Development of a Thermophilic Ethanologen, *Thermoanaerobacterium saccharolyticum*

Background

The US Department of Energy Biomass Program and Mascoma Corporation funded the development of *T. saccaharolyticum* for the conversion of lignocellulose to ethanol. The following data are taken from the Biomass Program Platform Review 2011 and the Final Report available at:

http://www.osti.gov/scitech/biblio/1033560/

In October of 2014, Mascoma's name and yeast business were sold to Lallemand LLC, with the IP and knowhow related to bacterial processing – including those stemming from the project described herein - transferred to Enchi Corporation (www.Enchicorp.com).

enchi

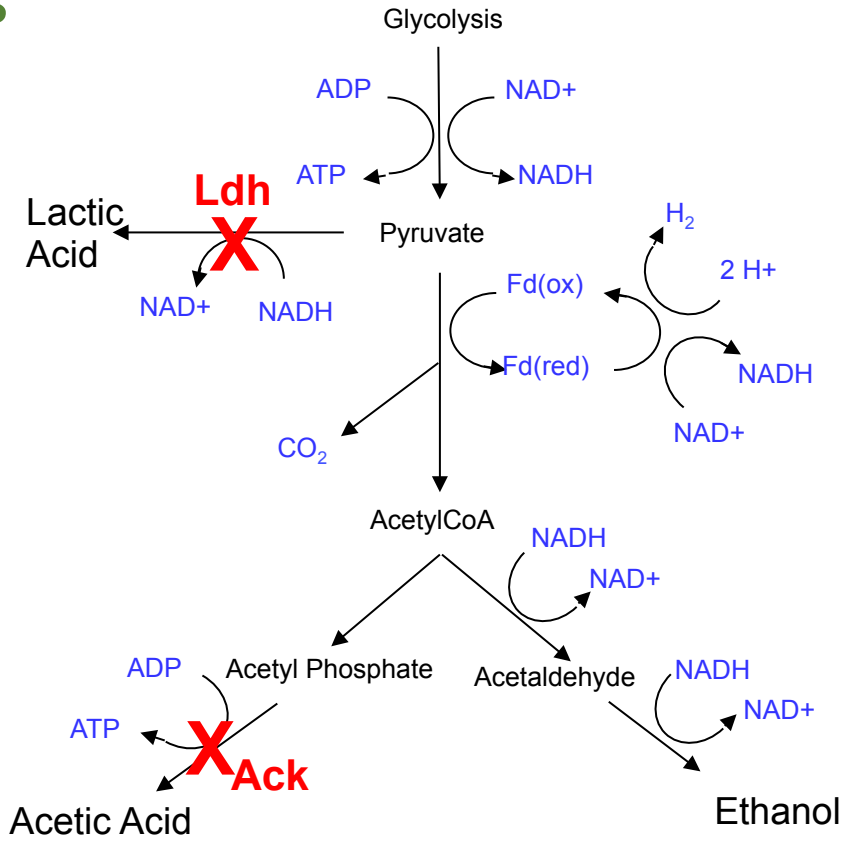
Goals and Objectives

- **Goal:** To develop a robust thermophilic bacterium capable of converting biomass sugars to ethanol at high yields and rates
- **Objectives**
  - To improve the characteristics of a genetically modified strain of *T. saccharolyticum* for ethanol production from pure mixed sugars
  - To develop methods for simultaneous saccharification and co-fermentation of pretreated hardwood chips by *T. saccharolyticum*

Project Overview - Context

T. saccharolyticum Attributes

- Thermophilic anaerobe isolated for growth on xylan at pH 4.5
- Readily grows on glucose, xylose, xylan, starch, pectin and other biomass sugars
- Performs well at pH and temperature optima of fungal cellulases
- Native strains produce ethanol, acetic and lactic acids



Initial Metabolic Engineering

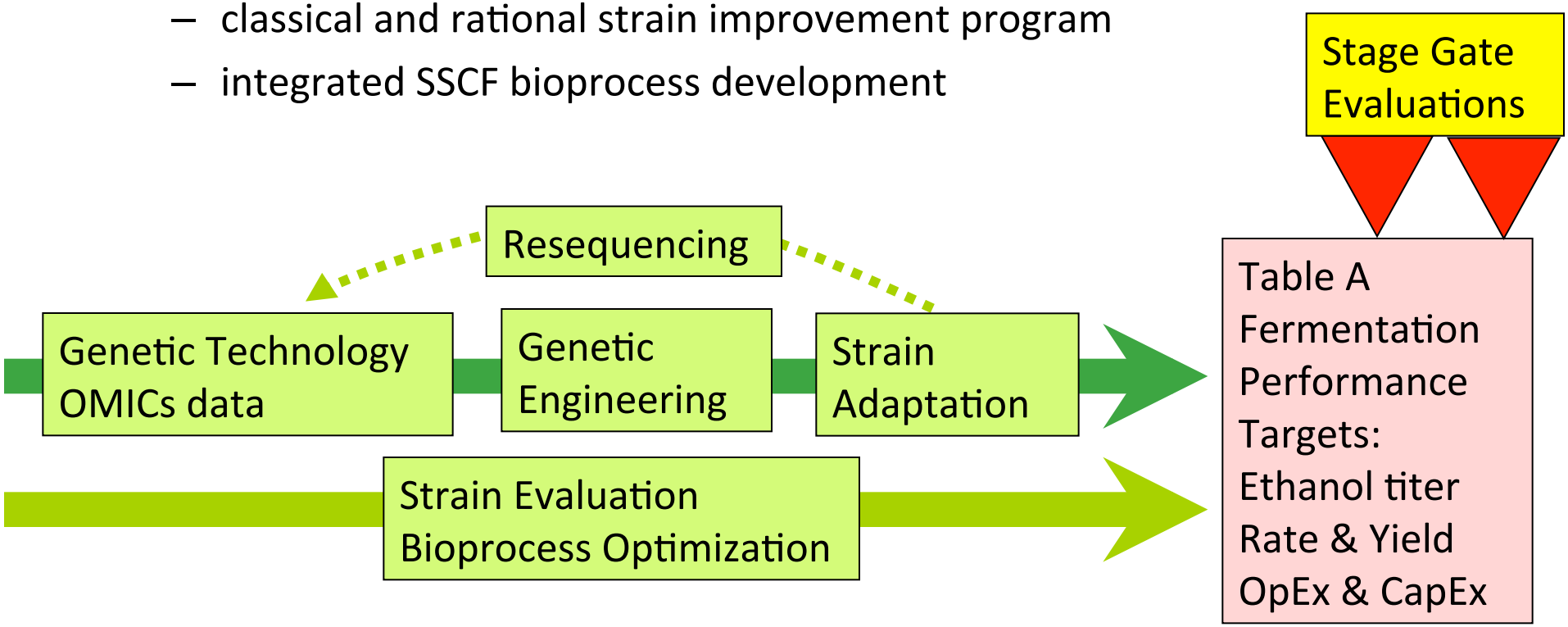
- Developed basic genetic tools and removed genes required for acetic and lactic acid production
- Engineered strain ALK2 produced ethanol at near theoretical yield
- Initial fermentations only reached low ethanol titer

Shaw *et al.* (2008) PNAS 105(37).

enchi

Approach

- Employ a proprietary bacterium capable of:
  - rapid, simultaneous utilization of C5 and C6 biomass-derived sugars
  - producing ethanol at high yield
  - growing at temperatures and pH optimal for cellulase activity
- Develop into commercial biocatalyst via:
  - classical and rational strain improvement program
  - integrated SSCF bioprocess development

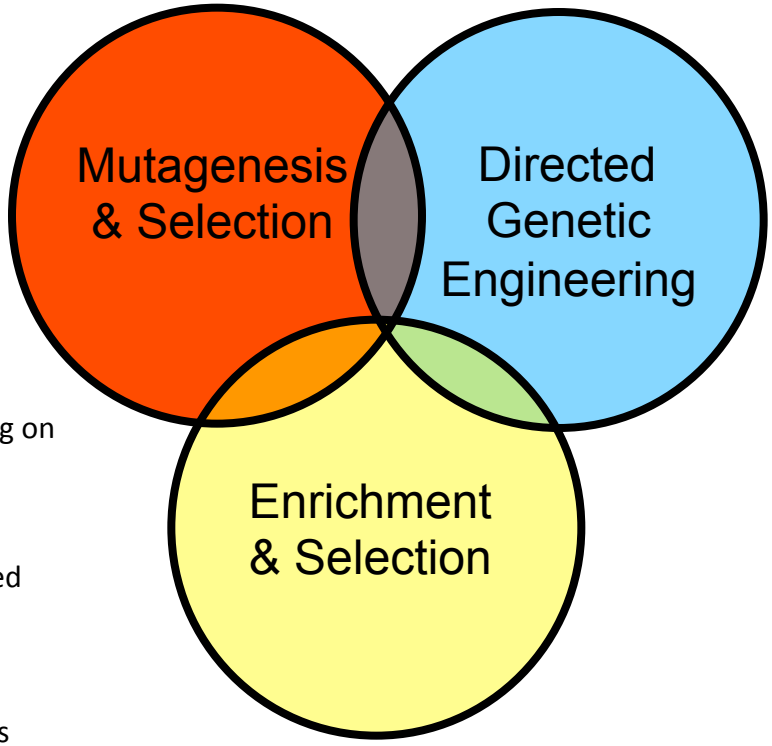


enchi

Strain Development for Intermediate Stage Gate

T. saccharolyticum JW/SL-YS485

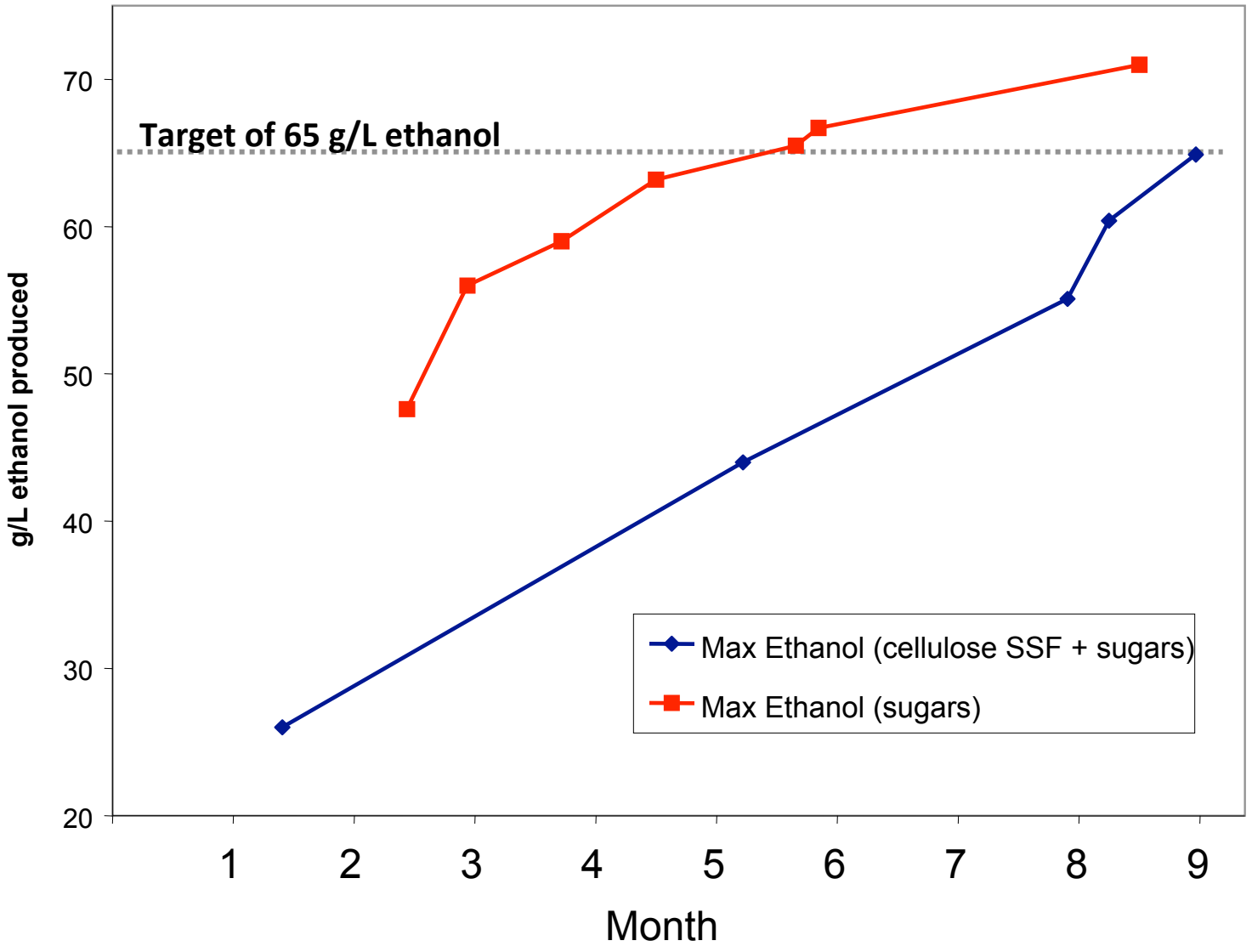
- M0355 — Markerless *pta:ack*, L-*ldh* deletion strain
- M0734 — Chemical mutagenesis and screening on pretreated hardwood hydrolysate
- M0863 — Selection for rapid growth on defined hydrolysate inhibitors
- M1151 — Introduction of urease+, other genes
- M1291 — ΔEPS (knockout in putative Exopolysaccharide operon)
- M1442 — Selection for rapid growth in minimal media



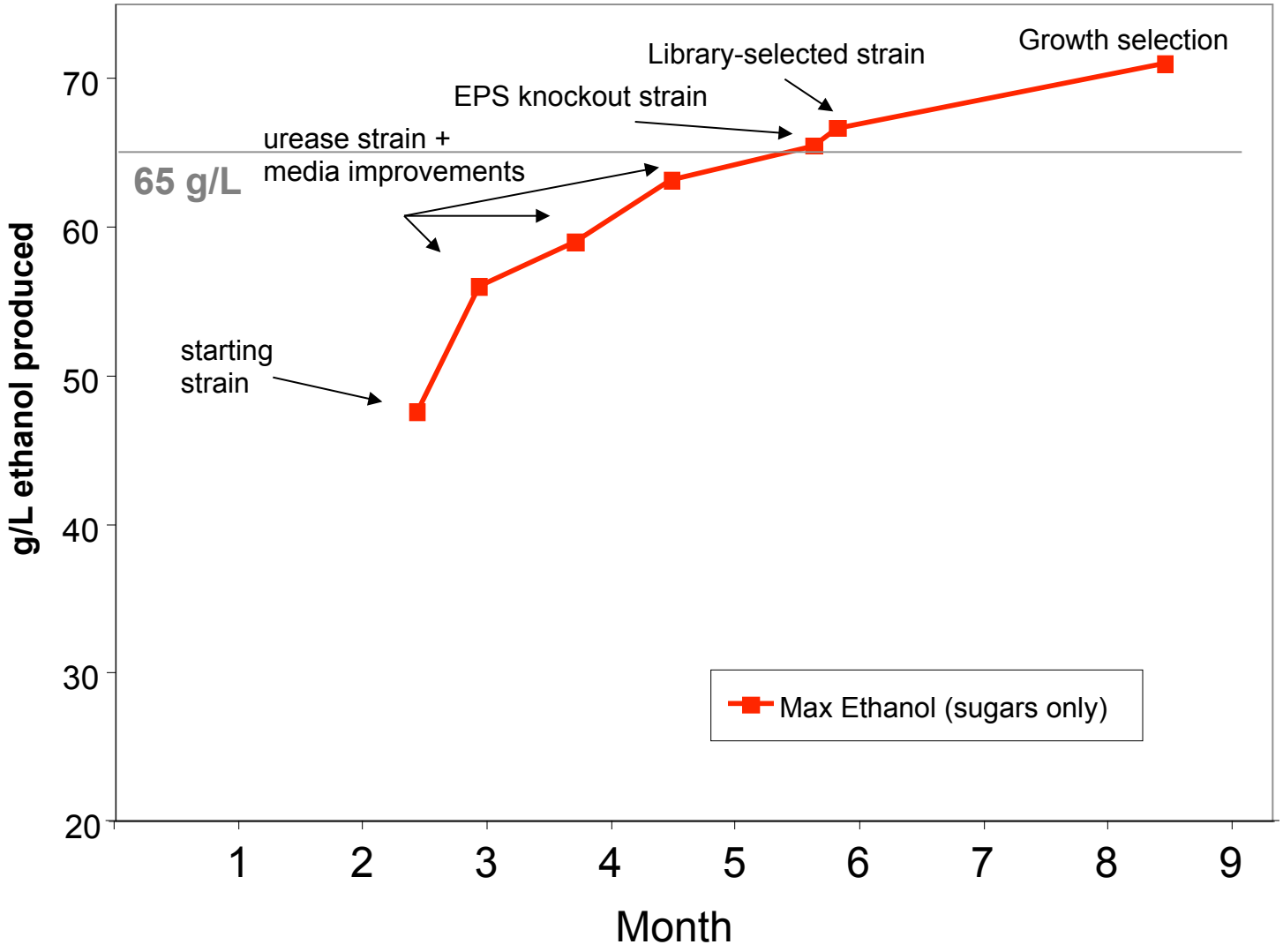
- 30 strains were screened for DOE stage gate evaluation
- Scale-down SSF tests in sealed bottles

enchi

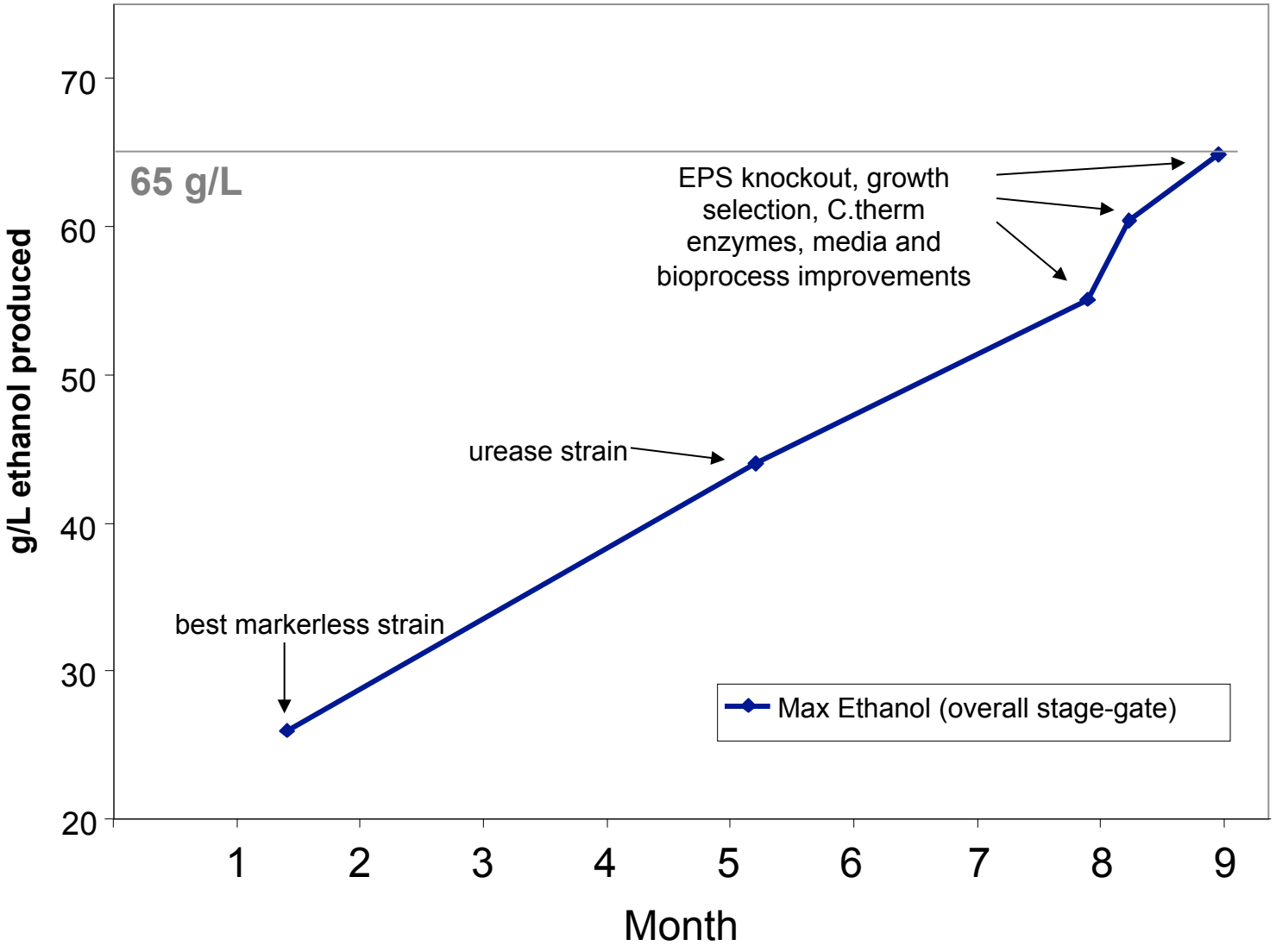
Strain Improvement Progress



Strain Improvement – Polysaccharide Fermentation

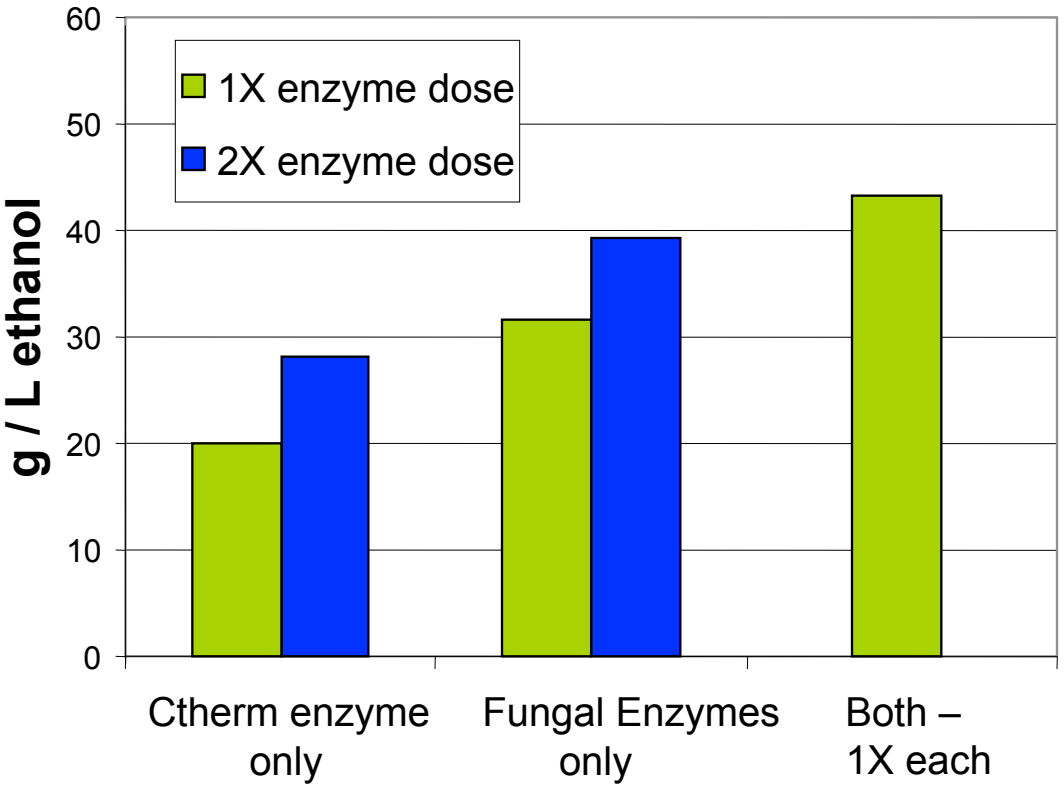


Strain Improvement – SSCF with Acetate



Demonstrated Synergy of Fungal & Bacterial Cellulases

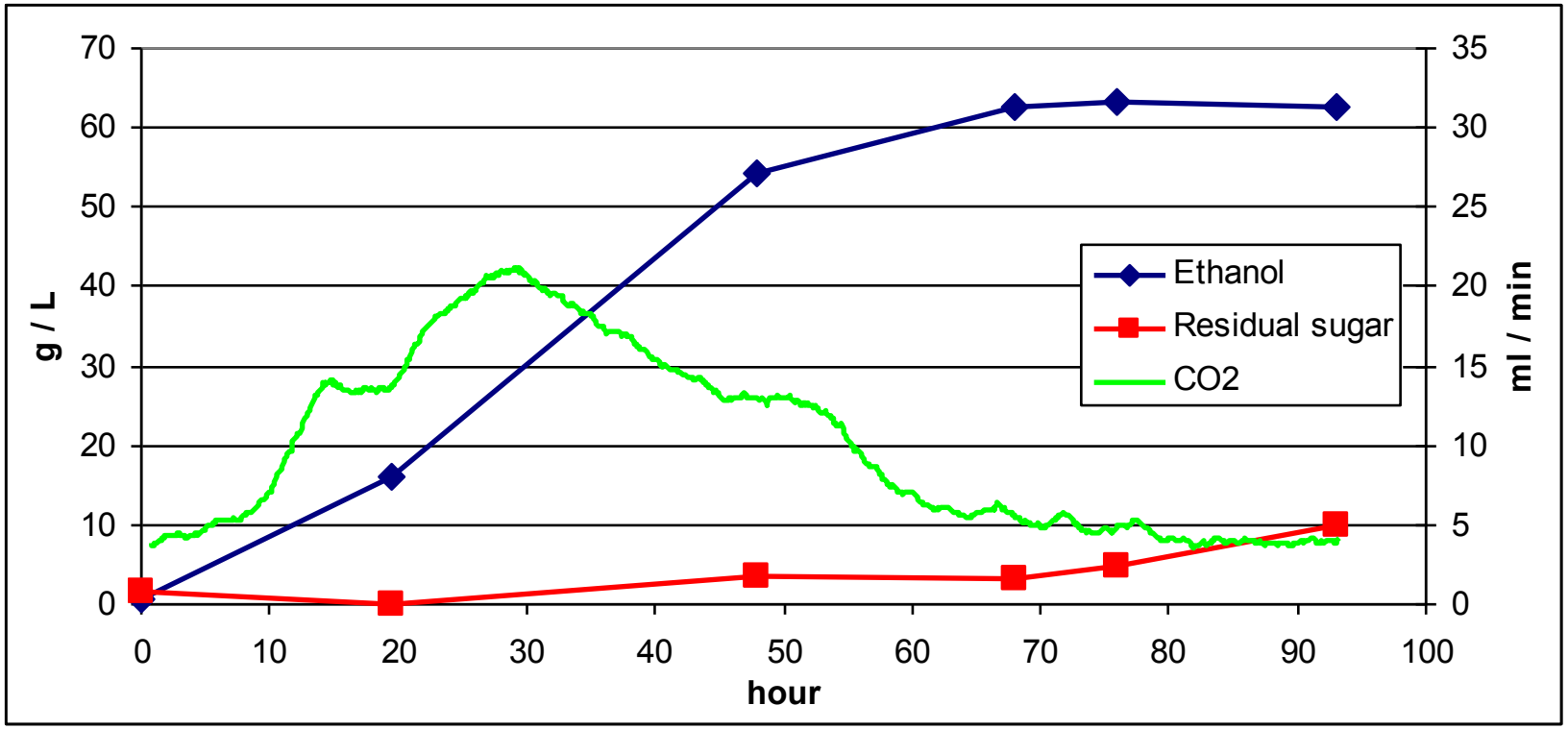
SSCF with strain M1291 in 150 g/L Sigmacell + 10 g/L acetate
51 degC, 96 hrs



1X = 0.4 Avicelase U/gTS for C. thermocellum enzyme or 5 mg/gTS for fungal enzymes

Intermediate Stage Gate: “Mock Hydrolysate”

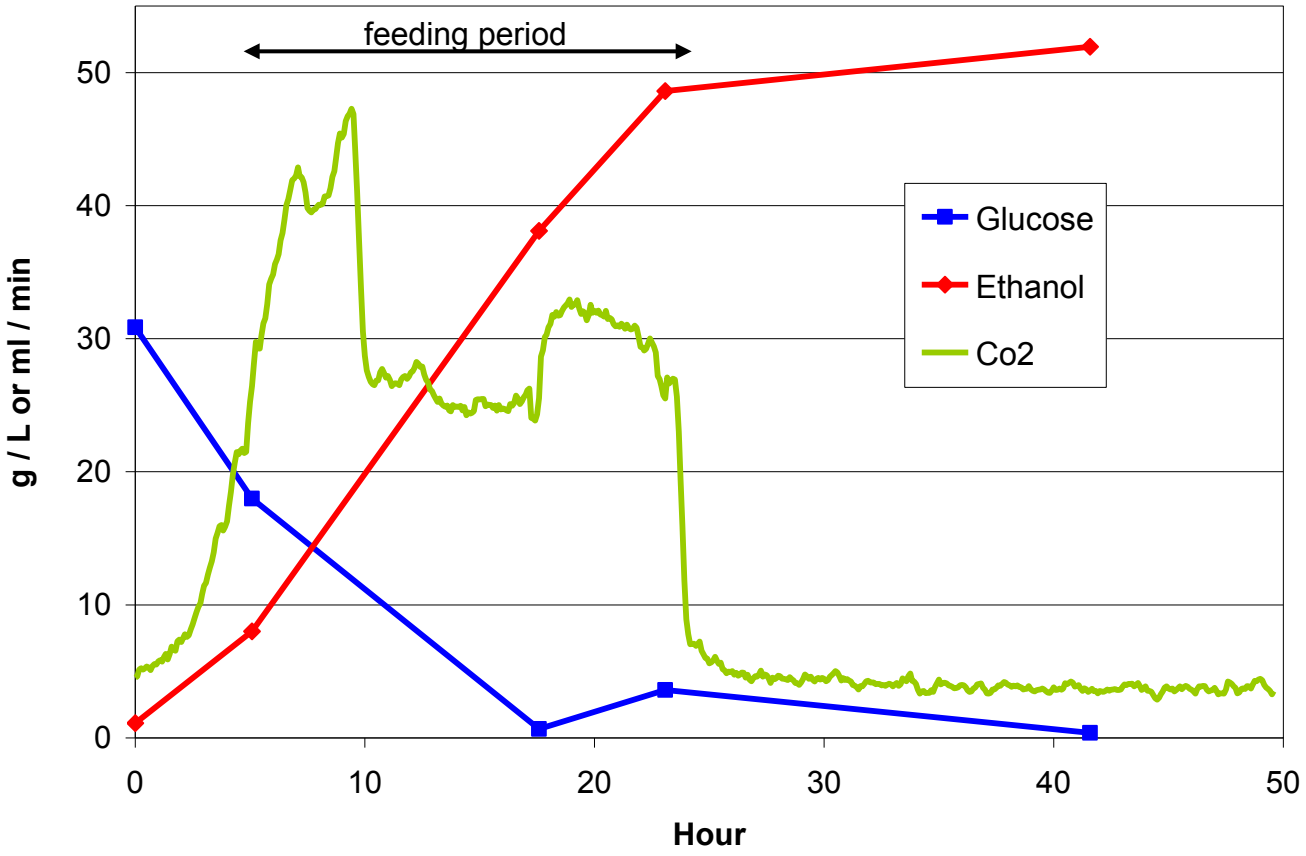
Fed batch fermentation of 100 g/L cellulose and 55 g/L mixed sugars in the presence of 10 g/L acetic acid. Fungal + Bacterial cellulases.



- **> 93% utilization of available monomer sugars (>99% for xylose)**
- **Cellulose hydrolysis limited overall rates, titers**

Fermentation of Cellulose Fraction (C6)

- Fed-batch fermentation of enzymatic hydrolysate from pretreated hardwood

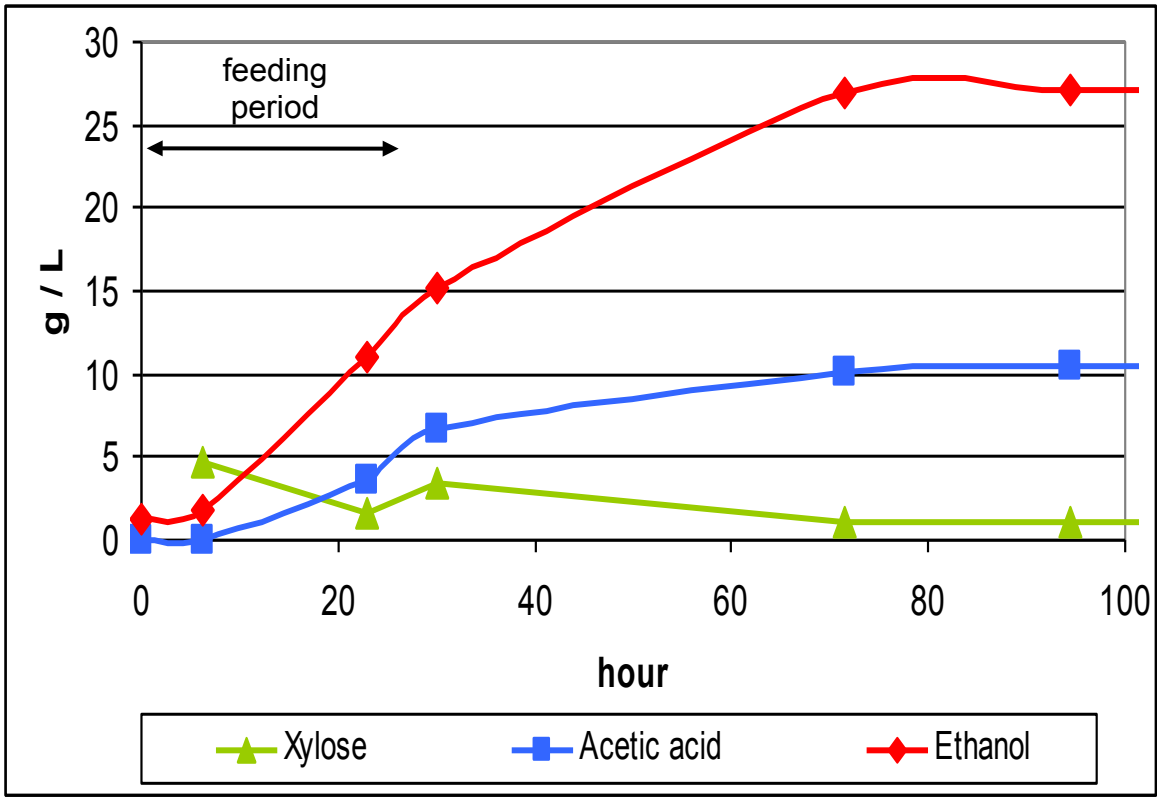


- > 2 g/L/hr productivity
- > 99% sugar utilization

enchi

Fermentation of Hemicellulose (C5) Fraction

- Fed batch fermentation of oligosaccharide-rich hemicellulose hydrolysate from pretreated hardwood



- 90 % metabolic yield
- 86 % process yield
- > 95 % sugar utilization
- No added enzyme or acid

enchi

Summary of Key Milestones & Deliverables

1	Characterized pretreated material	complete
2	Improved genome sequence & annotation, generate OMICs data	complete
3	Identified conditions to induce stasis	complete
4	Commercial enzymes tested	complete
5	Markerless strain	complete
6	Benchmarks of performance in hydrolysates	complete
7	Low cost medium	complete
8	Strain that produces 5.5 % ethanol in the presence of inhibitors at 50% nominal levels	complete
9	Identified mutations that lead to better performance	complete
10	5.0% ethanol titer at 10L scale from hydrolysate	3.2% ethanol in 60 hrs at 100 L scale & 78% yield
11	Improved Strain, capable of 6.5 % ethanol at 95% yield and 90% sugar utilization in the presence of acetic acid and other inhibitors	6.1% ethanol in stage gate test (SSF)
12	Improved Bioprocess, 6.5% (w/v) ethanol titer in < 60 hours, with 95% yield and 90% sugar utilization	5.0% ethanol in 60 hrs with 90% yield

enchi

Relevance

Addresses Biochemical Platform Goal of Reducing Processing costs by providing a new technology with

1.	High yield:	86-92% theoretical ethanol in Stage Gate tests
		native xylan / hemicellulose degrader
		co-utilization of glucose and xylose
2.	High titer:	61 g/L ethanol in Stage Gate tests
		52 g/L ethanol from C6 hydrolysate of hardwood
3.	High productivity:	60 g/L ethanol in 60 hours in Stage Gate tests
		49 g/L ethanol in 23 hours from C6 hydrolysate

- NREL-validated economic model of 400 ton/day plant using fermentation data from intermediate Stage Gate showed:
  - >25 % reduction in Capital Expenses by increasing annual production
  - >20 % reduction in Operating Expenses

enchi

Summary

- **Valuable Native Attributes of *T. saccharolyticum***
  - Thermophilic operating temperatures
  - Broad sugar utilization
  - Native xylan / hemicellulose degrader
  - Easy to genetically manipulate
- **Advanced strains show significant improvement in key areas**
  - Ethanol production rate, yield and titer
  - Growth and ethanol production in the presence of inhibitors
  - Cellulose conversion under thermophilic SSCF conditions
  - Utilization of commercially-relevant feedstocks

enchi

Publications

1. Shaw AJ, Podkaminer KK, Desai SG, Bardsley JS, Rogers SR, Thorne PG, Hogsett DA, Lynd LR. Metabolic engineering of a thermophilic bacterium to produce ethanol at high yield. Proc Natl Acad Sci U S A. 2008 Sep 16;105(37):13769-74.
2. Shaw AJ, Covalla SF, Hogsett DA, Herring CD. A marker removal system for *Thermoanaerobacterium saccharolyticum* and development of a markerless ethanologen. Appl Environ Microbiol. 2011 Apr;77(7):2534-6.
3. Shaw AJ, Hogsett DA, Lynd LR. Natural competence in *Thermoanaerobacter* and *Thermoanaerobacterium* species. Appl Environ Microbiol. 2010; 76:4713-9.
4. Shaw AJ, Covalla SF, Miller BB, Firliet BT, Hogsett DA, Herring CD. Urease expression in a *Thermoanaerobacterium saccharolyticum* ethanologen allows high titer ethanol production. Metab Eng. 2012 Sep; 14(5):528-32.
5. Tsakraklides V, Shaw AJ, Miller BB, Hogsett DA, Herring CD. Carbon catabolite repression in *Thermoanaerobacterium saccharolyticum*. Biotechnol Biofuels. 2012 Nov 26;5(1):85.
6. Podkaminer KK, Kenealy WR, Herring CD, Hogsett DA, Lynd LR. Ethanol and anaerobic conditions reversibly inhibit commercial cellulase activity in thermophilic simultaneous saccharification and fermentation (tSSF). Biotechnol Biofuels. 2012 Jun 15;5(1):43.
7. Lee JM, Venditti RA, Jameela H, Kenealy WR. Detoxification of woody hydrolyzates with activated carbon for bioconversion to ethanol by the thermophilic anaerobic bacterium *Thermoanaerobacterium saccharolyticum.* Biomass and Bioenergy Jan. 2011, 35:626-636.
8. Currie DH, Raman B, Gowen CM, Tschaplinski TJ, Land ML, Brown SD, Covalla SF, Klingeman DM, Yang ZK, Engle NL, Johnson CM, Rodriguez M, Shaw AJ, Kenealy WR, Lynd LR, Fong SS, Mielenz JR, Davison BH, Hogsett DA, Herring CD. Genome-scale resources for *Thermoanaerobacterium saccharolyticum*. BMC Syst Biol. 2015 Jun 26;9(1):30.
9. Shaw, AJ, Miller BB, Rogers SR, Kenealy WR, Meola A, Bhandiwad A, Sillers WR, Shikhare I, Hogsett DA, and Herring CD. Anaerobic detoxification of acetic acid in a thermophilic ethanologen. Biotechnology for Biofuels 8, no. 75 (2015).