs

Kinetic data: T=30°C, pH=6.0, and 8 g/L Ac
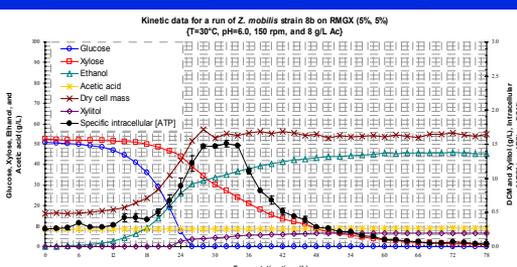
Conclusions

✿ Demonstrated that the luciferase-based ATP measurement method is effective and reliable for determining intracellular ATP concentration in *Z. mobilis* culture samples during fermentation processes.

✿ Experiments were performed and kinetic parameters determined over a pH range of 5.0-6.0, and in the presence of varying initial amounts of acetic acid.

✿ Through this work, a substantially better understanding of the intracellular ATP concentration profile during this type of fermentation processes was accomplished for several operating conditions. This will lead to apply this intracellular ATP measurement protocol to help validate a previously developed kinetic model that incorporates an ATP balance.

Acknowledgements