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

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NREL/PR-510-35288

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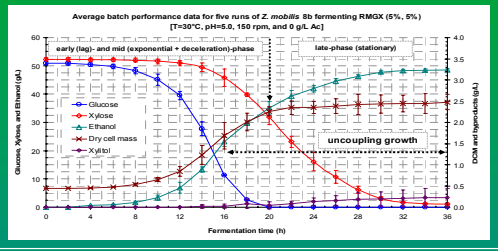
Abstract

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. Ideally such a microorganism will also tolerate acetic acid and other inhibitory components typically present in biomass hydrolyzates. In the present study, the fermentation and growth characteristics of a metabolically engineered strain of *Zymomonas mobilis* capable of fermenting both glucose and xylose to ethanol have been investigated in batch mixed sugar fermentations. Fermentations were carried out at a temperature of 30 °C, over a pH range of 5.0-6.0, and in the presence of varying initial concentrations of acetic acid (0-8 g/L). Concentrations of the following components were measured: (i) intracellular adenosine-5'-triphosphate (ATP), (ii) glucose, (iii) xylose, (iv) ethanol, (v) dry cell mass (DCM), (vi) xylitol, and (vii) acetic acid. In order to measure ATP in whole broth fermentation samples, we applied a sensitive method based on the bioluminescent firefly reaction of luciferin, O2, Mg+2, luciferase, and ATP, in which light production is linearly related to ATP concentration. 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. Results demonstrate the capability to determine the levels of intracellular ATP during the course of pure and mixed sugar fermentations using 10% (w/v) total sugar concentration (pure glucose, pure xylose, or glucose/xylose mixtures). High ethanol process yields (≥ 85% of theoretical) were achieved for all conditions studied, whereas maximum levels of free ATP varied between 1.5 and 3.0 μg ATP/mg DCM. This work also illustrates how pH strongly influences the inhibitory effect of acetic acid on specific sugar uptake and product formation rates and how the nature of the sugar substrate influences the utilization of ATP for glucose- and xylose-mediated growth processes. Xylose fermentations accumulated ATP at a much slower rate than the mixed sugar fermentations (5% glucose/5% xylose) and mixed sugar fermentations at a slightly slower rate than the glucose system. These findings provide quantitative information about how fermentation conditions such as substrate type, pH, and acetic acid concentration affect the rate of free ATP accumulation, and are being used to try to validate a previously developed kinetic model that incorporates an intracellular ATP-balance.

Introduction: Fermentation data (uncoupling)



Introduction: ATP-related equations

$$\mu = \frac{\mu}{\mu_{max}} \quad \text{where} \quad \mu = \frac{1}{[C]}\frac{d[C]}{dt}$$

ν: growth potential
μ_{max}: maximum specific growth rate (h^{-1})
μ: specific growth rate (h^{-1})
C: cell mass concentration (g/L)
(General definition)

$$\nu = \frac{[ATP]_{specific}}{K_{ATP} + [ATP]_{specific}}$$

$[ATP]_{specific}$: specific ATP concentration (μg ATP/mg DCM)
K_{ATP}: saturation constant for the ATP-governed growth
(Suggested by Kompala and coworkers, 2002)

$$\frac{d[ATP]_{specific}}{dt} = r_{ATP,prod} - \mu\,[ATP]_{specific} - r_{ATP,cons}$$

Consumption of ATP for all non-growth-related maintenance activities

Utilization of ATP for glucose- and xylose-mediated growth processes

$d[ATP]_{specific}/dt$: net rate of specific [ATP] accumulation (g ATP/ g DCM – h)
$r_{ATP,cons}$: specific [ATP] consumption rate (g ATP/ g DCM – h)
(Suggested by Kompala and coworkers, 2002)

Objectives

- Demonstrate the effectiveness and reliability of the bioluminescence method based on luciferin-firefly luciferase for determining intracellular ATP concentrations in recombinant *Z. mobilis* cells samples during pure and mixed sugar ethanol fermentations.
- Apply specific ATP concentration data to help validate/reformulate a previously developed kinetic model (Kompala and coworkers, 2002) for *Z. mobilis* fermentation of glucose/xylose mixtures that incorporates an intracellular ATP balance to describe the growth uncoupling phenomenon.
- Expand the ATP-based kinetic model of a metabolically-engineered strain of *Z. mobilis* to better describe multiple sugar utilization, the phenomenon of cell growth uncoupling from ethanol production, and the ATP maintenance requirements at different fermentation pHs and concentrations of acetic acid.

Materials and Methods

- Microorganism
  - A genomic DNA-integrated glucose and xylose fermenting strain of *Z. mobilis*
- Fermentation system
  - Bioflo 3000 fermentors and MX3 Biosamplers
- Fermentation conditions
  - sugar substrate concentration (100 g/L)
  - temperature 30°C, agitation rate 150 rpm, anaerobic
  - at different pH (5.0-6.0)
  - at different initial 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 {using 2 min extraction time} (luminometer)

Intracellular ATP measurements

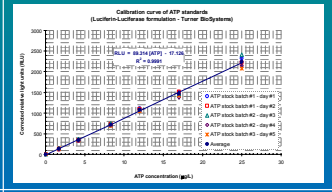
- A luminometer combined with ATP luciferin-firefly luciferase reagent, appears to provide a simple and sensitive method for quantifying the amount of ATP in *Z. mobilis* cultures samples.

$$\text{Luciferin} + \text{ATP} + O_2 \xrightarrow[Mg^{2+}]{\text{Luciferase}} \text{Oxyluciferin} + \text{AMP} + PP_i + CO_2 + \text{Light}$$

- 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.

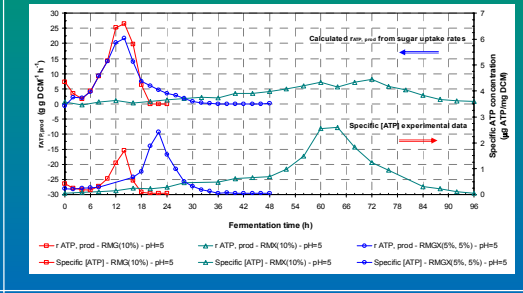
Extraction and Calibration Curve

- The extraction of 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.

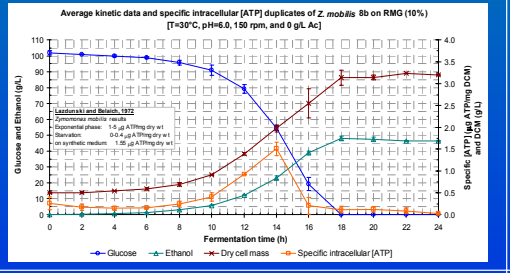


- A cell extraction time of 2 minutes appears to be reasonable for releasing intracellular ATP from *Z. mobilis* fermentation samples.
- A standard calibration curve is prepared by plotting the average blank-corrected relative light units (RLU) reading for each ATP standard versus its respective ATP concentration.

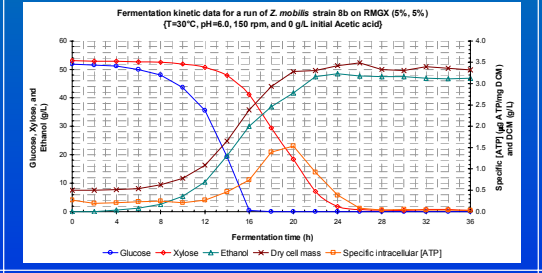
Specific [ATP] and rates: Effect of [S], pH=5



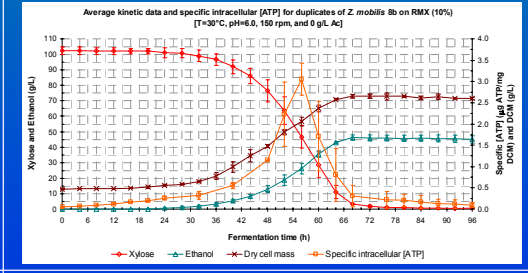
Fermentation kinetic data: Glucose



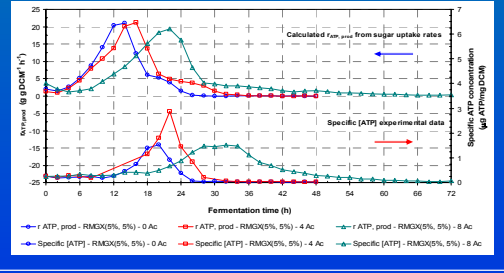
Fermentation kinetic data: Glucose/Xylose



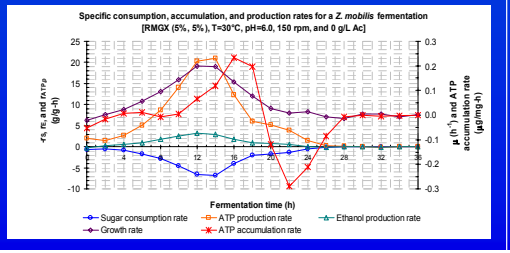
Fermentation kinetic data: Xylose



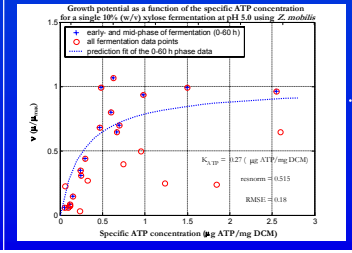
Specific [ATP] and rates: Effect of initial [Ac], pH=6



Fermentation specific rates: Glucose/Xylose



ATP-related analyses: ν ($[ATP]_{specific}$) – Xylose run



Prediction fit

$$\nu = \frac{[ATP]_{specific}}{K_{ATP} + [ATP]_{specific}}$$

Conclusions

- The luciferase-based ATP measurement method appears to be effective and reliable for determining intracellular [ATP] in *Z. mobilis* culture samples during fermentation processes.
- Substantial difference was observed in [ATP] profiles depending upon fermentation conditions (substrate type, pH, and initial [Ac]), although absolute maximum accumulation levels of free ATP varied within a relatively narrow range (1.5-3.5 μg ATP/mg DCM).
- Xylose fermentations accumulated ATP at a much slower rate than the mixed sugar fermentations (5% glucose/5% xylose) and mixed sugar fermentations at a slightly slower rate than the glucose system.
- Higher levels of acetic acid appeared to delay the onset and perhaps influence the extent of accumulation of free ATP.
- These findings will be used to try to validate and expand upon a previously developed kinetic model that incorporates an intracellular ATP balance.

Acknowledgements

- This work was funded by the Office of the Biomass Program of the U.S. Department of Energy (DOE) via the Biofuels Research Initiative for Student Advancement (BRISA) program.
- JCS was also funded by the National Science Foundation-Experimental Program to Stimulate Competitive Research in Puerto Rico (NSF-EPSCoR), Chemical Engineering Department, and the AGEP Program from University of Puerto Rico, at Mayagüez.
- The authors thank Christina Eddy, Min Zhang, Raymond Ruiz, Ali Mohagheghi, Daniel Schell, Gary McMillen, Bob Lyons, and Rafael Nieves from NREL, Dhinakar Kompala, Mehmet Altintas from University of Colorado – Boulder, and Gerson Santos-León of DOE (now with Abengoa Bioenergy Corporation), and the UPRM-ChE graduate students and faculty for their assistance and advise during this work.

Presented at the 15th AAAS/PR-EPSCoR Annual Meeting, San Juan, Puerto Rico, November 2003