PRODUCTION OF LIGNOCELLULOSIC ISOBUTANOL BY FERMENTATION AND CONVERSION TO BIOJET

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LIST OF ACRONYMS

ASTM	ASTM International
ATJ	alcohol-to-jet
C	carbon
CDW	cell dry weight
CL	CatchLight Energy
CMB	combined mild bisulfite
°C	degrees Celsius
FPL	Forest Products Laboratory
g	gram
Gal	gallon
GC	gas chromatography
GIFT®	Gevo Integrated Fermentation Technology
GRAS	generally regarded as safe
HPLC	High pressure liquid chromatography
h or hrs	hours
iBuOH	isobutanol
IC	ion chromatography
L or l	liter
MBS	mild bisulfite
mL	milliliter
MS or mass spec	mass spectrometer
MW	willed wood
NARA	Northwest Advanced Renewables Alliance
NIFA	National Institute of Food and Agriculture
PNW	Pacific Northwest
SHF	separate hydrolysis and fermentation
SPK	synthetic paraffinic kerosene
SPORL	Sulfite Pretreatment to Overcome Recalcitrance of Lignocelluloses
SSF	simultaneous saccharification and fermentation
TEA	techno-economic assessment
UMW	unconcentrated milled wood
USDA	United States Department of Agriculture
WO	wet oxidation
WSU	Washington State University
WY	Weyerhaeuser Company
YSI	Yellow Springs, Incorporated

EXECUTIVE SUMMARY

Gevo developed fermentation and process technology to convert biomass sugars to isobutanol and further into renewable jet fuel through chemical processing. Gevo concurrently developed GIFT®, Gevo Integrated Fermentation Technology, to produce isobutanol at targeted productivity, titer, and yield using a yeast biocatalyst adapted to hydrolyzate. The goal of this project was met. Gevo produced isobutanol according to a specification developed by Gevo that ensured the isobutanol will be converted into renewable biojet using existing Gevo technology. Quantities of about 1,000 gallons of biojet were prepared and validated as suitable jet fuel blend stock using ASTM's fit for purpose testing protocol and input from stakeholders. The details of the scale-up portion of the work are found in a separate final report entitled "Production of 1,000 Gallons of Biojet in the NARA Consortium" by Dr. Robert J. Wooley (Wooley et al., 2016). The specific tasks completed during this project are: (1) Characterize toxicity of a representative sample of pre-treated woody biomass (Douglas-fir) for fermentation; (2) Adapt yeast biocatalyst to pretreated biomass hydrolyzate; (3) Produce isobutanol in a 1L batch fermentation from pretreated biomass sugars using the adapted yeast biocatalyst; (4) Economic assessment of wood to isobutanol, jet; (5) Produce isobutanol in a 1L GIFT® fermentation from pretreated biomass sugars using the adapted yeast biocatalyst; (6) Analysis of isobutanol to close the mass balance and determine potential low-level impurities.

INTRODUCTION

Sustainable production of jet fuel from renewable, regionally specific feedstocks, addresses the issue of reducing carbon emissions from aircraft. The Pacific Northwest (PNW) is home to several large companies in the aerospace industry as well as several major commercial airports. These stakeholders have a strong interest in moving the aviation industry to lower carbon emissions (SAFN, 2011). Woody biomass represents the only realistic, sustainable, feedstock for developing fuels at scale and cost in PNW. The NARA project has brought together Weyerhaeuser, a major producer of woody biomass in the PNW, and the USDA Forest Products Laboratory (FPL), the only major research lab in the USDA system focused on production of value-added products from forests, and Gevo, a producer of low carbon and renewable fuels.

The USDA FPL has developed an economical and effective chemical pretreatment process for softwood biomass to liberate both sugars and lignin for downstream use. That pretreatment process is known as SPORL or **S**ulfite **P**retreatment to **O**vercome **R**ecalcitrance of **L**ignocelluloses. This pretreatment technology is novel, yet similar enough to current low pH wood pulping technology that it might be implemented in stranded pulp and paper industry assets in the PNW.

Gevo has developed fermentation and process technology to convert biomass sugars into a four-carbon alcohol, called isobutanol (iBuOH), by fermentation. Isobutanol is a natural product that provides food and beverages flavor and aroma. It is made in tiny quantities naturally by yeast. Gevo has developed a commercial process based on GRAS, generally regarded as safe, genetically modified yeast that produce iBuOH at high fermentation rates, titers, and yields. Further, Gevo has developed an effective and high yield process to convert iBuOH further into renewable jet fuel through chemical processing.

Within the NARA project, Gevo focused on adapting an existing line of yeast biocatalysts that Gevo developed outside of the NARA project for its commercial operation in Luverne, MN, to convert the sugars derived from the SPORL process on forest residues from the PNW. Gevo further developed its patented GIFT® technology and process to produce iBuOH at a specification that permitted conversion of that renewable, cellulosic iBuOH into renewable, synthetic biojet fuel, called synthetic paraffinic kerosene (SPK). Gevo's alcohol-to-jet synthetic paraffinic kerosene (ATJ-SPK) process turns its bio-based iBuOH into jet fuel that meets the requirements of the recently revised ASTM D7566 (Standard Specification for Aviation Turbine Fuel Containing Synthesized Hydrocarbons) for up to a 30 percent fuel blend. Outside of NARA, Gevo spent about eight years and millions of dollars collecting the necessary scientific and technical data to support the revision of the ASTM D7566 specification to include Gevo's ATJ process.

TASK 1: CHARACTERIZE HYDROLYZATE AND COMPLETE BENCHMARKING OF PRE-TREATED BIOMASS FOR FERMENTATION INTO ISOBUTANOL

The goals of this task were to chemically characterize and understand the sugar, inhibitor, and nutrient composition of various hydrolyzates and complete benchmarking experiments for each feedstock/hydrolyzate combination from the NARA project. Gevo also collected data and results on hydrolysis effectiveness of various feedstock and pretreatment parameters and tested these sugars as a feedstock for benchmark ethanol and iBuOH fermentation at various scales. Early in the NARA project, there were three different pretreatment technologies that were examined: SPORL, Wet Oxidation, and Milled Wood. Woody biomass feedstocks were mainly Douglas-fir and Douglas-fir forest residuals, but in the later years of the project, waste fibers from western hemlock were also characterized.

The original plan within the project was for pre-treatment partners, such as the USDA FPL and WSU Tri-Cities Labs, to conduct the hydrolysis of their pretreated material. However, as we progressed within the project, we agreed that Gevo should also do hydrolysis of pre-treated material for comparative purposes with the other partners. Further, and specifically for SPORL pre-treated material, it was easier to ship this pretreated feedstock as a low pH (2-3) wet solid then hydrolyze at Gevo for use in characterization and fermentation experiments. Thus, Gevo agreed to add this work to our scope under Task 1, which was expanded to include conducting the hydrolysis of the pre-treated materials.

Further, NARA partners agreed that while a representative Douglas-fir forest residual feedstock was collected and made available for use in all laboratory experiments among NARA partners for pre-treatment, hydrolysis, and for use in fermentation, the NARA partners would use a reference (positive control) pulp-quality wood chip of Douglas-fir that was named FS-01. This feedstock is pictured below in Figure LIF- 1.1.

Early isobutanol production strains, such as Gevo strains LB2 and LB3, could not tolerate growth and fermentation in 100% hydrolyzate. Because of this, a reference ethanol-producing yeast strain was used as a benchmark for growth and fermentation performance in the first year of the project. The ethanol producing yeast used was Gevo LB1. The project did not seek to produce ethanol from the pre-treated and hydrolyzed feedstocks. The ethanol producing strain was used only as a positive control until more tolerant isobutanol producing strains were generated within Task 2 of the project (see Task 2: Adapt yeast biocatalyst to pretreated biomass hydrolysate, for more information).

For laboratory assessments and ease of handling at the small bench scale, all material was fully hydrolyzed and then clarified (insoluble solids were removed) prior to use for growth and fermentation. This is known as separate hydrolysis

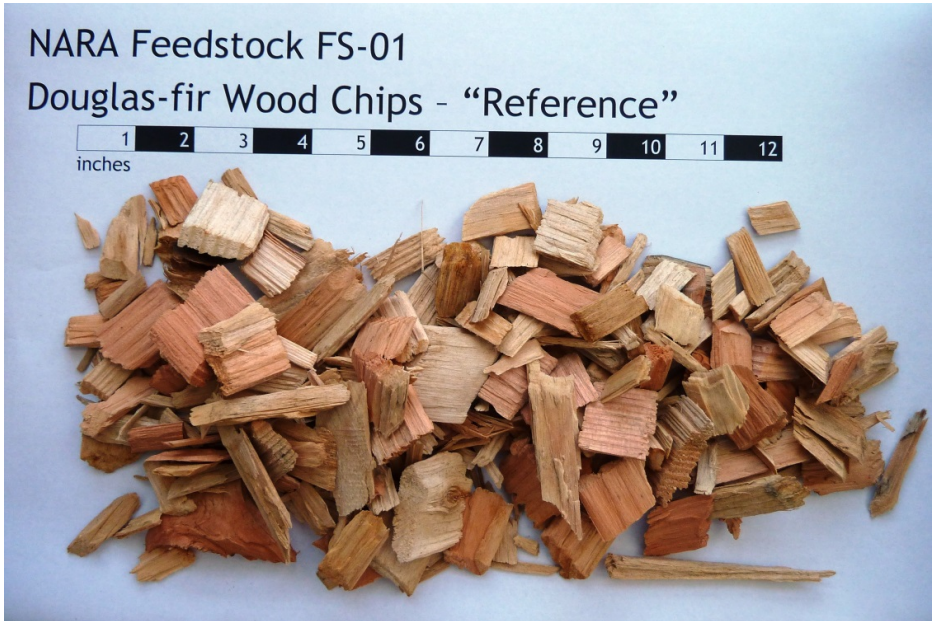

Figure LIF-1.1. Image of NARA Feedstock FS-01. Pulp-quality Douglas-fir wood chips used as a reference material until a representative sample of Douglas-fir forest residuals could be collected and distributed to the project.

and fermentation (SHF). The goal of the project was to maintain flexibility for a larger scale process and be able to implement simultaneous saccharification and fermentation (SSF) methods or SHF methods as needed. However, hydrolyzed and insoluble solids-free materials are more practical for handling at the laboratory scale in test tubes, small fermenters, Gevo's small GIFT® units, and shake flasks.

The first material received by Gevo was SPORL-treated material from Dr. Junyong Zhu at the USDA Forest Products Laboratory. The second material received by Gevo was pretreated by Wet Oxidation by Dr. Birgitte Ahring's laboratory at Washington State University, Tri-Cities (Richland, WA). Material from the wet oxidation pretreatment was received in two forms: as pretreated biomass (24% solids) and as pretreated and enzymatically hydrolyzed liquid (no solids, clarified). Both materials were generated from pulp quality Douglas-fir wood chips provided by Weyerhaeuser and the NARA Feedstock Logistics team. A list of the pretreated feedstocks that Gevo characterized during the NARA project is show in Table LIF-1.1. Additional feedstocks were sometimes analyzed for pretreatment partners in order to cross validate analytical methods or techniques, but are not included in Table LIF-1.1.

Table LIF-1.1. Sugar and inhibitor concentrations in FS-01, FS-03, FS-10, and hemlock feedstocks from various pretreatment methods. Concentrations of sugars and inhibitors were determined using HPLC at Gevo. (n.d. = not detected)

	% solids in hydrolysis	Glucose (g/L)	Xylose (g/L)	Galactose (g/L)	Arabinose (g/L)	Mannose (g/L)	Acetate (g/L)	HMF (g/L)	Furfural (g/L)	Total Hexose (g/L)
FS-01 Wet Oxidation Hydrolyzate		57.20	6.67	5.12	1.58	20.87	7.27	3.90	0.99	83.19
FS-03 Wet Oxidation Hydrolyzate (Batch A)	19.9	87.54	4.67	5.14	0.76	10.06	12.46	3.66	0.81	102.74
FS-03 Wet Oxidation Hydrolyzate (Batch B)	23.19	89.58	5.25	3.00	n.d.	7.66	7.00	n.d.	n.d.	100.24
FS-10 Wet Oxidation Hydrolyzate (Batch A)	15.09	44.76	6.61	4.18	6.00	16.84	7.94	2.42	0.56	65.78
FS-10 Wet Oxidation Hydrolyzate (Batch B)	24.99	67.22	4.57	2.37	n.d.	9.04	3.90	n.d.	n.d.	78.63
FS-10 Wet Oxidation Hydrolyzate (Batch C)	21.78	54.79	12.01	5.38	4.43	9.59	7.99	3.53	0.26	69.76
FS-01 SPORL Hydrolyzate		93.65	6.89	4.94	1.24	23.01	4.56	0.79	0.10	121.60
FS-03 SPORL Hydrolyzate	36.43	81.81	5.79	3.82	0.40	7.02	5.78	1.84	0.63	92.65
FS-10 SPORL-Na^+ Pretreated SSL		7.83	7.32	5.29	2.21	18.06	3.61	n.d.	n.d.	31.18
FS-10 SPORL-Na^+ Pretreated Washed Solids Hydrolyzate	24.48	63.88	3.77	2.07	0.68	7.52	1.65	n.d.	n.d.	73.47
FS-10 SPORL-Na^+ Pretreated Unwashed Solids Hydrolyzate	29.19	84.22	10.14	5.26	2.02	20.93	3.44	n.d.	n.d.	110.41
FS-10 SPORL-Ca^{2+} Pretreated Hydrolyzate	26.29	62.74	6.84	5.07	n.d.	11.83	0.62	n.d.	n.d.	79.64
FS-10 SPORL-Mg^{2+} Pretreated Hydrolyzate	24%	71.59	8.89	4.23	14.39	0.61	3.00	n.d.	n.d.	76.43
FS-10 SPORL-Mg^{2+} Pretreated Solids Hydrolyzate	27.29	88.56	8.39	3.47	12.68	0.66	2.71	n.d.	n.d.	92.70
FS-10 SPORL-Mg^{2+} Pretreated Liquor	As rec'd	7.11	12.38	7.82	24.09	0.00	4.57	n.d.	n.d.	14.94
FS-03 Catchlight Combined Hydrolyzate	19.27	164.12	8.56	5.24	0.94	13.34	3.45	0.15	0.14	182.70
FS-03 Catchlight Clean Hydrolyzate	28.81	130.89	1.84	0.49	n.d.	1.34	0.26	0.14	0.01	132.72
FS-10 Unconcentrated Milled Wood Hydrolyzate	12.9%	39.84	7.61	1.87	2.04	11.40	0.74	n.d.	n.d.	53.11
FS-10 Concentrated Milled Wood Hydrolyzate	As rec'd	61.84	9.21	0.46	1.36	12.76	0.77	n.d.	n.d.	75.06
FS-01 Milled Wood (40 min. Grind)	~25%	90.34	9.77	0.61	2.12	10.67	1.58	n.d.	n.d	101.61
EW-01 Milled Wood (80 min. Grind)	~25%	28.83	3.54	0.63	1.22	6.09	0.99	0.05	n.d.	35.55
EW-01 Milled Wood (120 min. Grind)	~25%	92.30	10.86	1.25	2.60	15.01	2.27	0.06	n.d.	108.57
Cosmo Reject Solids Hydrolyzate	24.86	111.35	2.02	0.15	1.45	0.79	0.27	n.d.	n.d.	112.29
FS-10 SPORL-Mg2+ Pretreated Concentrate	As rec'd	282.63	44.05	18.25	7.18	75.28	10.39	n.d.	n.d.	376.2

Sugar and inhibitor concentrations were determined by high performance liquid chromatography (HPLC) analysis for each feedstock and pretreatment method received (Table LIF-1.1). To date, Gevo has received and characterized the following: FS-01, FS-03, and FS-10 SPORL. Characterization work of FS-10 SPORL has involved several varieties of SPORL pretreatment. Included in the characterization work was sodium pretreated FS-10 SPORL material, which was previously denoted as FS-10 mild bisulfite. Washed and unwashed solids variations of SPORL material have been characterized and the spent sulfite liquor has also been characterized. Calcium and magnesium pretreated SPORL material has also been included in the characterization work conducted by GEVO. SPORL pretreated materials were obtained from Dr. Junyong Zhu at the USDA Forest Products Laboratory (Madison, WI). FS-01, FS-03, and FS-10 wet oxidation pretreated materials were obtained from Dr. Birgitte Ahring at Washington State University – Tri-Cities (Richland, WA).

FS-03 pretreated material (Clean and Combined) was obtained from Catchlight Energy (Federal Way, WA). FS-10 milled wood pretreated material (Concentrated and Unconcentrated) was obtained from Dr. Johnway Gao at Catchlight Energy. Hemlock reject fibers that were washed solids were produced by Cosmo Specialty Fibers and milled by Dr. Junyong Zhu, and FS-01 (40 minute grind time) and EW-01 (80 and 120 minute grind time) Milled Wood (MW) samples were from Jinwu Wang at Washington State University (Pullman, WA).

Wet Oxidation Hydrolyzate

Wet oxidation material was received by Gevo from Dr. Birgitte Ahring's lab at Washington State University, Tri-Cities (Richland, WA). The material received was in one of two forms: pretreated biomass that was hydrolyzed and clarified by Dr. Birgitte Ahring's laboratory or pretreated biomass that was unhydrolyzed and still contained insoluble solids. The unhydrolyzed pretreated biomass was determined to be 24% solids by weight and had 10 g/L free glucose and 4.3 g/L acetate, as measured at Gevo by HPLC. Dr. Birgitte Ahring's laboratory provided a raw material compositional analysis along with the material (see Table LIF-1.2). The raw material compositional analysis provided was used to base enzyme dosing for hydrolysis by enzyme cocktails provided by commercial enzyme manufacturer Novozymes.

Table LIF-1.2. Raw material compositional analysis of the Douglas fir pulp quality chips. Analysis was provided by Dr. Birgitte Ahring's laboratory at Washington State University, Tri-Cities (Richland, WA).

Douglas fir									
% Glucan	% Xylan	% Galactan	% Arabinan	% Mannan	% Lignin	%Extractives	% Acetate	% Ash	Sum
44.7	2.5	2.0	2.1	11.8	29.6	5.0	2.3	0.5	100.2

Enzymatic digestibility of the unhydrolyzed pretreated material was tested by hydrolyzing in a pH controlled 2L fermentor using CTec2 and HTec2 over 50 hours. Acetate concentrations remained constant and glucose concentration reached 50 g/L (see Figure LIF-1.2). Both the materials shipped had similar glucose (50 g/L), xylose (7.5 g/L), and acetate (5 g/L) after hydrolyzing and clarification (see Table LIF-1.3). Therefore, similar hydrolysis results and efficiencies were achieved in both locations.

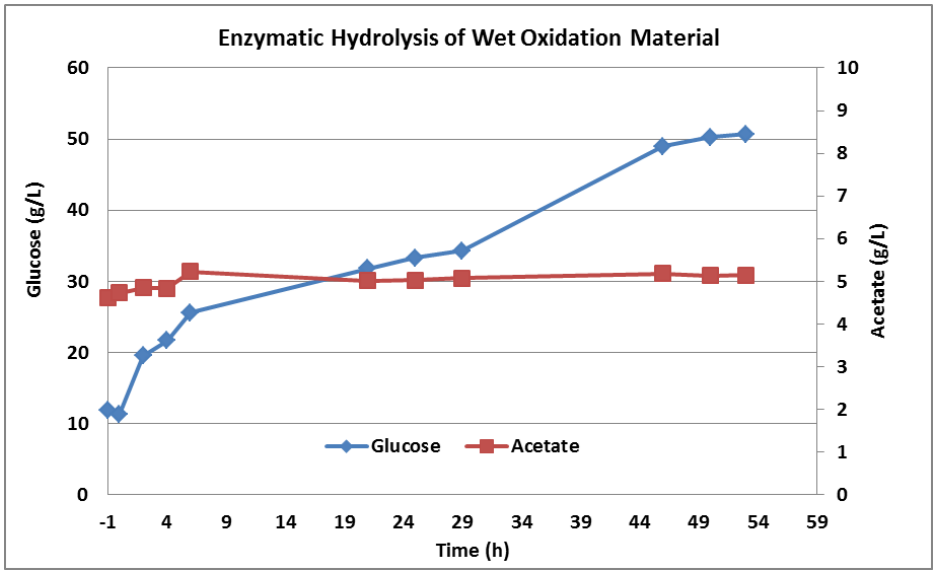

Figure LIF-1.2. Enzymatic hydrolysis test for 24% wet oxidation pretreated biomass. Hydrolysis was done in a pH-controlled 2L fermentor (pH5) at 50°C. Addition of 0.1 ml CTec2 (Novozymes) per gram glucan at 0 hours and 24 hours.

Table LIF-1.3. Comparison of clarified wet oxidation biomass hydrolyzates. Material 1 is the clarified biomass hydrolyzate as received by Gevo. Material 2 is the clarified biomass hydrolyzate from the enzymatic test shown above (see Figure LIF-1.2) produced in the Gevo laboratory.

	Glucose (g/L)	Xylose (g/L)	Acetate (g/L)	Pyruvate (g/L)
Material 1 clarified biomass hydrolyzate from WSU Tri-Cities	51.7	7.7	5.2	0.1
Material 2 clarified biomass hydrolyzate produced at Gevo	50.7	8.2	5.1	0.3

An ethanol-producing strain, LB1, was used as a benchmark for growth and fermentation performance. Hydrolyzed and clarified material was inoculated, and growth and product formation monitored (Figure LIF-1.3). LB1 consumed 50 g/L glucose and produced 30 g/L ethanol and 4 g/L glycerol in 13 hours of fermentation in shake flasks (see Figure LIF-1.3). Other fermentable sugars like galactose/ mannose were not determined in this experiment but are expected to be present. The analytical methods required for detection and quantitation of other sugars and additional compounds present in the hydrolyzate are being implemented and validated.

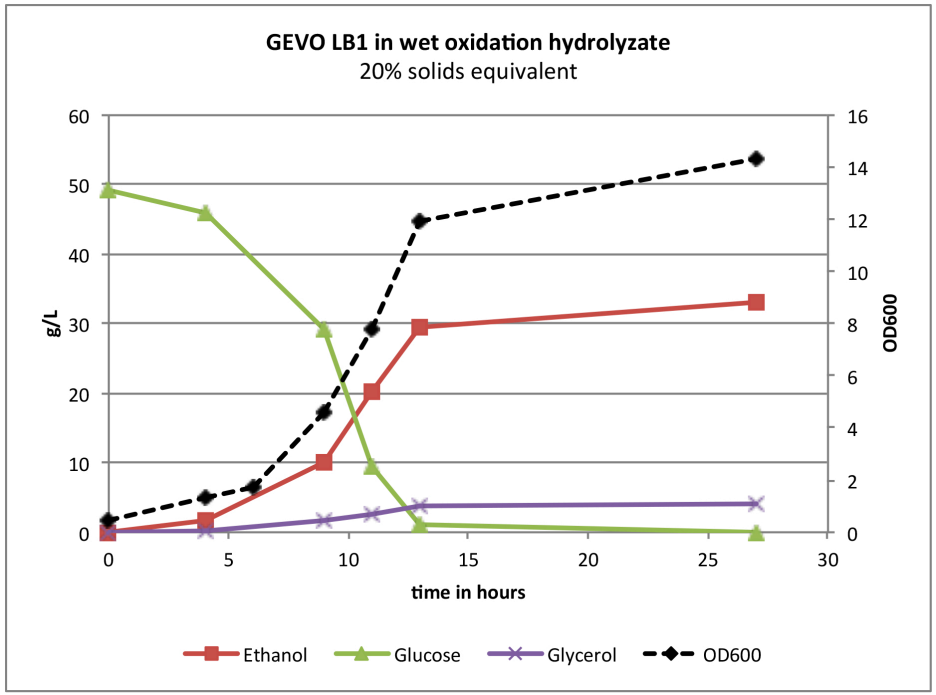

Figure LIF-1.3. Benchmark fermentation for wet oxidation hydrolyzate. The hydrolyzate was supplemented with a nutrient package, salts, and a buffering agent and was sterile-filtered. Fermentation was carried out by inoculating with LB1 (starting OD600 ~0.4) and incubating at 33°C. Fermentation progress was monitored by GC and HPLC analysis.

An un-evolved, unimproved isobutanol producing strain, GEVO LB2, was also used as a benchmark strain for growth in Wet Oxidation hydrolyzate samples diluted to a various extent with pure sugar streams (produced in lab with no inhibitors from sugar stock solutions). The growth and fermentation inhibition as compared to pure sugar solutions of the wet oxidation hydrolyzate for an isobutanol producing yeast (LB2) was tested by culturing the strain in increasing concentrations of wet oxidation hydrolyzate (0%, 20%, 40%, and 60%) in shake flasks. LB2 grew in all wet oxidation hydrolyzate concentrations tested (see Figure LIF-1.4). Growth rates at 0%, 20%, and 40% hydrolyzate were similar and slightly reduced at 60%. The biomass yield was similar for all concentrations with a 20% decrease in 60% hydrolyzate compared to the 0% control medium.

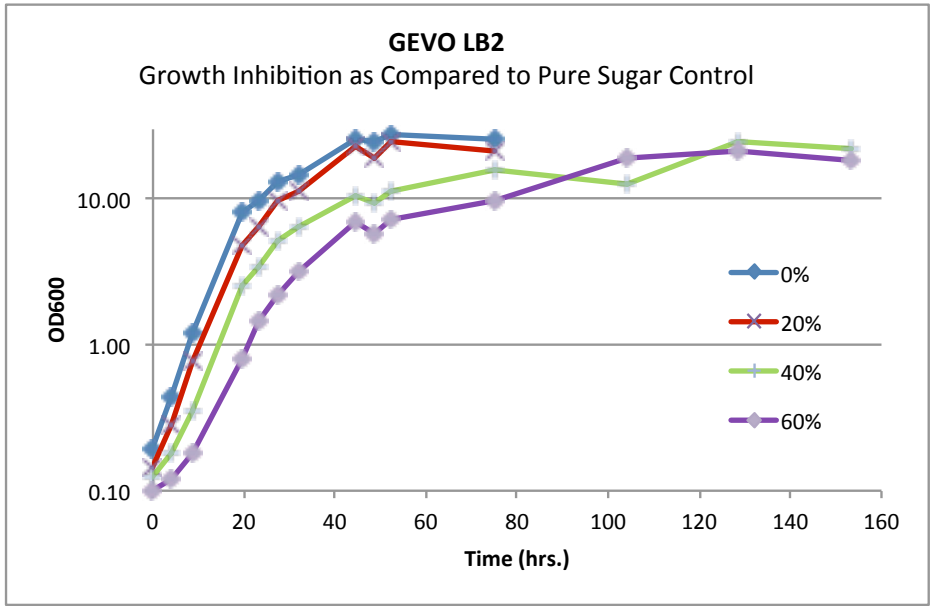

Figure LIF-1.4. Growth of isobutanol-producing strain in enzymatically hydrolyzed Wet Oxidation biomass in shake flask. The hydrolyzate was supplemented with a nutrient package, salts, and a buffering agent. Control medium contained equal amounts of supplements and 50 g/l glucose. Growth media were inoculated at 0.3 OD from preculture and incubated at 33°C. 0, 20, 40, and 60% v/v hydrolyzate concentrations were tested corresponding to 0, 5, 10, and 15% solids equivalent respectively. 0% represents the pure sugar control condition.

A second batch of wet oxidation-treated biomass was received from Dr. Birgitte Ahring's laboratory at Washington State University, Tri-Cities. The material was generated from Douglas fir forest residuals (NARA Feedstock FS-03). The material was received as partially clarified liquids. Larger particles were removed by Dr. Birgitte Ahring's lab using a screw press. Smaller particles were still present in the sample.

Table LIF-1.4. Pretreated Douglas fir biomass used during this trimester or expected in the future.

Pretreated Material	Hydrolyzed	Received	Comments
FS-10 Wet Oxidation (Batch A)	Yes	7/17/2013	Not optimized
FS-10 Wet Oxidation (Batch B)	Yes	11/19/2013	Batch will NOT be used for down selection evaluation
FS-10 Wet Oxidation (Batch C)	No	No	Batch will be evaluated in mini GIFT® systems for down selection

FS-01, FS-03, and FS-10 wet oxidation pretreated materials were from Dr. Birgitte Ahring at Washington State University–Tricities (Richland, WA), and two hydrolyzate types (Clean and Combined) of pretreated FS-03 material were from Catchlight Energy.

SPORL (*Sulfite Pretreatment to Overcome Recalcitrance of Lignocellulose*) and MBS (*mild bisulfite*) Pretreatment Methods

SPORL-pretreated biomass was received as washed solids with a solid content of about 22% (w/w). No free glucose or xylose was measured in the liquid fraction by high pressure liquid chromatography (HPLC) analytical methods employed at Gevo. Based on Gevo's understanding of the SPORL process, it was presumed that the solids represented available glucose only in the form of cellulose. Analysis by HPLC showed 0.6 g/L acetate was present in the SPORL material. To assess enzymatically available sugars in the material, the biomass was enzymatically hydrolyzed to completion (see Table LIF-1.5) using enzyme cocktails from Novozymes. The Cellic CTec2 enzyme cocktail from Novozymes were used. 61.2 g/L and 93.5 g/L glucose were released from 10% and 20% solids material, respectively, after 48 hours of hydrolysis.

Table LIF-1.5. Enzymatic hydrolysis test for SPORL pretreated biomass. Hydrolysis was done in sodium-acetate buffer (pH 5) at 50°C in a shake flask. 0.1 ml CTec2 (Novozymes) was added per gram glucan. Glucose levels were determined by a rapid assay using a YSI Biochemistry Analyzer. (https://www.ysi.com/products/biochemistry-analyzers).

Biomass (% solids)	Free Glucose 24h of digestion	Free Glucose 48h of digestion
10%	50.9 g/L	61.2 g/L
20%	74.5 g/L	93.5 g/L

Enzymatically hydrolyzed and clarified (insoluble solids removed) feedstock was inoculated, and growth and product formation was monitored (Figure LIF-1.5). 51 g/L ethanol and 4.3 g/L glycerol were produced in 12 hours. 97 g/L of glucose was consumed. Other fermentable sugars like galactose and mannose were not monitored in this experiment, but were expected to be present. The analytical methods required for detection and quantitation of other sugars and additional compounds present in hydrolyzate were being implemented and validated at Gevo at the time of this work.

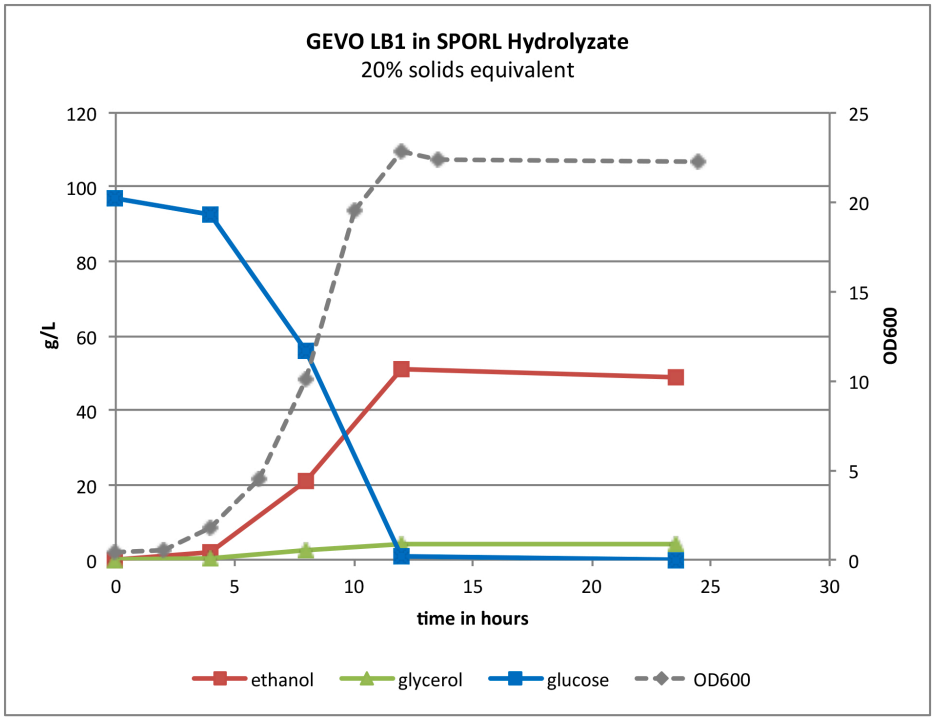

Figure LIF-1.5. Benchmark fermentation for SPORL pretreated Douglas fir pulp quality chip hydrolyzate. SPORL hydrolyzate (22% solids) was enzymatically hydrolyzed with ~0.1 ml CTec2 loading at 50°C in a shake flask (300 rpm). After hydrolysis was complete, the insoluble solids were removed by centrifugation and a nutrient package, salts, and a buffering agent were added. The medium was subsequently sterile-filtered. Fermentation was carried out by inoculating with LB1 (starting OD600 ~0.4) and incubating at 33°C. Fermentation progress was monitored by GC and HPLC analysis.

Growth and fermentation inhibition in hydrolysate, compared to pure sugar solution experiments in test tubes, showed growth of Gevo's isobutanol-producing yeast strain, named LB2, in enzymatically hydrolyzed and clarified material up to 20% solids equivalent (this was material as received). Growth was then monitored in shake flasks and Gevo strains grew as well or better in washed SPORL clarified hydrolyzate (20% solids equivalent) compared to a standard medium with similar sugar concentrations (see Figure LIF-1.6). While growth rates were similar, the overall biomass yield was reduced somewhat at the hydrolyzate levels tested. It is important to note that washed SPORL pre-treated material includes an extra step of water washing to remove inhibitory molecules. This may or may not be economically viable at a commercial scale. Thus, NARA partners agree that ideally, the project and process would use unwashed solids.

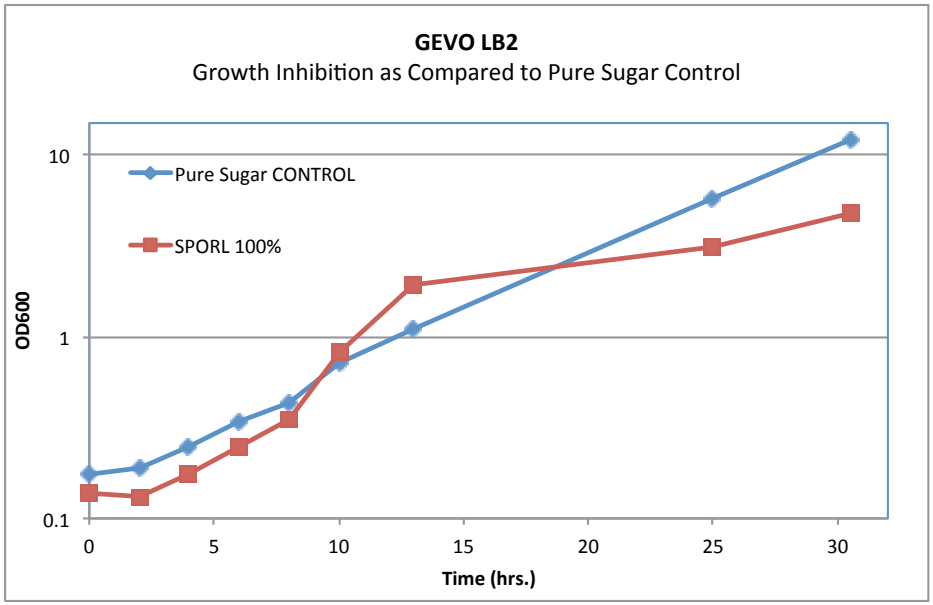

Figure LIF-1.6. Growth of a Gevo isobutanol-producing strain in enzymatically hydrolyzed SPORL biomass (here, washed solids to remove inhibitors) in shake flask fermentations. The hydrolyzate was supplemented with a nutrient package, salts, and a buffering agent. Control medium contained equal amounts of supplements and 100 g/L glucose. Growth media were inoculated from precultures and incubated at 33°C.

A second batch of SPORL-treated biomass was received from Dr. JY Zhu at the USDA Forest Products Laboratory (Madison, WI). The material was generated from Douglas fir forest residuals (NARA Feedstock FS-03). The pretreated biomass was received as unwashed solids (40% w/w) and a non-detoxified liquid hydrolyzate (liquor). Appearance of the pre-treated biomass was similar to the batch received earlier based on NARA FS-01 material (Figure LIF-1.7).

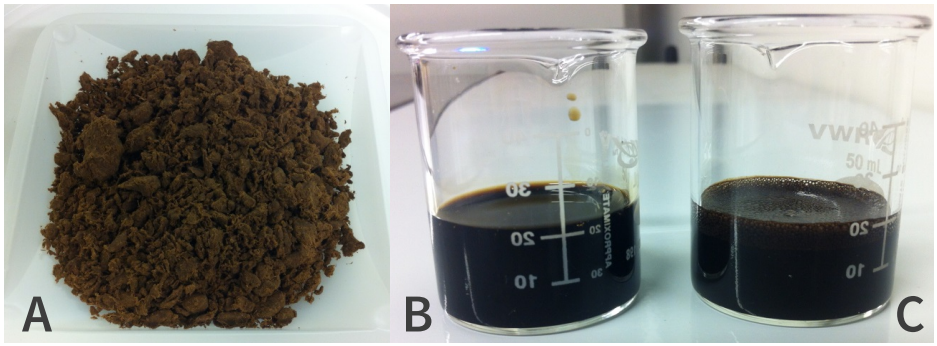

Figure LIF-1.7. Second batch of SPORL pretreated biomass as received by Gevo. Dr. JY Zhu and team at the USDA Forest Products Laboratory kindly provided the products of the SPORL pretreatment process as two separate streams: pressed solids (A) as shown in a large weigh boat and separate liquor stream (B). A portion of the two streams were combined (C) to achieve a slurry stream with approx. 20% solids that could be enzymatically hydrolyzed and used in subsequent fermentations.

The sugar concentration of the liquid stream was determined by high-pressure liquid chromatography (HPLC) analysis. Both streams were combined to generate a slurry stream with approx. 20% solids. The slurry was enzymatically hydrolyzed and then sugar concentrations in the hydrolyzed slurry were determined. The sugar concentrations were compared to the enzymatically hydrolyzed material from the second batch of SPORL pretreated material (NARA feedstock, FS-01). Results are shown in Table LIF-1.6.

Table LIF-1.6. Sugar concentrations in the liquid stream and slurry after enzymatic hydrolysis in shake flasks. FS-03 SPORL Liquor is the non-detoxified liquid stream as received, the first row indicates the compositional analysis as received from Dr. JY Zhu and the second row indicates the compositional analysis results done at Gevo. "FS-03 SPORL Slurry" is a slurry derived from mixing the received solids and liquor stream in equal amount (g/g). Enzymatic hydrolysis of the slurry was carried out in shake flasks at 50°C and pH 5 for 72h using Novozymes Cellic CTec3 (0.1ml/g glucan) and HTec3 (0.01ml/g glucan). Final sugar concentrations were determined by HPLC analysis. FS-01 SPORL Slurry data were obtained previously under identical conditions and are shown for comparison. N.d. = not detected.

	Cellobiose (g/l)	Glucose (g/l)	Xylose (g/l)	Galactose (g/l)	Arabinose (g/l)	Mannose (g/l)
FS-03 SPORL Liquor (Dr. Zhu's lab data)	n.d.	4.35	4.37	4.35	0.13	7.95
FS-03 SPORL Liquor (in house)	0.12	6.26	3.30	4.10	0.39	6.97
FS-03 SPORL Slurry	14.14	81.81	5.79	3.82	0.40	7.02
FS-01 SPORL Slurry	10.47	93.65	6.89	4.94	1.24	23.01

Growth inhibition experiments in test tubes showed that the Gevo's isobutanol-producing yeast strains were sensitive to the SPORL pretreated biomass hydrolyzate. A high-throughput microfermentation system (BioLector, m2p-labs) was used to screen the growth profiles and rates at different hydrolyzate percentages. A first experiment was set up to with one of Gevo's isobutanol producing strains (LB3) in the liquid hydrolyzate stream. Figure LIF-1.8 shows growth at 20-100% of SPORL derived liquid hydrolyzate.

Concentrations of all sugars were lower in FS-03 derived SPORL slurry than observed in previous batches of SPORL-pretreated FS-01 material. Data generated at Gevo corresponded well with the measurements in Dr. JY Zhu's lab. Noteworthy, the concentration of mannose is significant lower in FS-03 derived SPORL material (<7g/l) than seen in FS-01 derived SPORL material at >20 g/l. (Hawkins et al, 2013). After enzymatic hydrolysis of 20% pretreated material, concentrations of cellobiose, xylose, galactose and arabinose were similar between the two batches. As expected, mannose concentrations did not differ from the liquid stream and remained at ~7 g/l. Glucose concentrations of FS-03 derived SPORL material was ~87% compared to FS-01 derived SPORL material. This could be explained by a lower glucan concentration of FS-03 biomass compared to FS-01 biomass. Total sugar concentrations were still favorable, with 106.8 g/l and 6.2 g/l for hexoses and pentoses, respectively.

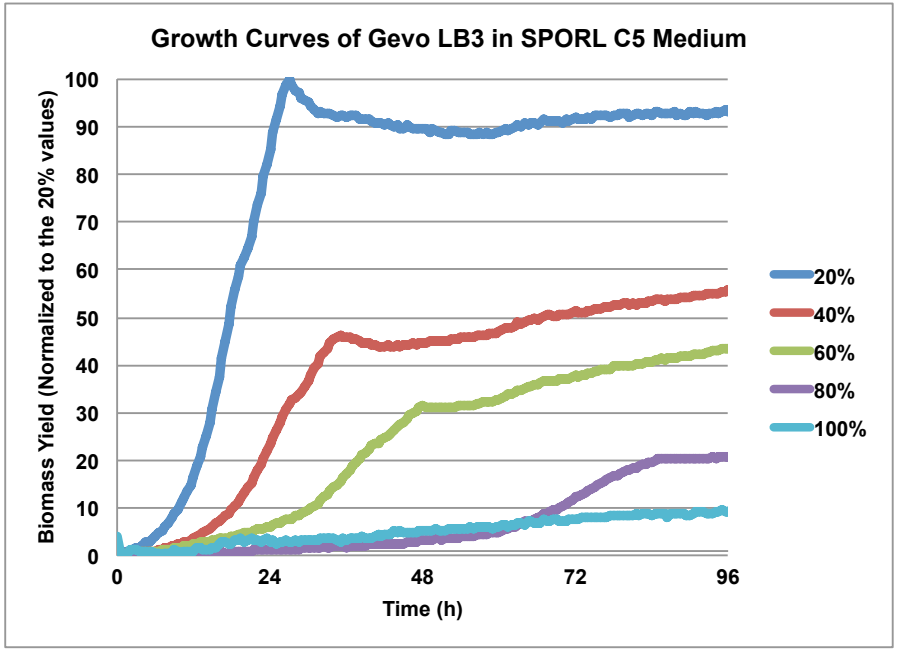

Figure LIF-1.8. Growth of Gevo LB3 (isobutanol-producing) strain in liquid hydrolyzate derived from SPORL pretreated biomass in a high-throughput microfermentation system (BioLector, m2p-labs). The liquid hydrolyzate stream (composition shown in Table LIF-1.6) was supplemented with a nutrient package, salts, and a buffering agent. Different percentages of SPORL C5 media contained equal amounts of corresponding sugars and supplements. Growth media were inoculated with Gevo LB3 and incubated at 33°C.

Gevo has worked to optimize the hydrolysis efficiency of the various pretreated feedstocks that have been received. Gevo partnered directly with Novozymes, who kindly provided the Cellic CTec2 and HTec2 and thereafter Cellic CTec3 and HTec3 enzymes for use in the NARA project. Novozymes also advised Gevo on temperatures, pH, and enzyme loading to use for the various pretreated feedstocks. Various solids loading and enzyme addition rates were explored in order to optimize hydrolysis efficiency.

Gevo received FS-10 SPORL material on October 22, 2014, and this was the first time material has been received as a combined liquor and solids material, 25% solids (w/w). In order to accommodate the combined liquor and solids sample, process conditions for hydrolysis needed to be adjusted. Hydrolysis was initiated using half the amount of recommended Novozymes Cellic CTec3 and HTec3 enzymes in 2L Fernbach flasks at 50° C incubators with agitation but without pH control until the material was slightly liquefied so that mixing would be possible in 2L fermenters (24-36 hours). The pH of the SPORL material (pH 2-3) is below the optimum pH range for enzyme activity (pH 5-6) so hydrolysis was completed in 2L fermenters at pH 6 with a final concentration of Novozymes Cellic CTec3 at 0.1ml/g glucan and HTec3 at 0.01ml/g glucan (48 hours). Dr. Zhu observed that enzymatic hydrolysis in lignosulfonate containing material is more efficient at pH 6 (Lou et al, 2013), and

the enzyme concentrations followed Dr. Zhu's recommendations. During previous hydrolysis efforts, Rushton impellers were used for mixing in the 2L bioreactors but problems with mixing were observed. To achieve better mixing, marine type impellers were utilized and found to work more efficiently. The marine-type impellers were used in all hydrolysis work thereafter.

Comparison of SPORL/MBS and WO Pretreatment Methods

Growth performance of the current best isobutanol producing biocatalyst (LB4) was compared in different pretreated hydrolyzates using the NARA feedstock FS-03 and FS-10. All of the WO and SPORL/MBS pretreated hydrolyzates were clarified and supplemented with a nutrient package prior to the experiments. The comparative growth of Gevo strain LB4 is show below in each of these different pretreated feed-stocks (Figure LIF-1.9).

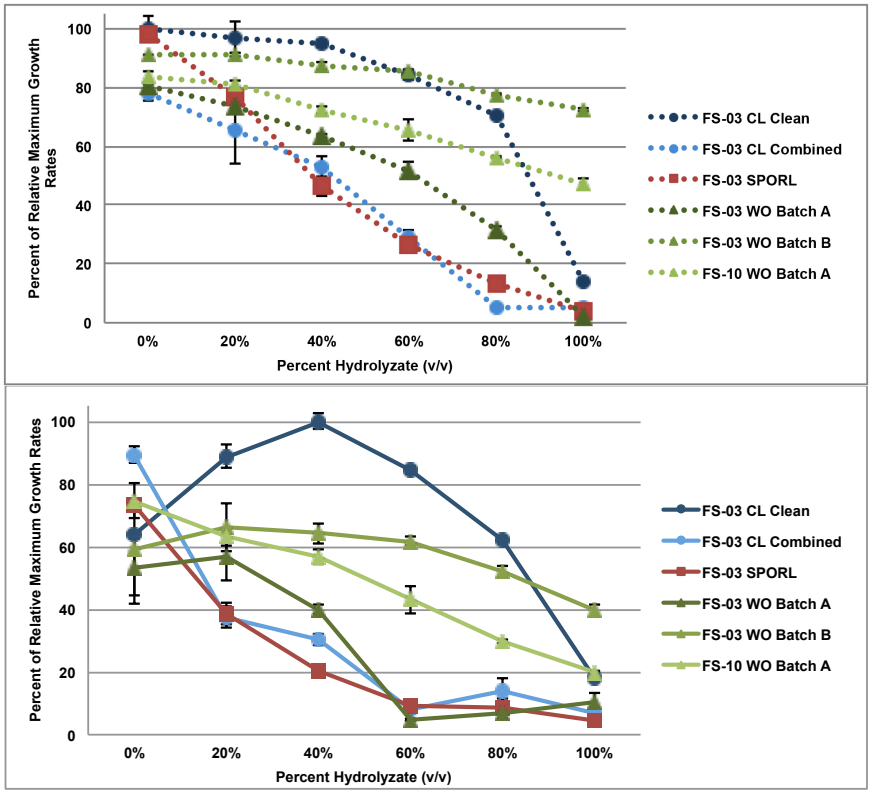

Figure LIF-1.9. High throughput growth screening showing the percent of relative growth rates of the benchmark ethanol producing biocatalyst LB1 (Top) and the current best performing hydrolyzate adapted biocatalyst, LB4 (Bottom). Growth rate data was obtained using a high-throughput microfermentation system (BioLector, m2p-labs). All hydrolyzates were clarified to remove solids and all were supplemented with a nutrient package, salts, and a buffering agent. The different percentages of hydrolyzate and mock media contained equal amounts of corresponding sugars, acetate, and supplements. 100% (v/v) hydrolyzate is equal to approximately 30-36% equivalent solids for all biomass materials. Error bars represent one standard deviation of replicates.

After analysis, characterization of new feedstock hydrolyzates occured through benchmarking growth and fermentation using an isobutanol producing biocatalyst. A review of hydrolyzate samples tested in the shake flask benchmarking process is presented in Figure LIF-1.10. All of the pretreated hydrolyzate media were clarified and supplemented with a nutrient package prior to the experiments. Growth and fermentation performance were compared in batch shake flask fermentations using the current best isobutanol producing hydrolysate-adapted biocatalyst (LB4) in different hydrolyzates from NARA feedstock FS-03 and FS-10.

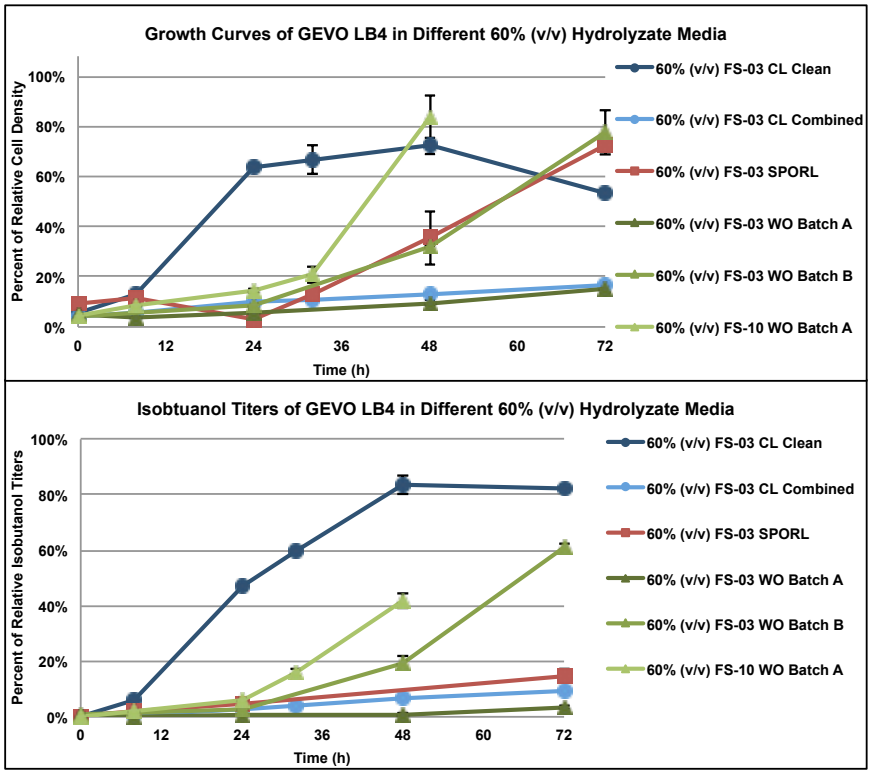

Figure LIF-1.10. Relative growth (Top) and relative isobutanol titers (Bottom) using the current best hydrolyzate adapted biocatalyst (LB4) in 60% (v/v) hydrolyzates derived from WO, SPORL, and Catchlight Energy pretreated materials (FS-03 and FS-10) in shake flask fermentations. Data was combined from multiple experiments. All hydrolyzates were clarified to remove solids and were supplemented with a nutrient package, salts, and a buffering agent. The 60% (v/v) mixtures have sugars and acetate equivalent to 100% of the hydrolyzate. 100% hydrolyzate is equal to approximately 30-36% equivalent solids for all biomass materials. Fermentation was carried out at 33°C. Cell density was measured using a spectrophotometer and isobutanol levels were determined by GC analysis. Error bars represent the standard deviation. Abbreviations: WO, wet oxidation; SPORL, sulfite pretreatment to overcome recalcitrance of lignocellulose; CL, Catchlight Energy.

Milled Wood Pretreatment Method

The growth and isobutanol production data using the Concentrated Milled Wood hydrolysate (Table LIF-1.7) showed excellent results using the 100% hydrolyzate. Growth of LB4 in the 100% hydrolyzate media was only slightly lower than the mock (control) media and isobutanol production was comparable with higher maximum titers (Figure LIF-1.11). Based on these results, additional testing should be performed with Concentrated Milled Wood hydrolyzates if the process is economical and scalable.

Table LIF-1.7. Pretreated Douglas fir biomass used during the NARA Year 2, first trimester.

Pretreated Material	Hydrolyzed	Received	Comments
FS-10 Milled Wood	Yes	11/25/2013	First batch for small scale evaluation

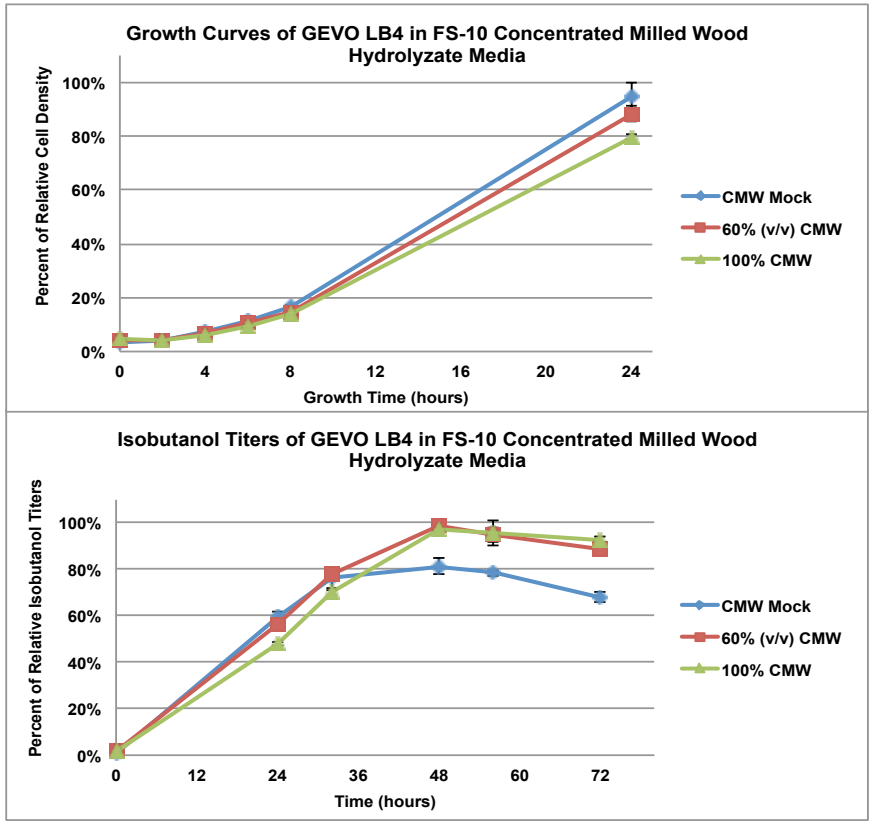

Figure LIF-1.11. Relative growth (Top) and relative isobutanol titers (Bottom) of the current best hydrolyzate adapted biocatalyst (LB4) in mock, 60% (v/v), and 100% hydrolyzates derived from FS-10 Concentrated Milled Wood in shake flask fermentations. The material was received as a clarified hydrolyzate and all concentrations were supplemented with an equivalent nutrient package, salts, and a buffering agent. All media contained equal amounts of corresponding sugars and acetate. Fermentation was carried out at 33°C. Cell density was measured using a spectrophotometer and isobutanol levels were determined by GC analysis. Error bars represent the standard deviation. Abbreviations: CMW, Concentrated Milled Wood.

1,000 gal IPK Task - Scale-Up Feedstock Hydrolysis Characterization

During Year-5 of NARA, Gevo focused on characterizing material that will be used in the 1,000 gallon IPK pilot trial. Gevo received FS-10 SPORL material that was pretreated with [SO_2 + $Mg(OH)_2$] and with [MgBs + H_2SO_4] from Dr. Junyong Zhu at the USDA Forest Products Laboratory (FPL). Hydrolysis of the material was carried out, and the performance of the yeast biocatalyst in the hydrolysates was evaluated in 1L GIFT® fermentations. Gevo received pretreated FS-20 material from Andritz demonstration runs (35 minute and 45 minute pretreatment times), which subse-quently pressed and washed. The pressed and washed material was then hydro-lyzed and evaluated in 1L GIFT® fermentations using next generation biocatalyst and commercial nutrient package. In addition, Gevo has also provided fermenta-tion support for the 1000 gallon demonstration at ICM and has provided analysis of isobutanol produced at ICM.

Feedstock Analysis

Gevo analyzed several feedstocks for use in the 1,000 gal IPK scale-up task. Below, analyses and physical images show properties (such as chip size, color, moisture, etc.) for each of these feedstocks. The NARA NR-03 material was the third batch of pretreated material using NARA feedstock, FS-20, at ZeaChem, Inc. in Boardman, Oregon (Figure LIF-1.12, LIF-1.13, LIF-1.14. LIF-1.15). While the majority of the feedstock used in the 1,000 gal IPK task within NARA was planned to be FS-20 feedstock pretreated by ZeaChem, Inc., additional feedstock from Cosmo Specialty Fibers (Cosmopolis, Washington) was also made available for laboratory characterization and testing. The Cosmo material was process reject fibers of western hemlock, a softwood grown in the Pacific Northwest (Figure LIF-1.16). The fate of these reject fibers at the Cosmo mill would be to fuel a steam boiler. Thus, generating other products like isobutanol and biojet from the sugars within those fibers would add value.

Figure LIF-1.12. Gevo received 2x5 gallon buckets of NR-03 material pretreated at ZeaChem, Inc.

Figure LIF-1.13. FS-20 Feedstock (left) compared to FS-01 Pulp Quality Chips (right) prior to pretreatment and hydrolysis.

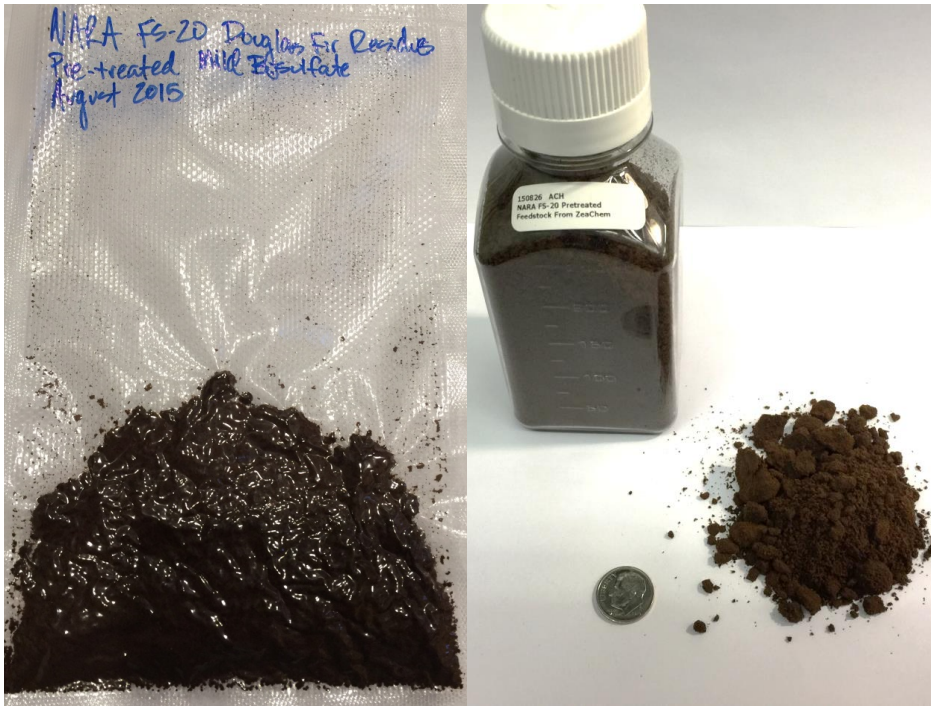

Figure LIF-1.14. Pretreated FS-20 feedstock, Lot NR-03 FP#5, Supersack B as received and used in hydrolysis.

Figure LIF-1.16. Cosmo Reject fibers, which have been pretreated by Cosmo Specialty Fibers, Inc. (left) compared to untreated FS-20 NARA Feedstock (right).

Figure LIF-1.16. Cosmo Reject fibers, which have been pretreated by Cosmo Specialty Fibers, Inc. (left) compared to untreated FS-20 NARA Feedstock (right).

Hydrolysis

Gevo completed several rounds of hydrolysis with these materials. The solids loading during hydrolysis was decreased in order to increase hydrolysis yield. During scale up and several lab experiments, hydrolyzed sugars were concentrated by evaporation in order reach a desired sugar concentration (Figure LIF-1.17). Hydrolysis was carried out under controlled conditions in 2L DasGip fermentation vessels fitted with three marine impellers and top drive motors. A photo of the setup is shown in Figure LIF-1.18.

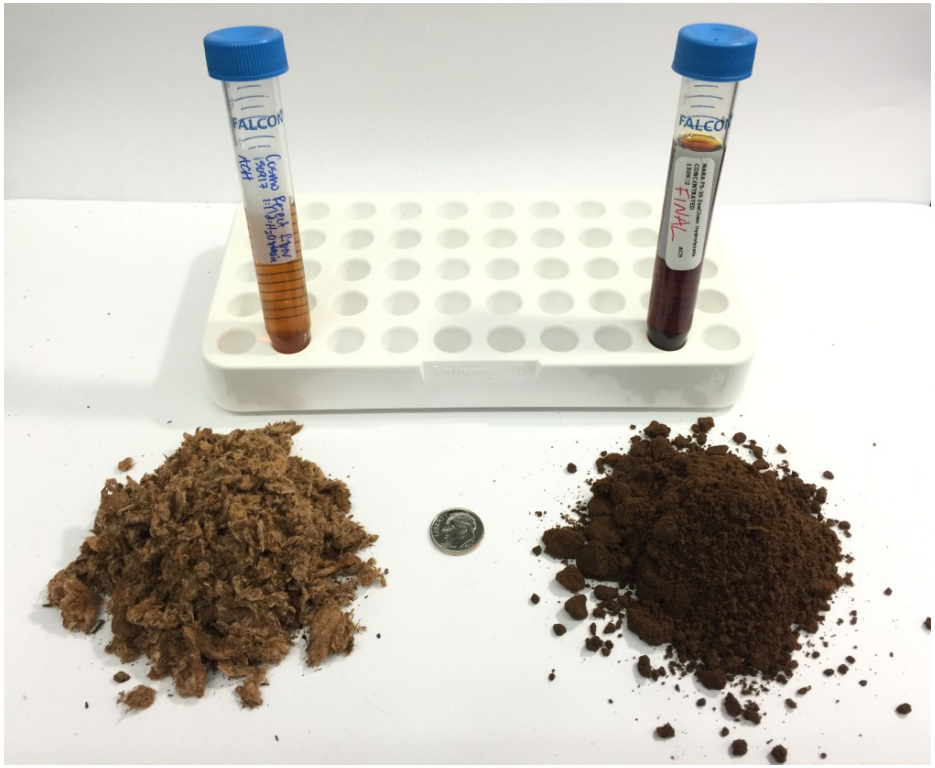

Figure LIF-1.17. Pretreated feedstocks used and their corresponding hydrolyzates. Left, Cosmo Reject Fibers (front) and the resulting filtered and concentrated hydrolyzate used in fermentations (rear). Right, NARA feedstock FS-20 NR-03 pretreated at ZeaChem (front) and the resulting filtered and concentrated hydrolyzate used in fermentations (rear).

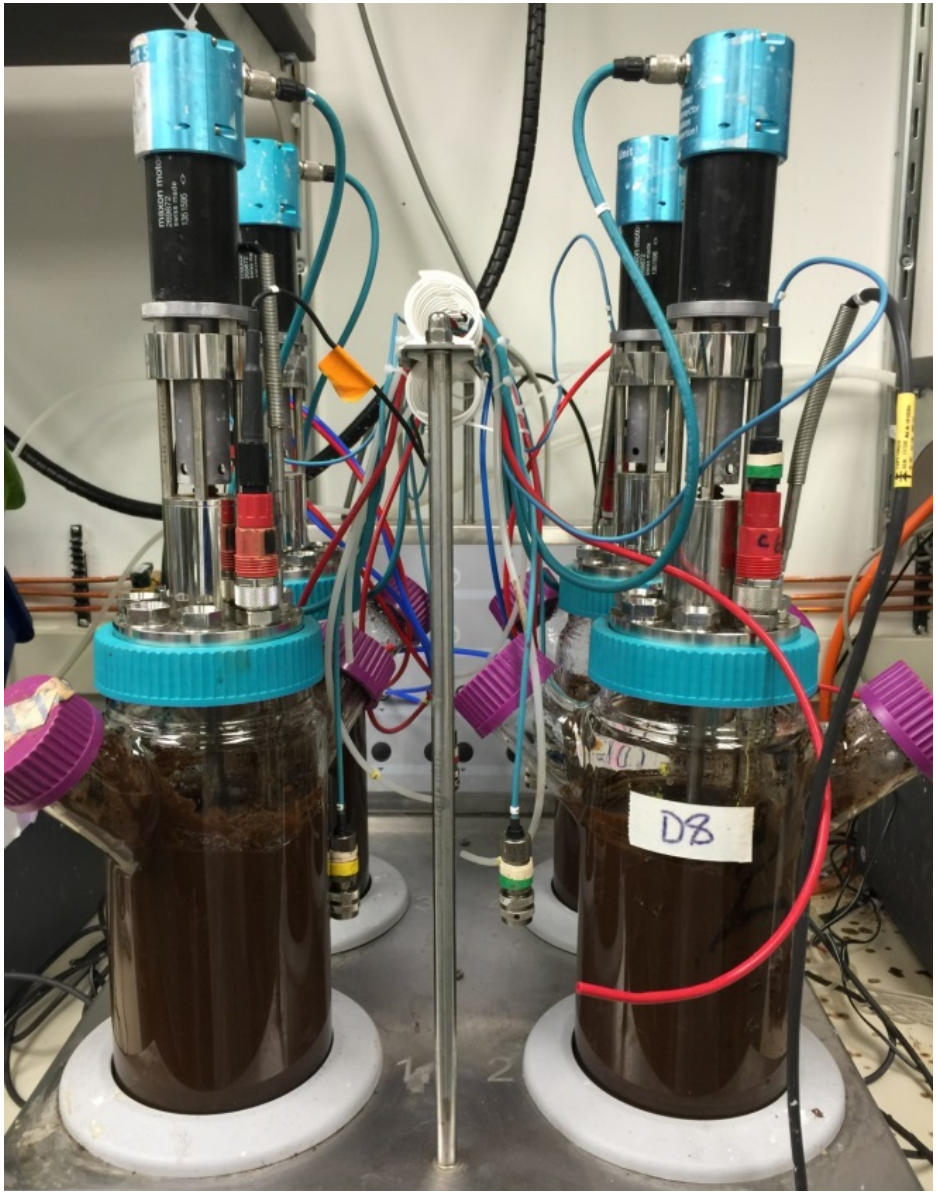

Figure LIF-1.18. 2L nominal volume DasGip bench-scale fermenters used for hydrolysis. The fermenters have top-drive motors with agitator shafts fitted with three marine-style impellers for superior mixing during hydrolysis.

Several batches of various feedstocks were hydrolyzed in the described fermenters shown in Figure LIF-1.18. A more detailed description of the hydrolysis conditions and feedstock used follows.

Hydrolyzate Batch 1 150907:

- NARA FS-20 NR-03 ZeaChem pretreated feedstock
- 18.7% solids at end of hydrolysis
- 0.08 g CTec3/g glucan and 0.008 g HTec3/g glucan used (June 2014 enzyme date)
- 73 g/L Free glucose at end of hydrolysis
- 47.33% glucan (w/w)
    - 187 g/L biomass * 0.4733 g glucan/ g biomass = 88.5 g glucan /L
    - 88.5 g glucan/L *1.11 = 98.24 theoretical glucose
- 73 g/L glucose / 98.24 theoretical = 75% yield.
    - Hydrolysis free glucose concentrations and yields based on HPLC data

Hydrolyzate Batch 2 150928:

- NARA FS-20 NR-03 ZeaChem pretreated feedstock
- 12% solids in the hydrolysis reaction
- 0.12 g CTec3/g glucan and 0.008 g HTec3/g glucan used (June 2014 enzyme date)
- Method used:
    - Sterilize 2 bench fermenters with pH probe and thermo well only. Plug all other head plate ports. Combine water and 200 grams of wet solids to vessels and connect to bioblock.
    - Heat vessels to 50°C and add the remaining wet solids via the side arm port. When all solids are added and temperature has reached set point, adjust pH to 5.0 with NaOH pellets.
    - When vessel is at target temperature (50°C) and pH (5), add enzymes to vessel. Adjust pH manually with NaOH pellets when needed.
    - Sample periodically for YSI glucose. After YSI glucose, boil native sample to inactivate CTec3/HTec3 enzymes, then store in Analytical submission plate in fridge for LC12 submission.
- Hydrolysis parameters are given in Table LIF-1.8. and glucose production is shown in Figure LIF-1.19.

Table LIF-1.8. Hydrolysis parameters of ZeaChem pretreated solids #NR03 from hydrolyzed batch 150928.

150928	Hydrolysis of Zeachem pretreated solids #NR03	
Target solids concentration:	12	% dry basis
Hydrolysis volume:	1500	mL
% dry solids:	39	%
C-tec enzyme dosing:	0.08	g/g glucan
H-tec enzyme dosing:	0.008	g/g glucan
Glucan concentration:	47.33	% w/w
Solids loading:	180	grams dry basis
Glucan loading:	85.2	grams dry basis
Solids loading:	462	grams wet basis
Water fraction from solids:	282	grams or mLs
Water addition:	1218	grams or mLs
C-tec dosage:	5.9	mL
H-tec dosage:	0.59	mL
Theoretical glucose conc.:	63.04	g/L

		YSI glucose		hydrolysis rate		hydrolysis yield		pH		Comments
		B5	B6	B5	B6	B5	B6	B5	B6	
Date/Time	Hours	g/L	g/L	g/L-h	g/L-h	% of theoretical				
9/28/2015 17:10	0	1.6	1.6					5.15	4.91	added 5.9 mL CTec3+0.59 mL HTec3 to each vessel
9/29/2015 7:40	14.5	44	42	2.93	2.79	69.8%	66.6%	4.8	4.76	added 1 pellet NaOH/ferm; adjusted agitation from 500->400 RPM
9/29/2015 11:40	18.5	46	44	0.50	0.38	73.0%	69.0%	4.9	4.88	
9/29/2015 15:10	22	46	45	0.00	0.29	73.0%	70.6%	4.89	4.85	
9/29/2015 17:10	24	47	44	0.50	-0.25	74.6%	69.8%	5.03	4.99	added 1 pellet NaOH/ferm; Added 2.5 mL of CTec3/vessel
9/30/2015 8:10	39	54	52	0.47	0.53	85.7%	82.5%	4.89	4.86	
9/30/2015 11:10	42	55.5	54.5	0.50	0.83	88.0%	86.4%	5.05	4.99	added 1 pellet NaOH/ferm 5 min before sampling
9/30/2015 15:10	46	56	54.5	0.13	0.00	88.8%	86.4%	4.98	4.94	

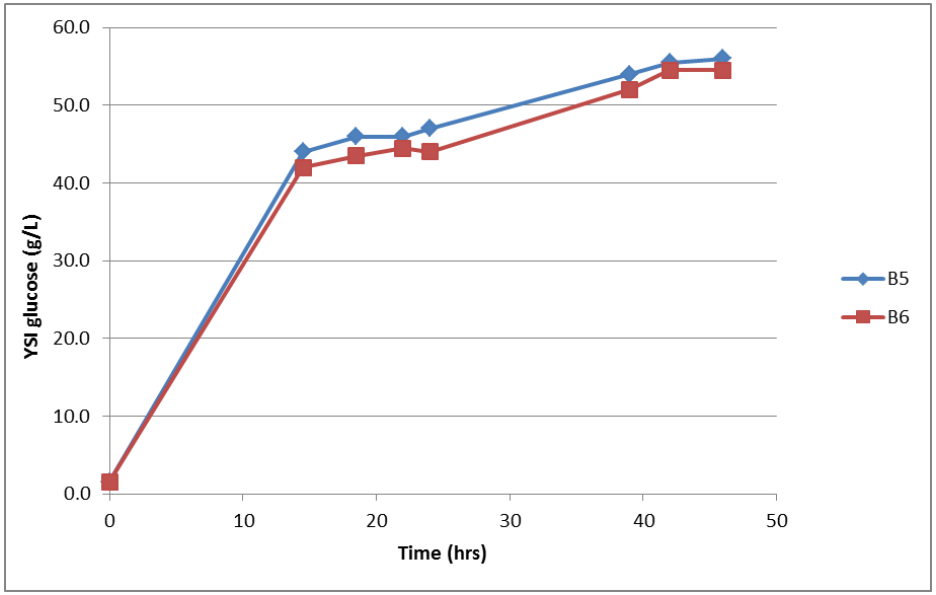

Figure LIF-1.19. Time course of free glucose during the 150928 hydrolysis as measured by YSI.

Hydrolyzate Batch 3 150930:

- NARA FS-20 NR-03 ZeaChem pretreated feedstock
- 15% solids in the hydrolysis reaction
- 0.24 g CTec3/g glucan and 0.008 g HTec3/g glucan used (June 2014 enzyme date)
- Method used:
    - Sterilize 2 bench fermenters with pH probe and thermo well only. Plug all other head plate ports. Combine water and 200 grams of wet solids to vessels and connect to bioblock.
    - Heat vessels to 50°C and add the remaining wet solids via the side arm port. When all solids are added and temperature has reached set point, adjust pH to 5.0 with NaOH pellets.
    - When vessel is at target temperature (50°C) and pH (5), add enzymes to vessel. Adjust pH manually with NaOH pellets when needed.
    - Sample periodically for YSI glucose. After YSI glucose, boil native sample to inactivate CTec3/HTec3 enzymes, then store in Analytical submission plate in fridge for LC12 submission.
- Hydrolysis parameters are given in Table LIF-1.9., and glucose production is shown in Figure LIF-1.20.

Table LIF-1.9. Hydrolysis parameters of ZeaChem pretreated solids #NR03 from hydrolyzed batch 150930.

Target solids concentration:	15	% dry basis
Hydrolysis volume:	1500	mL
% dry solids:	39	%
C-tec enzyme dosing:	0.112	g/g glucan
H-tec enzyme dosing:	0.008	g/g glucan
Glucan concentration:	47.33	% w/w
Solids loading:	225	grams dry basis
Glucan loading:	106.5	grams dry basis
Solids loading:	577	grams wet basis
Water fraction from solids:	352	grams or mLs
Water addition:	1148	grams or mLs
C-tec dosage:	10.4	mL
H-tec dosage:	0.74	mL
Theoretical glucose conc.:	78.80	g/L

			YSI glucose				hydrolysis rate				hydrolysis yield				pH				Comments
			B5	B6	B7	B8	B5	B6	B7	B8	B5	B6	B7	B8	B5	B6	B7	B8	
Init.	Date/Time	Hours	g/L	g/L	g/L	g/L	g/L-h	g/L-h	g/L-h	g/L-h	% of theoretical								
KWE	9/30/2015 18:15	0	1.8	1.8	1.8	1.8									5.03	4.92	5.04	4.92	
ACH	10/1/2015 9:00	14.75	50	51	52	57	3.27	3.34	3.37	3.74	63.4%	64.7%	65.4%	72.3%	5.12	4.44	4.47	4.8	Added 6 NaOH pellets to C6 & C7 because of lower pH; Added 2 NaOH pellets to C8; reduced agitation 700-->500
KWE	10/1/2015 12:15	18	54	53	55	60	1.23	0.46	1.08	0.77	68.5%	66.6%	69.8%	75.5%	5.1	4.94	4.98	4.98	Added 2.5 mL of additional C-tec per vessel
KWE	10/1/2015 16:15	22	59	59	59	64	1.25	1.62	1.00	1.12	74.9%	74.9%	74.9%	81.2%	5.06	4.89	4.93	4.96	Added 1 pellet NaOH
KWE	10/2/2015 8:00	37.75	70	69	69	72	0.70	0.63	0.60	0.48	88.8%	87.6%	86.9%	90.7%	5.1	4.88	4.96	4.94	
KWE	10/2/2015 11:30	41.25	70	69	68	75	0.00	0.00	-0.29	1.00	88.8%	87.6%	85.7%	95.2%	5.07	4.86	4.94	4.91	

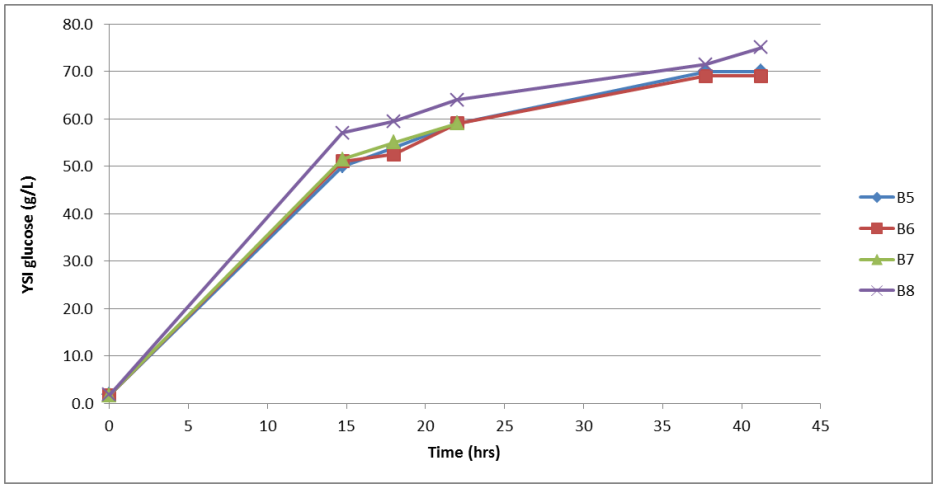

Figure LIF-1.20. Time course of free glucose during the 150930 hydrolysis as measured by YSI.

Cosmo Reject Fiber Hydrolysis 151020:

- Cosmo reject fibers, Cosmo Specialty Fibers, Cosmopolis, WA
- Fibers hydrolyzed using same methods as described above
- 13.69% solids in the hydrolysis reaction
- 0.24 g CTec3/g glucan and 0.008 g HTec3/g glucan used (June 2014 enzyme date)
- Hydrolysis resulted in 49 g/L free glucose (based on LC12 HPLC method) in 48 hours
    - 62% hydrolysis yield based on theoretical glucose concentration
    - Hydrolysis parameters are given in Table LIF-1.10., and glucose production is shown in Figure LIF-1.21.

Table LIF-1.10. Hydrolysis parameters of Cosmo pretreated solids from hydrolyzed batch 151020.

Hydrolysis of Cosmo Fibers		
Target solids concentration:	13.69	% dry basis
Hydrolysis volume:	1500	mL
% dry solids:	49.32	%
C-tec enzyme dosing:	0.112	g/g glucan
H-tec enzyme dosing:	0.008	g/g glucan
Glucan concentration:	55	% w/w
Solids loading:	205.35	grams dry basis
Glucan loading:	112.9	grams dry basis
Solids loading:	416	grams wet basis
Water fraction from solids:	211	grams or mLs
Water addition:	1289	grams or mLs
C-tec dosage:	10.6	mL
H-tec dosage:	0.76	mL
Theoretical glucose conc.:	83.58	g/L

		YSI glucose		hydrolysis rate		hydrolysis yield		pH		Comments
		C4	C5	C4	C5	C4	C5	C4	C5	
Date/Time	Hours	g/L	g/L	g/L-h	g/L-h	% of theoretical				
10/20/2015 16:00	0	1.2	1.1					5.03	4.92	
10/21/2015 9:00	17	42	44	2.42	2.50	50.7%	52.2%	4.86	4.88	
10/21/2015 15:00	23	54	52	1.87	1.47	64.1%	62.7%	5.12	5.05	
10/22/2015 10:00	42	62	59	0.42	0.35	73.6%	70.6%			
10/22/2015 16:00	48	61	58	0.30	0.22	73.0%	69.4%	5.1	5.04	13.69% solids
10/23/2015 9:00	65	62	60	0.06	0.11	74.2%	71.7%	5.05	5.07	
LC12 Analysis		50	49			59.8%	58.6%			LC12 Data

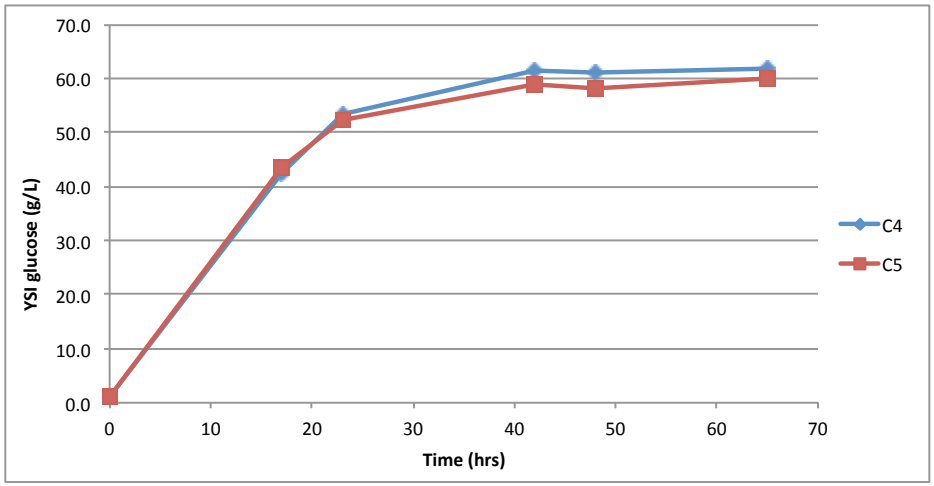

Figure LIF-1.21. Time course of free glucose during the 151020 hydrolysis as measured by YSI.

TASK 2: ADAPT YEAST BIOCATALYST TO PRETREATED BIOMASS HYDROLYZATE

The objective of this task was to take "virgin" isobutanol producing yeast that had been engineered at Gevo outside of the NARA project and for different, non-lignocellulosic feedstocks, and adapt them to grow in and produce isobutanol in hydrolyzate. Based on the data collected within (Task 1: Characterize hydrolyzate and complete benchmarking of pre-treated biomass for fermentation into isobutanol), each hydrolyzate was unique (FS-01/FS-03/FS-10, batch-to-batch, various pre-treatment methods) and a given isobutanol strain must be adapted to optimally tolerate the inhibitors present in and physical parameters of these unique hydrolyzates. Adapted yeast have some cross over benefits from one hydrolyzate type to another, meaning that a strain adapted to wet oxidation hydrolyzate performs better in SPORL hydrolyzate as compared to a virgin strain, and vice versa. However, it was clear that to achieve the goals of the NARA project, which were to produce 1,000 gal of IPK and move toward a commercially-viable process, a given isobutanol strain must be adapted to each specific hydrolyzate used in the NARA process. As the NARA project progressed, and pretreatment methods and feedstocks were down-selected to SPORL/MBS and Douglas-fir forest residuals and Cosmo Reject Fibers, evolution and adaptation migrated to using only those pretreatment methods/feedstocks.

Strain adaptation by serial transfers

Gevo's isobutanol producing biocatalyst LB3 was used as a starting strain for evolutionary engineering by serial transfers in rising concentrations of biomass hydrolyzate. Previous characterization had shown that Gevo LB3 was a superior isobutanol producing strain to Gevo LB2. As Gevo made progress on its isobutanol producing biocatalysts for its separate commercial purposes, the NARA project leveraged these improved strains and used them in laboratory and scale-up work.

A 96-well microplate protocol was developed to increase the number of independent cultures used in the adaptation process. A typical plate layout is shown in Figure LIF-2.1. The plate was inoculated with Gevo LB3 and incubated at 300 rpm in a microplate shaker (Minitron, Infors HT). Cell density was monitored by optical density, spectrophotometer (OD_{600}) and the cultures were continuously transferred to fresh media by dilution at certain time intervals.

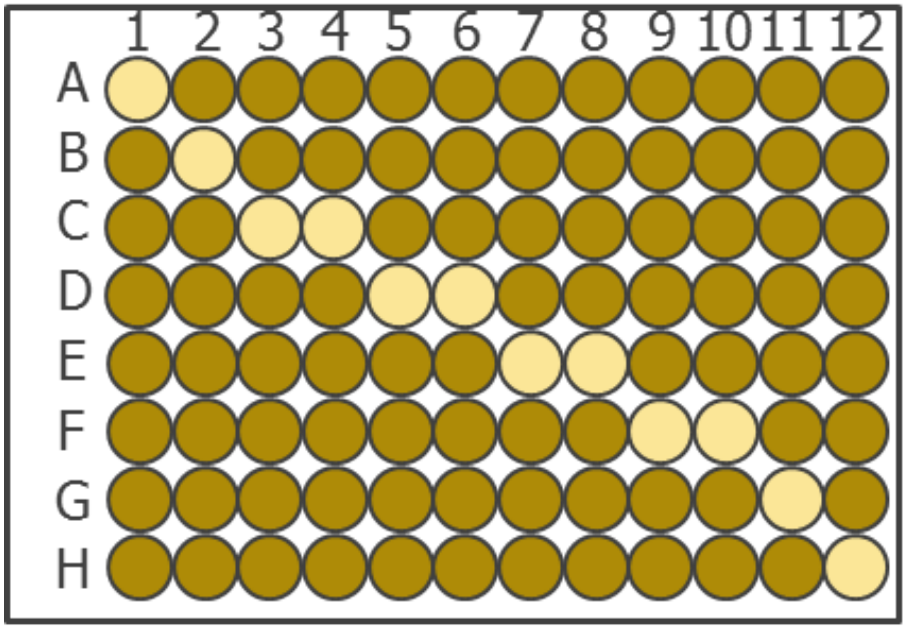

Figure LIF-2.1. Plate layout for strain adaptation. Gevo's isobutanol-producing yeast LB3 was used for serial transfers in 96-deepwell plates to improve growth within and tolerance of wet oxidation biomass hydrolyzate. 300 ul of clarified wet oxidation pretreated biomass hydrolyzate (50% v/v) was inoculated with Gevo LB3 (dark wells) or left uninoculated as negative control (light wells).

Wet Oxidation Hydrolyzate

The wet oxidation material used in the first adaptation experiments was described in detail in the previous section (Task 1: Characterize hydrolyzate and complete benchmarking of pre-treated biomass for fermentation into isobutanol) and is derived from pulp quality Douglas-fir wood chips (NARA feedstock FS-01). For the first adaptation experiments, all material was clarified and supplemented with a nutrient package prior to the experiments.

Improved growth was indicated by increased optical density. In this system, growth improvements were hypothesized to predict improved tolerance to the material. Candidate isolates for improvement were screened first in a high-throughput micro-fermentation system (BioLector, m2p-labs) which allowed online monitoring of growth performance. Figure LIF-2.2 shows a comparison of the parental strain LB3 to a previously adapted strain, LB4 and a newly identified candidate strain, LB5. While the parental strain LB3 does not grow at all in 80% (v/v) or 100% (v/v) of clarified biomass hydrolyzate (25% solids equivalent), both candidate strains

LB4 and LB5 derived from the adaptation process showed growth under these conditions.

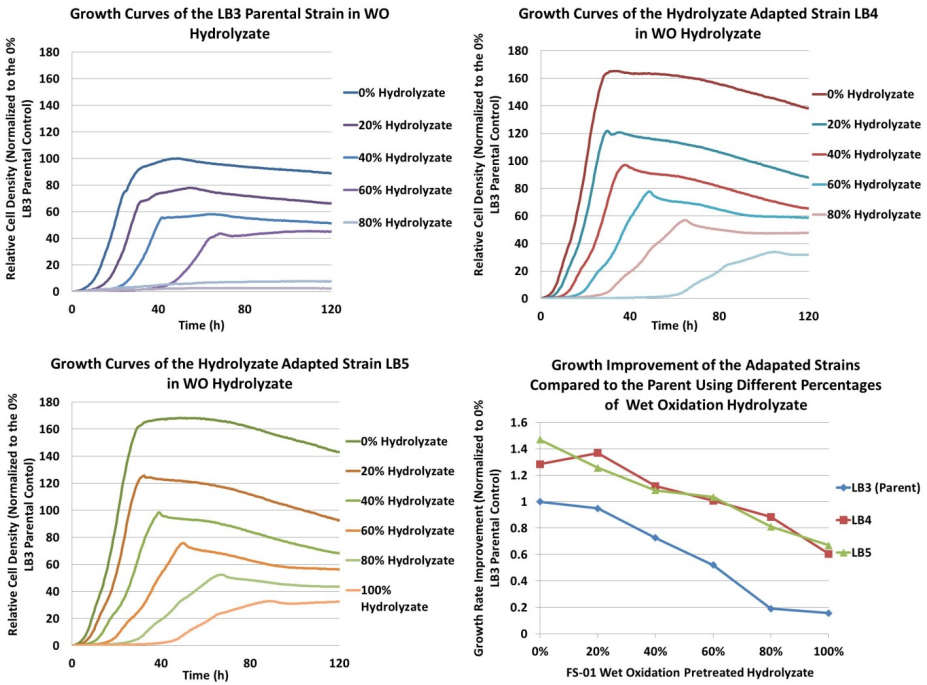

Figure LIF-2.2. Growth screening of wet oxidation pretreated hydrolyzate adapted strains using different percentages of FS-01 wet oxidation pretreated hydrolyzate. Growth of the isobutanol-producing parental strain LB3 (A), adapted strain LB4 (B), and adapted strain LB5 (C) were measured using a high-throughput microfermentation system (BioLector, m2p-labs). Relative cell densities in graphs A-C were normalized to the 0% hydrolyzate control. Graph D shows relative growth rates plotted against the different wet oxidation hydrolyzate concentrations. Values were normalized to the parental control (LB3) grown in 0% hydrolyzate. The clarified FS-01 wet oxidation pretreated hydrolyzate was supplemented with a nutrient package, salts, and a buffering agent. Different percentages of hydrolyzate media contained equal amounts of corresponding sugars and supplements. 100% (v/v) wet oxidation hydrolyzate was equal to 25% equivalent solids.

Growth and isobutanol production was tested for strain Gevo LB5 in a single-phase fermentation in clarified hydrolyzate derived from wet oxidation pretreated biomass (see Figure LIF-2.3). Gevo LB5 grew slower under these conditions than seen in the Biolector system. Growth and isobutanol production in this strain were correlated and more isobutanol was produced at 60% (v/v) hydrolyzate than at 80% (v/v). Little isobutanol production was observed at 100% (v/v) hydrolyzate. Comparatively, Gevo LB5 strain grew similarly to Gevo LB4, a strain developed using the same evolutionary strategy. However, comparison of Gevo LB4 and LB5 under isobutanol fermentation conditions indicated that Gevo LB5 did not perform as well as Gevo LB4 in wet oxidation material. This difference exemplifies the diversity of strain phenotypes possible when evolutionary engineering strategies are employed to adapt strains to new biomass materials.

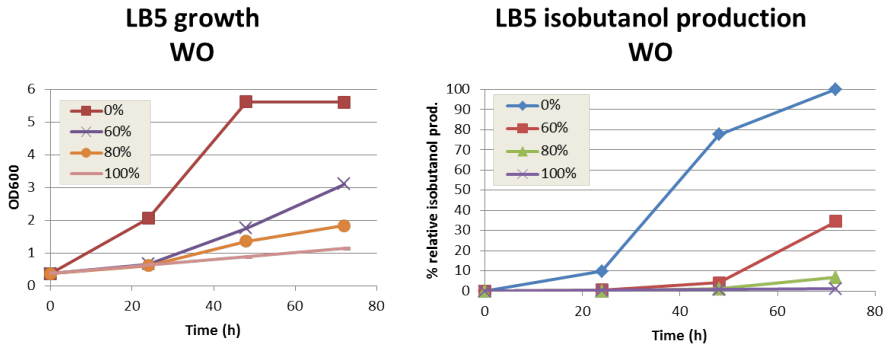

Figure LIF-2.3. Growth and isobutanol production by LB5 in clarified liquid hydrolyzate derived from wet oxidation pretreated biomass (FS-01) in batch fermentation. The liquid hydrolyzate stream was supplemented with a nutrient package, salts, and a buffering agent. Control medium contained equal amounts of corresponding sugars and supplements. 60% and 80% fermentation media was prepared by mixing corresponding control and liquid hydrolyzate stream (v/v). Fermentation was carried out at 33°C. Isobutanol levels were followed by GC analysis.

Restoration and production of a known biomass detoxification gene increased hydrolyzate tolerance using 100% (v/v) FS-01 wet oxidation pretreated hydrolyzate (25% solids equivalent) for the hydrolyzate adapted strains. In Figure LIF-2.4, tolerance is shown by an increase in relative cell density over time. Relative cell density was measured using a high-throughput micro-fermentation system (Biolector, m2p-labs) and shows that while the parental strain LB3, and the parental adapted strains LB4 and LB5 did not grow in 100% (v/v) hydrolyzate, the adapted strains LB9 and LB10 containing the detoxification genes, derived from LB4 and LB5 respectively, had increased tolerance to 100% (v/v) wet oxidation hydrolyzate.

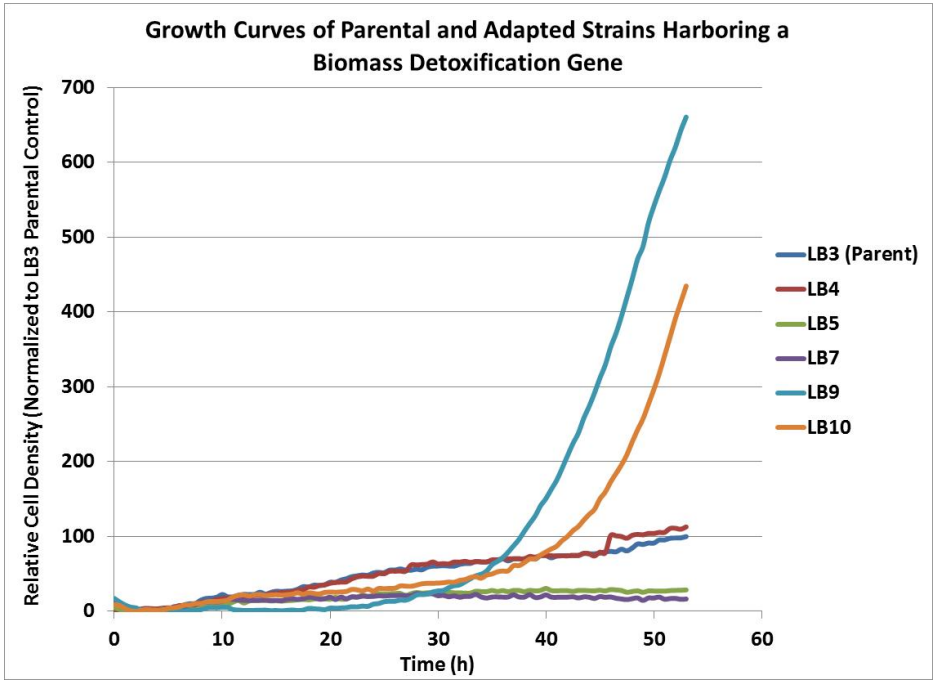

Figure LIF-2.4. Growth screening of strains producing a gene known for biomass detoxification in clarified 100% (v/v) wet oxidation pretreated biomass (FS-01) using a high-throughput micro-fermentation system (Biolector, m2p-labs). The LB3 parental control (blue), LB4 parental adapted strain (red), LB5 parental adapted strain (green), and LB7, an LB3 derived strain with the detoxification gene (purple) were not tolerant of the 100% (v/v) hydrolyzate. However, LB4 and LB5 derived strains with the biomass detoxification gene, LB9 (turquoise) and LB10 (orange) respectively, had increased tolerance to the 100% (v/v) hydrolyzate. Here, increased tolerance is exemplified by dramatically improved growth under similar conditions and hydrolyzate concentration. The liquid hydrolyzate stream was supplemented with a nutrient package, salts, and a buffering agent. The experiment was carried out at 33°C.

Inhibitor concentrations in biomass pretreatments can vary widely depending on the pretreatment method. To generate robust biocatalysts, adaptation to a specific pretreated hydrolyzate is needed. A biocatalyst adaptation program is ongoing to generate better performing isobutanol producing biocatalysts to the different hydrolyzates. Wet oxidation material used in the following experiments is described in detail in the previous quarterly report. Hydrolyzed pretreated biomass derived from pulp quality Douglas-fir (NARA feedstock FS-01) was used for fermentation experiments with Gevo's isobutanol producing strain LB4 (see Figure LF-2.5). At biomass hydrolyzate concentrations greater than 60%, a lag in isobutanol production was observed which also corresponds to an observed lag in growth. Rate of isobutanol production and final observed titer at 60% wet oxidation hydrolyzate was comparable to the corresponding pure sugar control. Gevo LB4 also produced isobutanol at 80% and 100% of biomass hydrolyzate, albeit at lower rate.

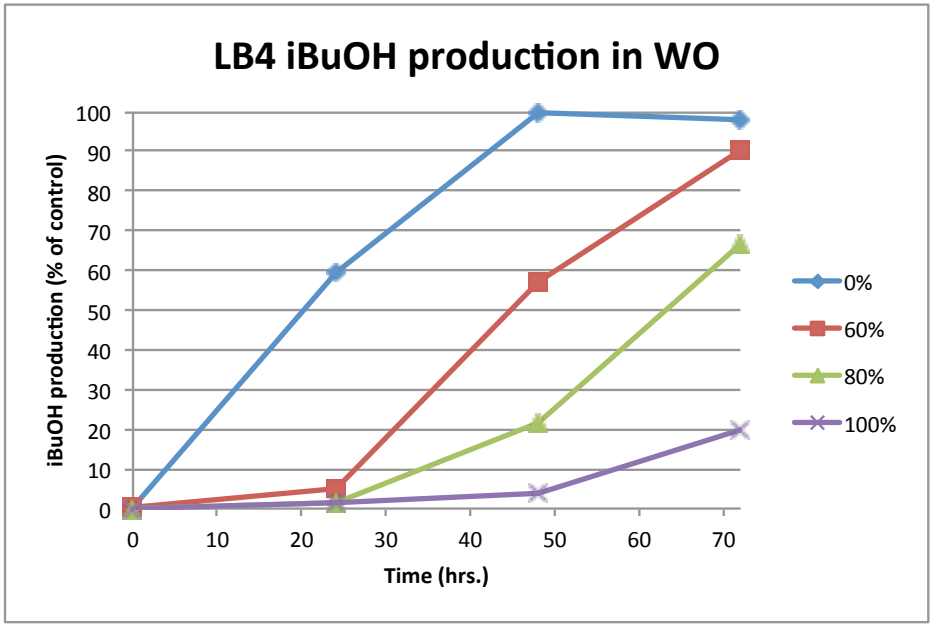

Figure LIF-2.5. Isobutanol production by Gevo isobutanol-producing strain LB4 in clarified liquid hydrolyzate derived from wet oxidation pretreated biomass (FS-01) in batch fermentation. The liquid hydrolyzate stream was supplemented with a nutrient package, salts, and a buffering agent. Control medium con-tained equal amounts of corresponding sugars and supplements. 60% and 80% fermentation media was prepared by mixing corresponding control and liquid hydrolyzate stream (v/v). Fermentation was carried out at 33°C. Isobutanol levels were followed by GC analysis.

SPORL adaptation

Inhibitor concentrations in biomass pretreatments can vary widely depending on the pretreatment method. To generate robust strains for fermentation in a given pretreated biomass, adaptation is needed in the particular pretreated biomass hydrolyzate. Previous work presented in this report described serial transfers in wet oxidation hydrolyzate yielding strains with improved fermentation performance. To further improve strains for fermentation of pretreated biomass, a similar adaptation program was carried out with SPORL hydrolyzate with the LB3 base strain and the LB4 strain that was evolved and adapted to wet oxidation material. These strains were inoculated in 96 deep-well plate containing 0.5 mL of 50% v/v SPORL black liquor medium in each well. Cultures were incubated at 33°C, shaking at 200 rpm for a specific time period before cultures were serial transferred into a new 96 deep-well plate. This process was repeated for approximately one month. Then, wells that had demonstrated improved growth were pooled and used to inoculate shake flask fermentations in 50% v/v SPORL black liquor medium. Figure LIF-2.6 shows the growth of the LB3 and LB4 parental strains and their respective adapted isolates post-adaptation, LB16 and LB17, in 50% v/v SPORL black liquor medium.

Growth was improved significantly for both LB16 and LB17 post-adaptation. Figure LIF-2.7 shows the isobutanol production for the parent LB4 as well as the adapted strain LB17. While LB16 showed improved growth over the parent, LB3, there was no improvement in isobutanol production. However, the LB4 adapted strain, LB17, showed significantly higher isobutanol titer and productivity post-adaptation in SPORL black liquor medium. Here again, the phenotypic diversity of LB16 and LB17 exemplifies the overall diversity of strains that can be generated using evolutionary engineering approaches.

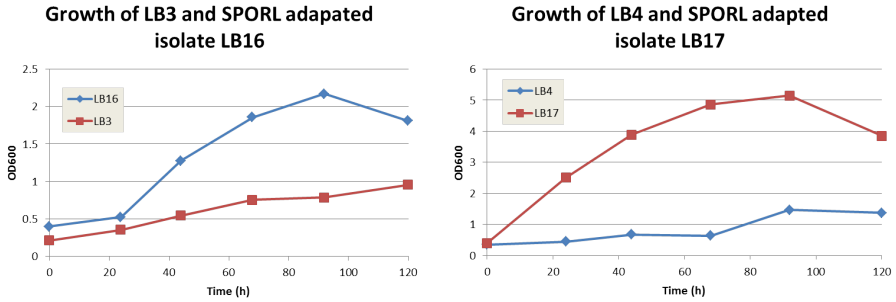

Figure LIF-2.6. Growth by LB3 and LB16 in the left-hand panel and LB4 and LB17 in the right-hand panel in SPORL black liquor (50% v/v) derived from SPORL pretreated biomass (FS-01). The liquid black liquor stream was supplemented with a nutrient package, salts, and a buffering agent. Fermentation was carried out at 33°C for 120 hours.

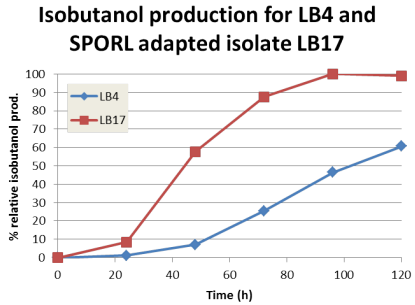

Figure LIF-2.7. Isobutanol production by LB4 and SPORL adapted isolate LB17 in SPORL black liquor (50% v/v) derived from SPORL pretreated biomass (FS-01). The liquid black liquor stream was supplemented with a nutrient package, salts, and a buffering agent. Fermentation was carried out at 33°C for 120 hours. Isobutanol levels were determined by GC analysis.

First generation hydrolyzate adapted biocatalysts with improved growth and isobutanol production performance have been isolated previously in both FS-03 (LB4) and FS-03 SPORL (LB21). A second generation biocatalyst, and the current best corn starch biocatalyst (LB23), was also selected for hydrolyzate adaptation after it demonstrated maximum growth rates similar to LB21 in FS-10 SPORL-Ca^{2+} and Mg^{2+} pretreated hydrolyzate (Figure LIF-2.8).

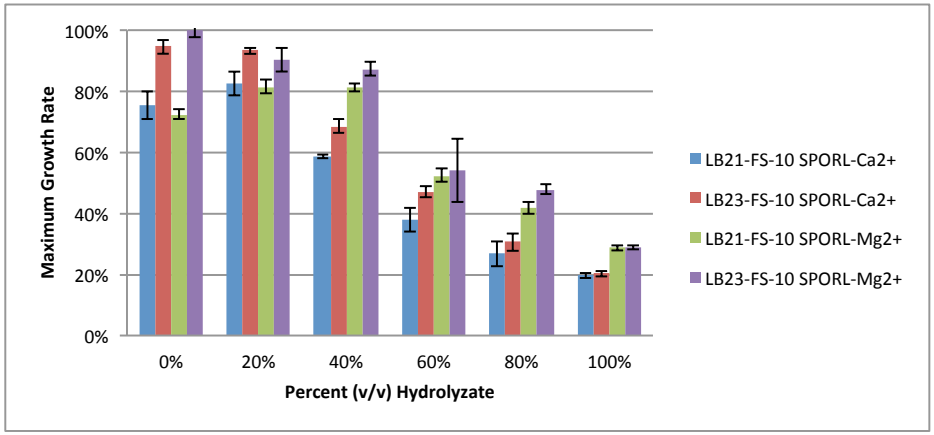

Figure LIF-2.8. Maximum growth rate of LB21 and LB23 in FS-10 SPORL-Ca2+ and Mg2+ pretreated hydrolyzate with NP 2.0. Mock medium made for each hydrolyzate is a combination of hexoses, pentoses and acetate supplemented with NP 2.0. Dilutions (volume/volume) of hydrolyzate were created using mock media. Growth carried out at 33°C and maximum growth rate measured for each percent (v/v) hydrolyzate.

Both LB21 and LB23 adaptation in FS-10 SPORL-Mg^{2+} pretreated hydrolyzate is currently being conducted. Most recently, an LB23 evolution was examined using high throughput analysis to compare growth rates of the LB23 parent to various evolved LB23 isolates (Figure LIF-2.9). Multiple LB23 evolved isolates had an improved maximum growth rate in 20% (v/v) FS-10 SPORL-Ca^{2+} pretreated hydrolyzate compared to the parent strain. Isolates 7, 9, and 14 shown in Figure LIF-2.9 warranted further characterization in order to demonstrate improved performance compared to LB23. However, a new strain, LB23, became available in parallel that was improved over these isolates.

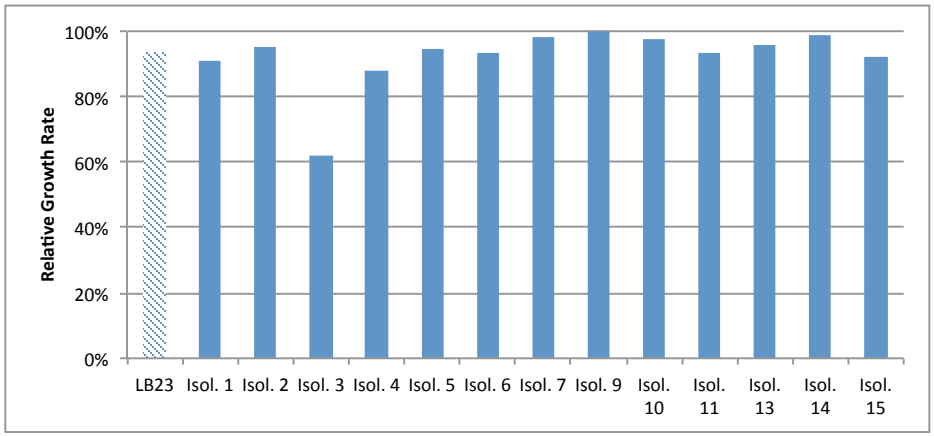

Figure LIF-2.9. Maximum growth rate of LB23 parent strain compared to LB23 evolved isolates in 20% v/v FS-10 SPORL-Ca2+ pretreated hydrolyzate with NP 2.0. 20% v/v mixture was created by diluting FS-10 SPORL-Ca2+ using buffered water. Growth was carried out at 33°C under high aeration conditions and maximum growth rate measured for each isolate.

The parent isobutanol producing biocatalyst LB3 was used to create the current best performing biocatalyst, LB4 (WO adapted LB3). Biocatalyst LB4 and a new strain from Gevo's Biocatalyst Engineering Group (LB20) are being used as starting biocatalysts for evolutionary adaptation to both FS-03 WO and SPORL pretreated hydrolyzates. FS-03 SPORL and WO hydrolyzate adapted isolates of LB4 (Figure LIF-2.10) and LB20 (Figure LIF-2.11) with improved growth are continuously being screened and selected through 1:10 serial dilutions of hydrolyzate medium approximately every week in 96 deep well plates. Cell density values in Figures LIF-2.10 and LIF-2.11 indicate the biocatalysts are adapting to the hydrolyzates over time. Adapted isolates are periodically tested for growth stability and contamination.

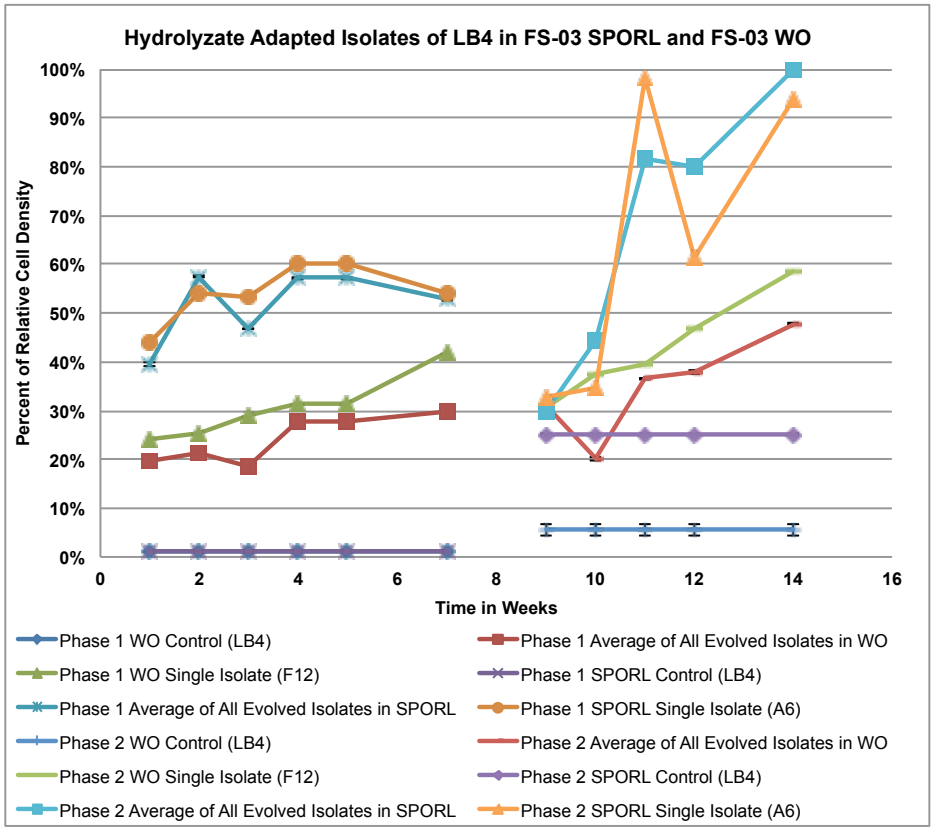

Figure LIF-2.10. Relative maximum cell densities of hydrolyzate adapted LB4 biocatalysts over time in 40% (v/v) FS-03 WO and SPORL pretreated hydrolyzates. The maximum cell density of the parental strain, LB4 (control), was compared to newly adapted isolates by following the maximum cell density approximately each week. Cell transfer and cell density measurements were performed using a Tecan Freedom Evo robotic system and Infinite M1000 Pro microplate reader, respectively. Relative cell densities in the graphs were normalized to the maximum cell density. The clarified FS-03 WO and SPORL hydrolyzates were supplemented with a nutrient package, salts, and a buffering agent.

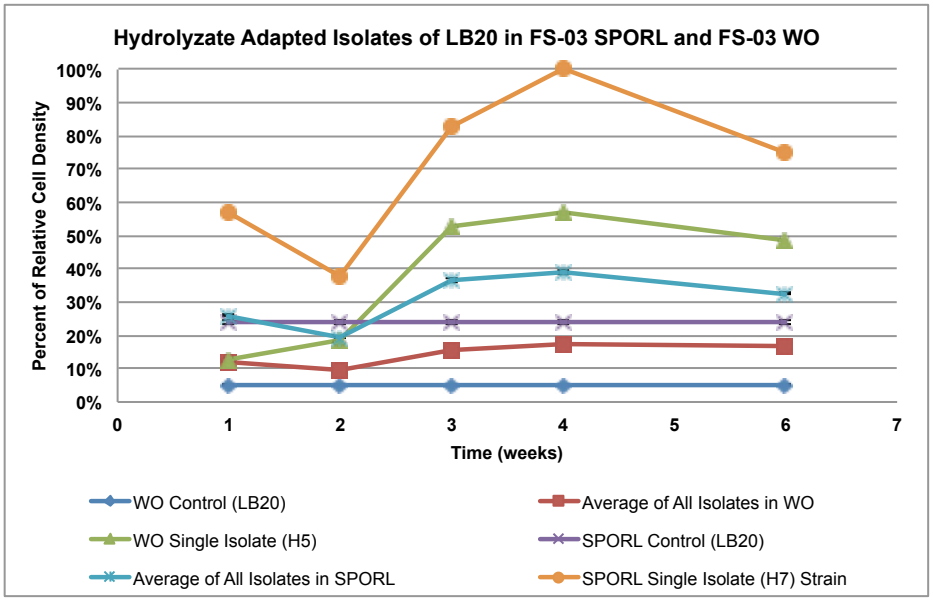

Figure LIF-2.11. Relative maximum cell densities of hydrolyzate adapted LB20 biocatalysts over time in 40% (v/v) FS-03 WO and SPORL pretreated hydrolyzates. The maximum cell density of the parental strain, LB20 (control), was compared to newly adapted isolates by following the maximum cell density approximately each week. Cell transfer and cell density measurements were performed using a Tecan Freedom Evo robotic system and Infinite M1000 Pro microplate reader, respectively. Relative cell densities in the graphs were normalized to the maximum cell density. The clarified FS-03 WO and SPORL hydrolyzates were supplemented with a nutrient package, salts, and a buffering agent.

Inhibitor concentrations in biomass pretreatments can vary widely depending on the pretreatment method. To generate robust biocatalysts, adaptation to a specific pretreated hydrolyzate is needed. A biocatalyst adaptation program is ongoing to generate better performing isobutanol producing biocatalysts to the different hydrolyzates. Hydrolyzate adapted strains with improved performance have been isolated from all hydrolyzates used in the adaptation program. The parent isobutanol producing biocatalyst LB3, the current best performing biocatalyst (LB4 (FS-01 WO adapted LB3)), the current best corn starch biocatalyst (LB20), and additional engineered strains from Gevo's strain collection have all been used as starting biocatalysts for evolutionary engineering using both FS-03 WO and SPORL pretreated hydrolyzates. Earlier, an improved SPORL hydrolyzate adapted isolate, LB19, showed improved growth rates in SPORL hydrolyzate media compared to the WO adapted LB4 strain (Figure LIF-2.12). LB19 proved to be an unstable isolate after reviving from frozen culture stocks (data not shown).

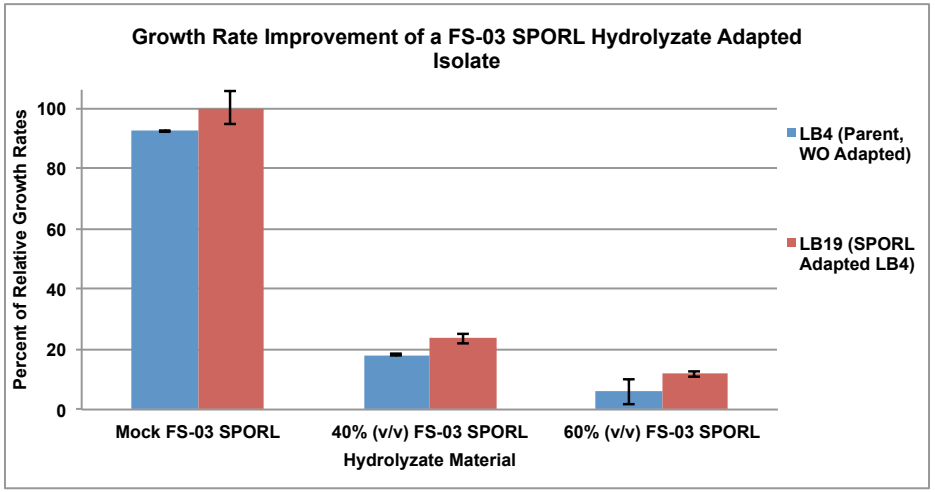

Figure LIF-2.12. High throughput growth screening showing the percent of relative growth rates of the current best performing hydrolyzate adapted biocatalyst (LB4) and a new SPORL adapted biocatalyst derived from LB4 (LB19). Percent relative growth rates were obtained using a high-throughput microfermentation system (BioLector, m2p-labs). All hydrolyzates were clarified to remove solids and all were supplemented with a nutrient package, salts, and a buffering agent. The different percentages of hydrolyzate and mock media contained equal amounts of corresponding sugars, acetate, and supplements. 100% (v/v) hydrolyzate is equal to approximately 30-36% equivalent solids for all biomass materials. Error bars represent the standard deviation. Abbreviations: WO, wet oxidation; SPORL, sulfite pretreatment to overcome recalcitrance of lignocellulose.

In addition, previously reported data showed the growth improvement of hydrolyzate adapted strains over time in WO and SPORL hydrolyzates starting with an improved isobutanol producing biocatalyst (LB20) from Gevo's biocatalyst engineering group. Over a six week timeframe, cell densities of individual hydrolyzate adapted strains and the average cell density of all isolates were compared to the parent biocatalyst (LB20) using 40% (v/v) FS-03 WO or SPORL hydrolyzate media, Figure LIF-2.13.

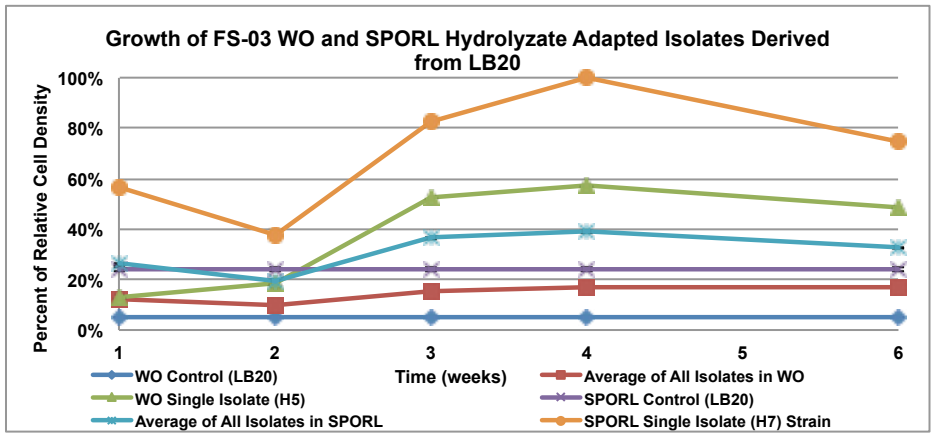

Figure LIF-2.13. Relative maximum cell densities of hydrolyzate adapted LB20 biocatalysts over time in 40% (v/v) FS-03 WO and SPORL pretreated hydrolyzates. The maximum cell density of the parental strain, LB20 (control), was compared to newly adapted isolates by following the maximum cell density approximately each week. Cell transfer and cell density measurements were performed using a Tecan Freedom Evo robotic system and Infinite M1000 Pro microplate reader, respectively. Relative cell densities in the graphs were normalized to the maximum cell density. The clarified FS-03 WO and SPORL hydrolyzates were supplemented with a nutrient package, salts, and a buffering agent. Abbreviations: WO, wet oxidation; SPORL, sulfite pretreatment to overcome recalcitrance of lignocellulose.

Previously unreported data using new FS-03 SPORL hydrolyzate adapted isolates shows an improvement in growth (Figure LIF-1.14) and specific isobutanol productivity (Figure LIF-1.15) compared to the parent strains. These new strains are very promising because they appear to be stable and not only show improved growth but also improved isobutanol productivity. They will continue to be characterized as well as other new isolates from the hydrolyzate adaptation program. New hydrolyzate adapted isolates are continuously being screened and selected through 1:10 serial dilutions of hydrolyzate medium approximately every week in 96 deep well plates. In addition, improved strains from Gevo's biocatalyst engineering group are routinely tested for hydrolyzate tolerance.

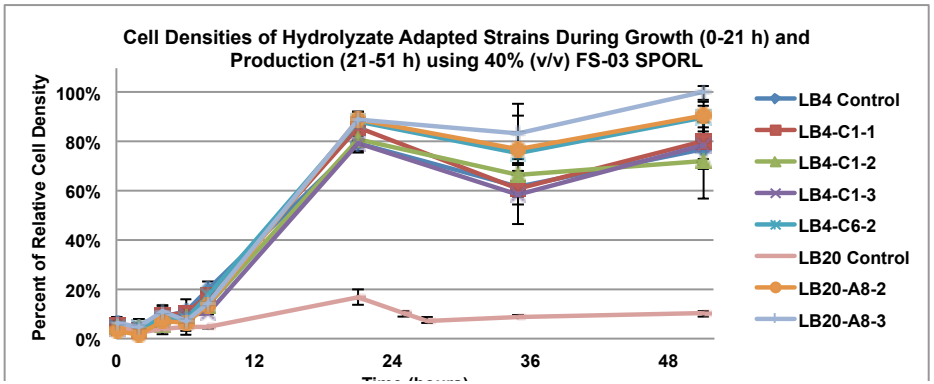

Figure LIF-1.14. Relative cell densities of hydrolyzate adapted LB4 and LB20 derived biocatalysts using 40% (v/v) FS-03 SPORL pretreated hydrolyzate medium in shake flask fermentations. All hydrolyzates were clarified to remove solids and were supplemented with a nutrient package, salts, and a buffering agent. The 40% (v/v) mixtures have sugars and acetate equivalent to 100% of the hydrolyzate. 100% hydrolyzate is equal to approximately 30-36% equivalent solids. Fermentation was carried out at 33°C. Cell density was measured using a spectrophotometer. Error bars represent the standard deviation. Abbreviations: SPORL, sulfite pretreatment to overcome recalcitrance of lignocellulose.

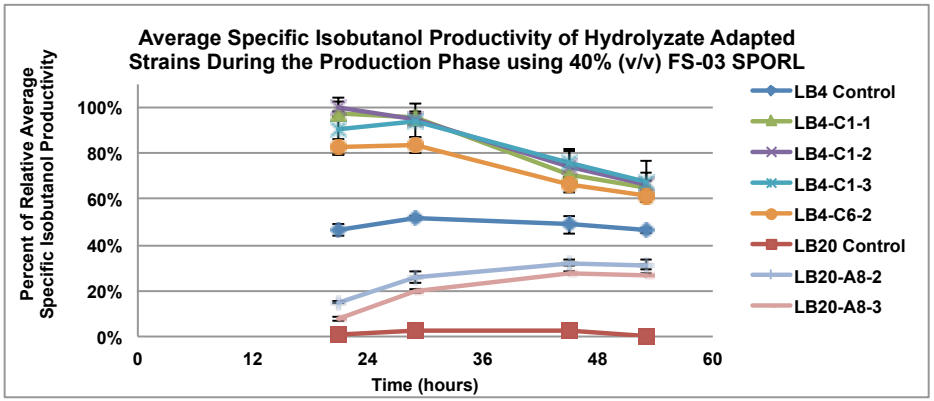

Figure LIF-1.15. Relative average specific isobutanol productivities of hydrolyzate adapted LB4 and LB20 derived biocatalysts using 40% (v/v) FS-03 SPORL pretreated hydrolyzate medium in shake flask fermentations. All hydrolyzates were clarified to remove solids and were supplemented with a nutrient package, salts, and a buffering agent. The 40% (v/v) mixtures have sugars and acetate equivalent to 100% of the hydrolyzate. 100% hydrolyzate is equal to approximately 30-36% equivalent solids. Fermentation was carried out at 33°C. Cell density and isobutanol titers were measured using a spectrophotometer and GC, respectively. Average specific productivity is the g CDW / (g/L/h of isobutanol). Error bars represent the standard deviation. Abbreviations: SPORL, sulfite pretreatment to overcome recalcitrance of lignocellulose.

TASK 3: PRODUCE ISOBUTANOL IN A 1L BATCH FERMENTATION FROM PRETREATED BIOMASS SUGARS USING THE ADAPTED YEAST BIOCATALYST.

During NARA Year-1, 4th quarter, work began on optimization of fermentation conditions for the isobutanol producing biocatalyst strain, Gevo LB3. This strain is the parent strain of adapted biocatalyst strains Gevo LB4, LB5, LB16, and LB17 and serves as a benchmark strain for establishing conditions that can be applied to adapted strains. Fermentation work in bioreactors was performed with adapted strains as they became available.

Learnings from fermentation process development include identifying impurities in the isobutanol and reducing or eliminating the impurity by using a different process control parameter, e.g the base used to control pH. Previously, ammonium hydroxide was used for pH control but changing to sodium hydroxide reduced the impurity significantly. Sodium concentrations above 0.5 g/L (9 mM) are known to inhibit yeast growth and fermentation so a test growth test with sodium chloride was used to measure the effects on the LB4 biocatalyst. An initial high-throughput growth experiment to test sodium levels was performed (Figure LIF-3.1). At the lowest level tested of 11.7 g/L (0.2 M) the final cell density decreased by 35% and the growth rate decreased by 20%. At 0.2 M sodium chloride, sodium is approximately 2 to 5 times the level added to fermenters when sodium hydroxide is used as the base.

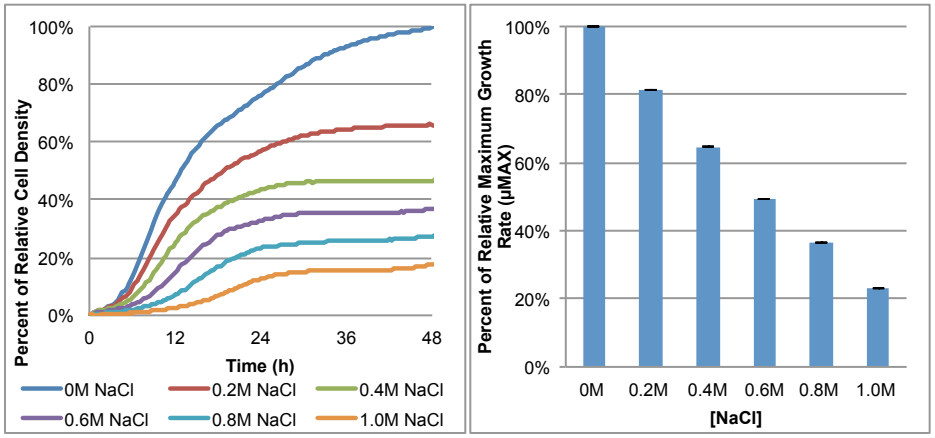

Figure LIF-3.1. High throughput growth screening showing the relative cell density (left) and percent of relative growth rates of the current best hydrolyzate adapted LB4 in different concentration of NaCl. Growth rate data was obtained using a high-throughput microfermentation system (BioLector, m2p-labs). The medium used for was a yeast extract based medium with glucose supplemented with salts and a buffering agent. Error bars represent the standard deviation.

Growth and isobutanol production in a 1L batch fermentation were compared using FS-03 WO (Batch A) and SPORL with the current best hydrolyzate biocatalyst available at that time, LB4 (WO adapted LB3). The fermentation was performed in two phases; growth using 20% (v/v) hydrolyzates and production using 60% (v/v) hydrolyzates. The growth in both 20% (v/v) hydrolyzates was similar to the mock hydrolyzates (Figure LIF-3.2, Left). There was continued growth during the production phase in the mock hydrolyzates but the cell density remained the same in the 60% (v/v) hydrolyzates (Figure LIF-3.2, Right).

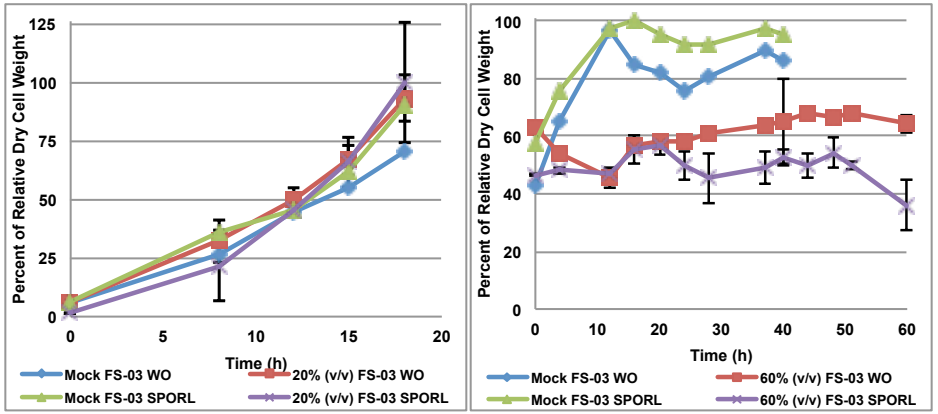

Figure LIF-3.2. One liter fermentation data using the current best isobutanol producing biocatalyst (LB4). The percent relative dry cell weight during the growth phase in 20% (v/v) WO and SPORL hydrolyzates (Left) and relative dry cell weight during the production phase in 60% (v/v) WO and SPORL (Right). The clarified FS-03 wet oxidation and SPORL pretreated hydrolyzates were supplemented with a nutrient package, salts, and pH adjusted during the fermentation. Different percentages of hydrolyzate media and mock media contained equal amounts of corresponding sugars and supplements for each pretreatment type. At 100% (v/v), not shown, wet oxidation pretreated hydrolyzate was equal to approximately 30-36% equivalent solids. Fermentation was carried out at 33°C. Dry Cell Weight was determined using a spectrophotometer and a cell weight conversion factor. Error bars on the 20% (v/v) and 60% (v/v) hydrolyzate data represent standard deviation of duplicates.

The total isobutanol titer (includes off-gas values) increased rapidly to a maximum at 24 hours in the production phase for the mock media while the total isobutanol titer slowly increased to a maximum at 60 hours that was higher than the mock media (Figure LIF-3.3).

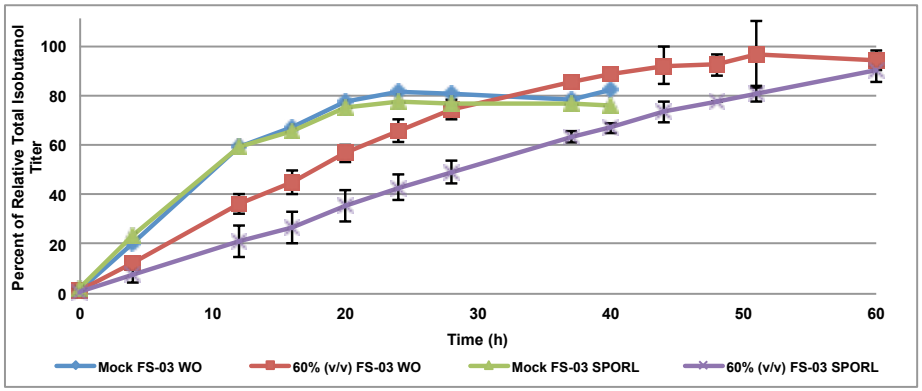

Figure LIF-3.3. Total isobutanol titer in one liter batch fermentation using the current best isobutanol producing biocatalyst (LB4). The percent relative total isobutanol titer during the production phase in 60% (v/v) WO and SPORL compared to mock media. The clarified FS-03 WO and SPORL pretreated hydrolyzates were supplemented with a nutrient package, salts, and pH adjusted during the fermentation. Different percentages of hydrolyzate media and mock media contained equal amounts of corresponding sugars and supplements for each pretreatment type. At 100% pretreated hydrolyzates were equal to approximately 24-36% equivalent solids. Fermentation was carried out at 33°C. Isobutanol levels were determined by GC analysis. Error bars represent standard deviation of duplicates

The average volumetric productivity and average specific productivity were higher with the mock hydrolyzates but decreased over-time to similar rates as the 60% (v/v) hydrolyzate rates (Figure LIF-3.4). Comparison between WO and SPORL pretreated hydrolyzates indicates that rates are higher in WO initially but over time the rates become similar (Figure LIF-3.4).

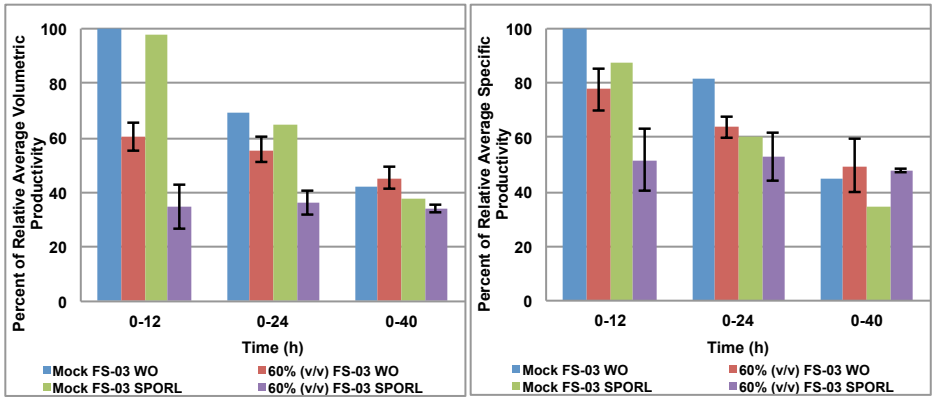

Figure LIF-3.4. One liter fermentation data using the current best isobutanol producing biocatalyst (LB4) showing the percent relative average volumetric productivity (Left) and percent relative average specific productivity (Right) during the production phase in 60% (v/v) WO and SPORL. The clarified FS-03 wet oxidation and SPORL pretreated hydrolyzates were supplemented with a nutrient package, salts, and pH adjusted during the fermentation. Different percentages of hydrolyzate media and mock media contained equal amounts of corresponding sugars and supplements for each pretreatment type. At 100% (v/v), not shown, wet oxidation pretreated hydrolyzate was equal to approximately 30-36% equivalent solids. Fermentation was carried out at 33°C. Isobutanol levels were determined by GC analysis and dry cell weight was determined using a spectrophotometer and a cell weight conversion factor. Error bars on 60% (v/v) hydrolyzate data represent standard deviation of duplicates.

TASK 4: ECONOMIC ASSESSMENT OF WOOD TO ISOBUTANOL, JET

In NARA Year-1, Quarter-2: Initial work was started on the development of a biomass to jet process model in ASPEN. Relevant Gevo process models were collected for integration into the final model and a common physical property dataset was developed. Background information on the USDA Forest Products Laboratory pretreatment method, SPORL, was reviewed.

In NARA Year-1, Quarter-3: In the previous quarter, work was started on the development of a biomass to jet process model in ASPEN. ASPEN is the leading computer software for process model development. From the manufacturer (ASPENTech):

Aspen Plus has a proven track record of providing substantial economic benefits throughout the process engineering lifecycle, from conceptual design and engineering to production. It brings the power of process simulation and optimization to the engineering desktop, and delivers a unique combination of modeling technology and ease of use. Aspen Plus enables companies to rapidly design new processes, deliver new products to market faster and optimize production.

Relevant Gevo process models were collected for integration into the final model, and a common physical property dataset was developed. Background information on the USDA Forest Products Laboratory pretreatment method, SPORL, was reviewed.

We visited the USDA Forest Products Laboratory and toured the pilot plant to better understand the SPORL process. OSBL areas (WWT, boiler/turbogenerator) were added to the base ASPEN model. An initial template for operating costs was developed. The isobutanol to jet ASPEN model was updated.

In Year-1, Quarter-4: Gevo Process Engineering team members held several teleconferences and multiple information exchanges with Gevan Marrs (Catchlight Energy) and Tom Spink (TSI, Inc.) over the last quarter to provide information for the NARA techno-economic analysis. Gevo has provided information on its production process unit operations to convert isobutanol to biojet (IPK, isoparaffinic kerosene). Gevo has also provided information and direction on how to model the lignocellulosic capital costs based to a large extent on the thorough analysis completed by NREL for the production of ethanol with insight on how to adapt this to the production of isobutanol. Finally, Gevo has worked collaboratively with Gevan and Tom to establish a basis for an operating expense model.

Gevo Process Engineering team finalized a modeling structure to supply material flows and capital equipment and operating costs to the NARA project. The general boundaries of the Gevo supplied information are illustrated in Figure LIF-4.1. An Aspen Plus model has been built to describe the Gevo technology included. This model will be used to generate the necessary outputs with the given inputs from the up-stream areas (primarily pretreatment) of the process. The inputs and outputs permit the NARA TEA and Aspen modeling team to assess and simulate Gevo proprietary information, without releasing that information specifically.

Figure LIF-4.1. Gevo approach to modeling material flows and capital and operating costs.

The Gevo process box can be summarized as follows. The saccharified biomass sugars are fermented and isobutanol recovered in a process essentially identical to the corn mash process being used currently at Gevo's plant in Luverne, MN. The process modeled here accommodates two feeds from the NARA mild bisulfite pretreatment, a liquid only stream separated from the mild bisulfite pretreatment by the NARA team and a solids containing stream where the cellulose has been enzymatically saccharified. Gevo discharges two whole stillage streams containing all the unreacted solids, insoluble and soluble, back to NARA for processing and recycling the water to pretreatment. Only a small amount of clean water, for vent scrubbers, is required by Gevo over what is present already in the hydrolyzate. Utility requirements include city water, steam, natural gas (for fired heaters and hydrogen production), cooling water, and electricity. No steam boilers or cooling towers have been assumed inside the Gevo box. Combined atmospheric vents (fermentation, fired heaters, etc.) were specified. Waste water was also specified as to flow and composition. Minor raw materials (other than biomass hydrolyzate) utilized in the process were specified as an operating cost amount. The material quantities are insignificant to the material balance. Hydrocarbon vents from the biojet (IPK) process are burned in the fired heaters and the combustion

products included in the combined vent along with the hydrogen reformer vent and fermentation vent. No other hydrocarbon products besides biojet (IPK) are produced in the process. All lower molecular weight materials (e.g., isobutylene and isooctane) are recycled and incorporated in jet range molecules. Byproducts from isobutanol fermentation are generally discharged in the whole stillage. Some lower molecular weight alcohols can be recycled to the fermentation.

The results of the Gevo iBuOH Fermentation and Conversion to IPK analysis and models were fed forward to the NARA Techno-Economic Assessment (TEA) team, lead by Tom Spink and Gevan Marrs. While Gevo's results are proprietary information to Gevo, they were included in a "black box" approach in the overall TEA work product. See the TEA team report (Marrs et al, 2016) for more details.

TASK 5: PRODUCE ISOBUTANOL IN 1L GIFT® FERMENTATION FROM PRE-TREATED BIOMASS SUGARS USING THE ADAPTED YEAST BIOCATALYST

The process parameters for producing isobutanol from pretreated biomass sugars in a 1L GIFT system were developed and identified using learnings from not only biomass based shake flask fermentations and 1L batch fermentations but also from fermentations using corn mash with strains produced by Gevo's Strain Development program. Furthermore, process development will continue as the pretreated biomass process improves, strains adapt to hydrolyzates, and additional learnings are transferred to Gevo members working on the NARA project from other internal Gevo teams.

Growth and isobutanol production of the current best hydrolyzate biocatalyst available at the time, LB4, was compared in a 1L GIFT® fermentation systems using FS-10 derived Wet Oxidation (Batch C), SPORL, Mild Bisulfite Solids, Mild Bisulfite Spent Sulfite Liquor (batch, not GIFT®), Combined Mild Bisulfite, Unconcentrated Milled Wood. The fermentation was performed in two phases; batch growth using 20% (v/v) hydrolyzates and production using 60% (v/v) hydrolyzates. The growth in all 20% (v/v) hydrolyzates was very similar (Figure LIF-5.1) considering they displayed a range of hexose concentrations. The growth during the production phase using 60% (v/v) hydrolyzates all had some growth except for the MB SSL which may have been a result of not using a GIFT® system (Figure LIF-5.2). The isobutanol titers had different maximum titers within a 24 hour timeframe with the highest coming from CMB, very similar titers from MB Solids, SPORL, and WO, and then the two lowest values coming from UMW and MB SSL (Figure LIF-5.3). The isobutanol titer differences are likely caused by one or two factors, inhibitors and hexose concentration.

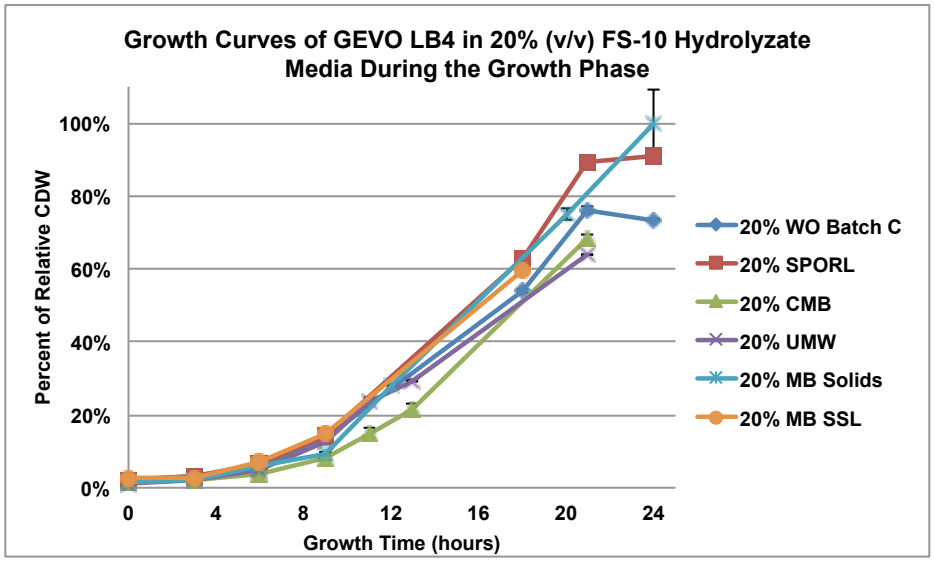

Figure LIF-5.1. Percent relative cell dry weight (growth) in one liter GIFT® system fermentation data using the current best hydrolyzate adapted isobutanol producing biocatalyst (LB4). The percent relative dry cell weight is shown during the growth phase in 20% (v/v) of each FS-10 hydrolyzate. The clarified hydrolyzates were supplemented with a nutrient package, salts, and pH controlled adjusted during the fermentation. The 20% (v/v) mixture of each hydrolyzate contained equal amounts of corresponding sugars to the 100% material and equivalent supplements were added to each hydrolyzate. Cell dry weight (CDW) was calculated using a conversion factor multiplied by the cell density measured on a spectrophotometer. All of the hydrolyzates were tested in GIFT® systems except the MB SSL. Error bars represent the standard deviation of duplicates. Abbreviations: WO, wet oxidation; SPORL, sulfite pretreatment to overcome recalcitrance of lignocellulose; CMB, combined mild bisulfite; UMW, unconcentrated milled wood; MB, mild bisulfite; SSL, spent sulfite liquor.

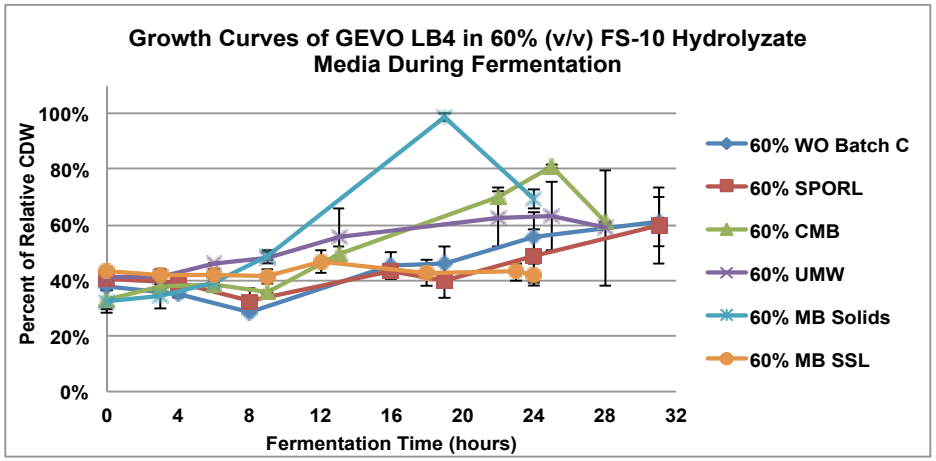

Figure LIF-5.2. One liter GIFT® system fermentation data using the current best hydrolyzate adapted isobutanol producing biocatalyst (LB4) showing the percent relative dry cell weight during the fermentation phase in 60% (v/v) of each FS-10 hydrolyzate. The clarified hydrolyzates were supplemented with a nutrient package, salts, and the pH was controlled during the fermentation. The 60% (v/v) mixture of each hydrolyzate contained equal amounts of corresponding sugars to the 100% material and equivalent supplements were added to each hydrolyzate. Cell dry weight (CDW) was calculated using a conversion factor multiplied by the cell density measured on a spectrophotometer. All of the hydrolyzates were tested in GIFT® systems except the MB SSL. Error bars represent the standard deviation of duplicates. Abbreviations: WO, wet oxidation; SPORL, sulfite pretreatment to overcome recalcitrance of lignocellulose; CMB, combined mild bisulfite; UMW, unconcentrated milled wood; MB, mild bisulfite; SSL, spent sulfite liquor.

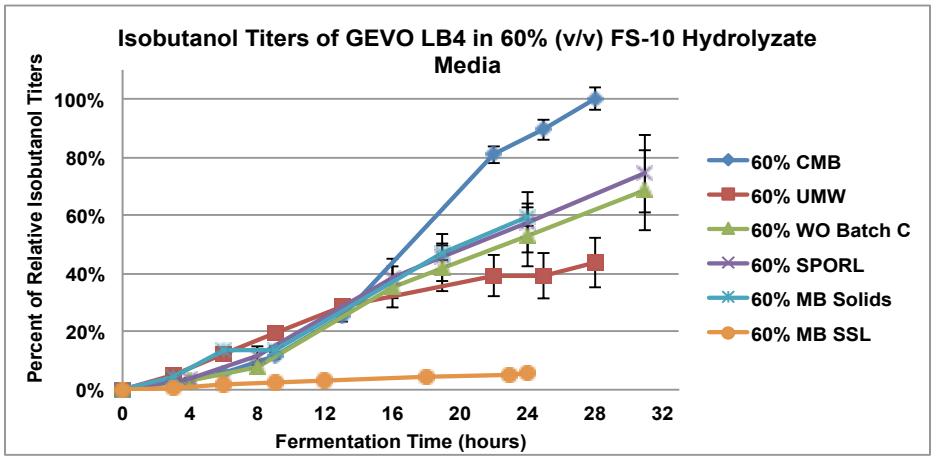

Figure LIF-5.3. One liter GIFT® system fermentation data using the current best hydrolyzate adapted isobutanol producing biocatalyst (LB4) showing the percent of relative isobutanol titers in 60% (v/v) of each FS-10 hydrolyzate. The clarified hydrolyzates were supplemented with a nutrient package, salts, and the pH was controlled during the fermentation. The 60% (v/v) mixture of each hydrolyzate contained equal amounts of corresponding sugars to the 100% material and equivalent supplements were added to each hydrolyzate. Isobutanol titers were measure using a GC. All of the hydrolyzates were tested in GIFT® systems except the MB SSL. Error bars represent the standard deviation of duplicates. Abbreviations: WO, wet oxidation; SPORL, sulfite pretreatment to overcome recalcitrance of lignocellulose; CMB, combined mild bisulfite; UMW, unconcentrated milled wood; MB, mild bisulfite; SSL, spent sulfite liquor.

The average specific isobutanol productivity can be separated into three categories, those near 100%, those in the 60-80% range, and those below 20% (Figure LIF-5.4). The CMB hydrolyzate had the highest rate at 100% while WO, SPORL, UMW, and MB Solids were all in the 60%-80% range. The only material below 20% was the MB SSL. This trend was the same the average volumetric productivity (Figure LIF-5.5). The differences in average specific productivity and average volumetric productivity results are likely caused by one or two factors, inhibitors, and hexose concentration.

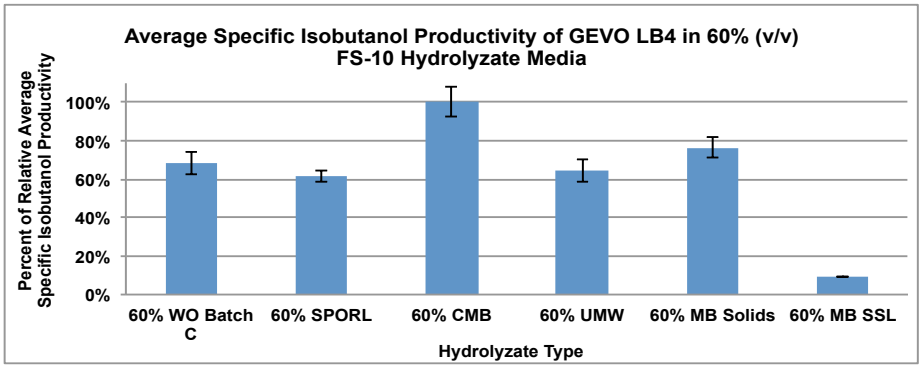

Figure LIF-5.4. One liter GIFT® system fermentation data using the current best hydrolyzate adapted isobutanol producing biocatalyst (LB4) showing the average specific isobutanol productivity in 60% (v/v) of each FS-10 hydrolyzate. The clarified hydrolyzates were supplemented with a nutrient package, salts, and the pH was controlled during the fermentation. The 60% (v/v) mixture of each hydrolyzate contained equal amounts of corresponding sugars to the 100% material and equivalent supplements were added to each hydrolyzate. Cell dry weight (CDW) was calculated using a conversion factor multiplied by the cell density measured on a spectrophotometer and isobutanol was measured using a GC. All of the hydrolyzates were tested in GIFT® systems except the MB SSL. Error bars represent the standard deviation of duplicates. Abbreviations: WO, wet oxidation; SPORL, sulfite pretreatment to overcome recalcitrance of lignocellulose; CMB, combined mild bisulfite; UMW, unconcentrated milled wood; MB, mild bisulfite; SSL, spent sulfite liquor.

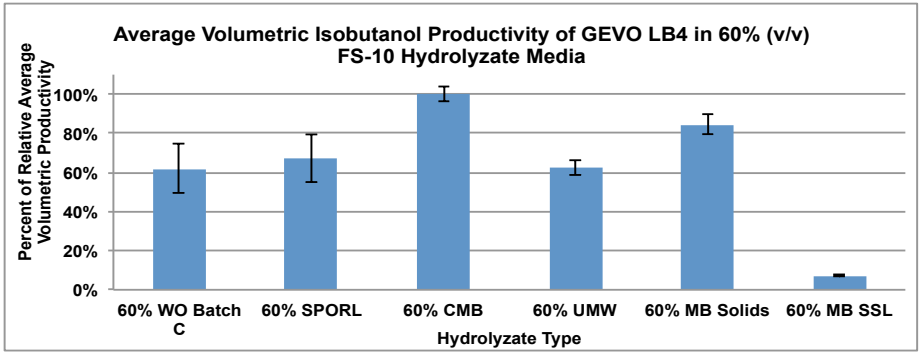

Figure LIF-5.5. One liter GIFT® system fermentation data using the current best hydrolyzate adapted isobutanol producing biocatalyst (LB4) showing the percent of relative average volumetric isobutanol productivity in 60% (v/v) of each FS-10 hydrolyzate. The clarified hydrolyzates were supplemented with a nutrient package, salts, and the pH was controlled during the fermentation. The 60% (v/v) mixture of each hydrolyzate contained equal amounts of corresponding sugars to the 100% material and equivalent supplements were added to each hydrolyzate. Isobutanol was measured using a GC. All of the hydrolyzates were tested in GIFT® systems except the MBS SSL. Error bars represent the standard deviation of duplicates. Abbreviations: WO, wet oxidation; SPORL, sulfite pretreatment to overcome recalcitrance of lignocellulose; CMB, combined mild bisulfite; UMW, unconcentrated milled wood; MBS, mild bisulfite; SSL, spent sulfite liquor.

The average hexose consumption rate can be separated into three categories, those in the 80 to 100% range, those in the 30-50% range, and those below 30% (Figure LIF-5.6). The CMB, UMW, and MB Solids hydrolyzate had the highest rates while WO and SPORL were in the 30%-50% range. The only material below 30% was the MB SSL. The percent of theoretical yields two categories, those above 50% and those below 50% (Figure LIF-5.7). The WO, SPORL, CMB, UMW, and MB Solids were all similar with WO having slightly better yields than the rest. The only material below 30% was the MB SSL. The differences in hexose consumption rates and percent of theoretical yields are likely caused by one or two factors: inhibitors and total hexose available.

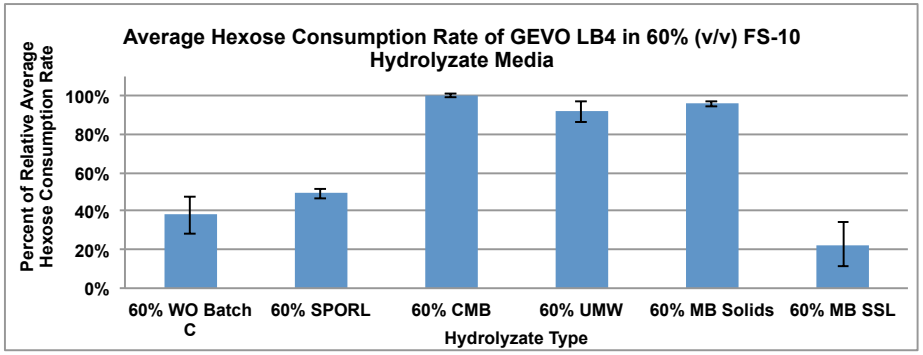

Figure LIF-5.6. One liter GIFT® system fermentation data using the current best hydrolyzate adapted isobutanol producing biocatalyst (LB4) showing the percent of relative hexose consumption rate in 60% (v/v) of each FS-10 hydrolyzate. The clarified hydrolyzates were supplemented with a nutrient package, salts, and the pH was controlled during the fermentation. The 60% (v/v) mixture of each hydrolyzate contained equal amounts of corresponding sugars to the 100% material and equivalent supplements were added to each hydrolyzate. Cell dry weight (CDW) was calculated using a conversion factor multiplied by the cell density measured on a spectrophotometer. All of the hydrolyzates were tested in GIFT® systems except the MB SSL. Error bars represent the standard deviation of duplicates. Abbreviations: WO, wet oxidation; SPORL, sulfite pretreatment to overcome recalcitrance of lignocellulose; CMB, combined mild bisulfite; UMW, unconcentrated milled wood; MB, mild bisulfite; SSL, spent sulfite liquor

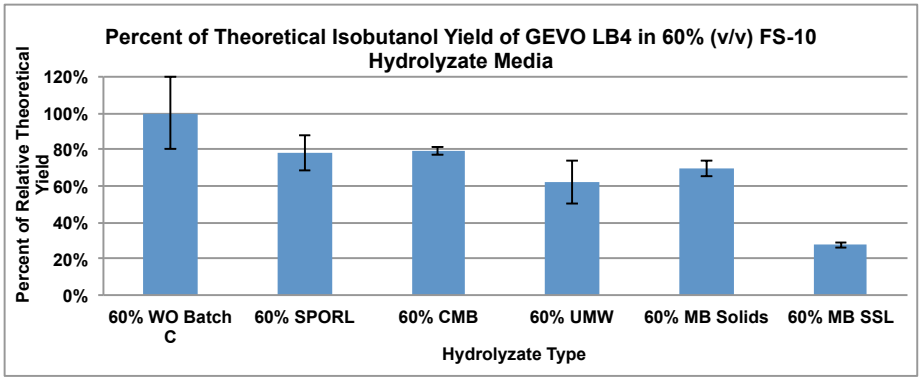

Figure LIF-5.7. One liter GIFT® system fermentation data using the current best hydrolyzate adapted isobutanol producing biocatalyst (LB4) showing the percent of relative isobutanol yields in 60% (v/v) of each FS-10 hydrolyzate. The clarified hydrolyzates were supplemented with a nutrient package, salts, and pH controlled adjusted during the fermentation. The 60% (v/v) mixture of each hydrolyzate contained equal amounts of corresponding sugars to the 100% material and equivalent supplements were added to each hydrolyzate. Cell dry weight (CDW) was calculated using a conversion factor multiplied by the cell density measured on a spectrophotometer. All of the hydrolyzates were tested in GIFT® systems except the MBS SSL. Error bars represent the standard deviation of duplicates. Abbreviations: WO, wet oxidation; SPORL, sulfite pretreatment to overcome recalcitrance of lignocellulose; CMB, combined mild bisulfite; UMW, unconcentrated milled wood; MBS, mild bisulfite; SSL, spent sulfite liquor.

To summarize the results of the various hydrolyzate types in the 1L GIFT® fermentation systems, under the conditions tested, the CMB was the highest performing material overall, while the WO, SPORL, MBS Solids, and UMW were very similar overall with one or the other having better results in one or two metrics. The lowest performing material overall was the MB SSL.

As improved, hydrolyzate adapted strains became available, like isobutanol biocatalyst LB21, they were used in the GIFT® fermentation work. A 1L GIFT® fermentation was performed using 100% (v/v) FS-10 concentrated milled wood (CMW) to further characterize the pretreatment and hydrolysis method used by Catchlight Energy. Catchlight and the USDA FPL (Drs. Gao and Zhu) worked together to perfect the SPORL/MBL pretreatment method. The volumetric rate of isobutanol production as well as the isobutanol titer achieved was comparable to 60% (v/v) FS-10 SPORL-Ca^{2+} pretreated hydrolyzate. n order to compare the milled wood process and SPORL pretreatment process, shipments of EW-01 and FS-01 MW samples were received from WSU and hydrolyzed at Gevo at similar solids percentages as previously hydrolyzed FS-10 SPORL-Mg^{2+} pretreated hydrolyzate. EW-01 MW (120 min. grind) hydrolyzed (23% solids, 108.6 g/L hexose sugars) under similar conditions as FS-10 SPORL-Mg^{2+} pretreated hydrolyzate (24% solids, 76.4 g/L hexose sugars) was the closest comparison in terms of hexose sugar concentration.

Growth of LB21 was nearly identical in 20% (v/v) FS-10 SPORL-Mg2+ pretreated hydrolyzate and 20% (v/v) EW-01 MW (120 min. grind) hydrolyzate using their corresponding mock media. The seed culture of LB21 from each condition was then used to inoculate 80% (v/v) of corresponding hydrolyzate using mock medium

as the balance and allowed to ferment for 24 hours when both hydrolyzates were depleted of hexose sugars. Volumetric isobutanol production of LB21 in 80% (v/v) FS-10 SPORL-Mg^{2+} pretreated hydrolyzate was an average of 26.4% higher than LB21 in 80% (v/v) EW-01 MW (120 min. grind) hydrolyzate after 24 hours. LB21 continued to produce isobutanol at similar rates in both hydrolyzates up to 12 hours before plateauing in EW-01 MW (120 min. grind) hydrolyzate (Figure LIF-5.8). The theoretical isobutanol yield of LB21 was 8.6% higher in 80% (v/v) FS-10 SPORL-Mg^{2+} pretreated hydrolyzate compared to the EW-10 MW (120 min. grind) hydrolyzate equivalent. Under the current pretreatment conditions, LB21 ferments better in FS-10 SPORL-Mg2+ pretreated hydrolyzate compared to EW-01 MW (120 min. grind) hydrolyzate under similar fermentation conditions. Production of isobutanol at a rate ≥0.3 g/L/h and ≥40% theoretical yield using 60% (v/v) hydrolyzates of every pretreated material tested in the 1L GIFT® fermentation systems was achieved using biocatalyst LB21.

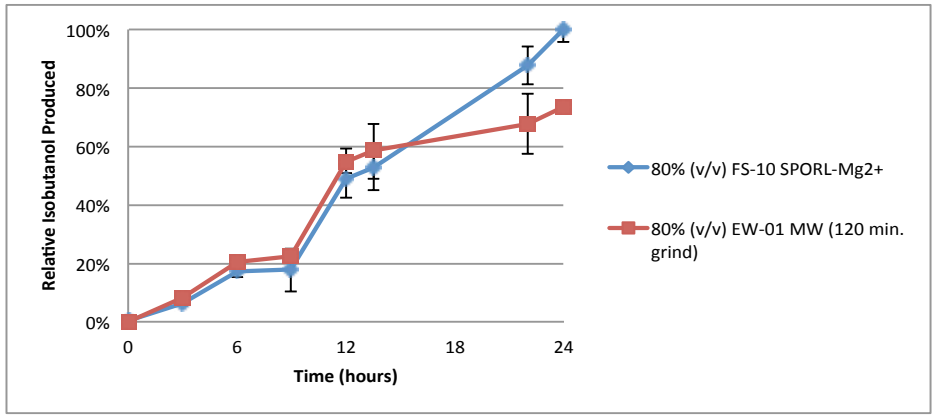

Figure LIF-5.8. Isobutanol produced by LB21 in 80% v/v FS-10 SPORL-Mg2+ pretreated and EW-01 MW (120 min. grind) hydrolyzate. Production of isobutanol was conducted in 1L fermentation vessels equipped with GIFT®, held at 33°C and pH controlled. Media was created using the corresponding mock media. Error bars represent the standard deviation.

Concentration of Hydrolyzate by RotoVap

In some circumstances, enzymatic hydrolysis was optimized for yield by conducting the hydrolysis at 10-15% solids. However, this leads to low sugar concentrations (but high yields). In some circumstances, it was desirable to have a higher concen-tration of sugar to maximize the benefit of Gevo's GIFT® technology. Thus, after hy-drolysis, the hydrolyzate was clarified by centrifugation to remove insoluble solids. The liquid hydrolyzate was decanted to a sterile bottle and then evaporated 500 mL at a time using a RotoVap, as shown in Figure LIF-5.8. The conditions used were:

- 500 mL native hydrolyzate
- 1L round bottom flask
- 85°C water bath for heat applied to the round bottom flask
- Rotational speed of 150-160 RPM
- Vacuum pressure of 350 mBar
- Evaporation took between 1.5-2h to concentrate the hydrolyzate 2-fold

Hydrolyzate was recovered, pooled, then filtered through a 0.45+0.20 µm Sartopore2 Midi Cartridge filter to further clarify (precipitant was formed during the concentration) and sterilize the hydrolyzate used prior to fermentation. A similar process was taken for each batch of hydrolyzate listed in Figure LIF-5.9.

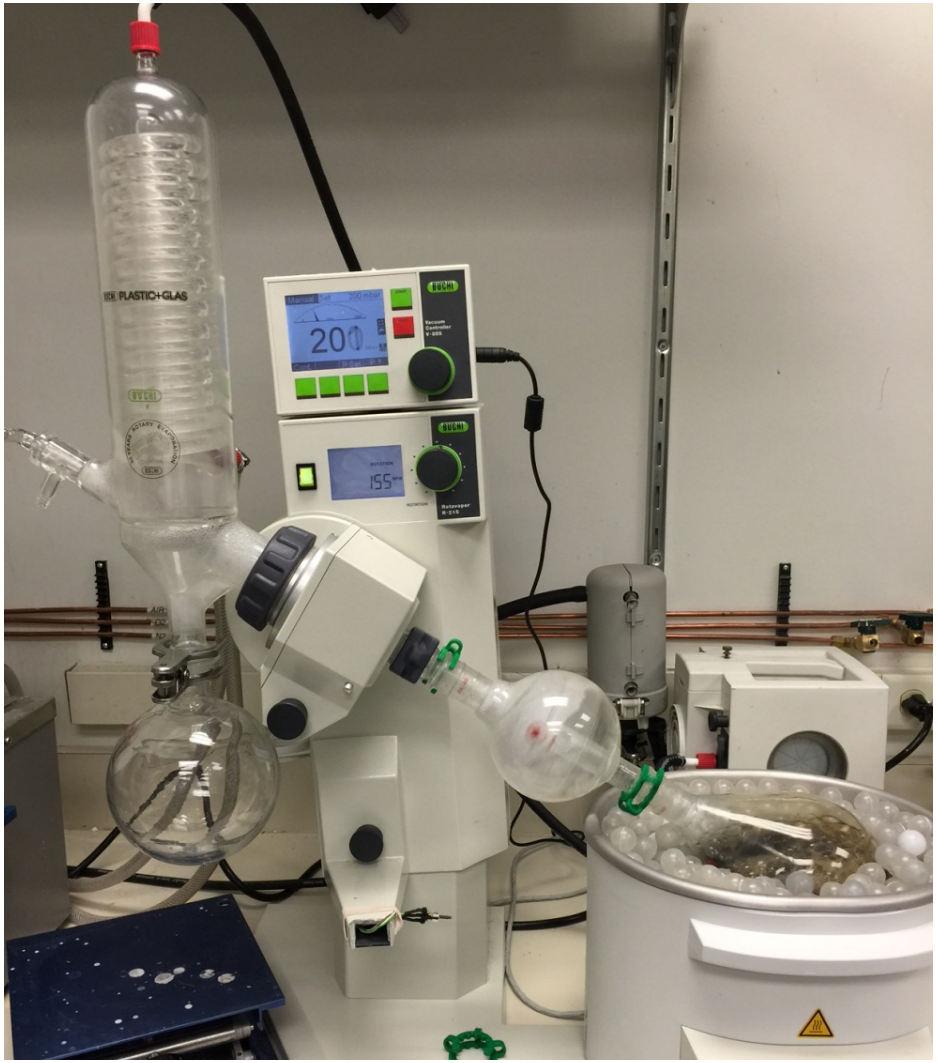

Figure LIF-5.9. RotoVap setup used to concentrate sugars in low solids hydrolyzate.

Fermentation of concentrated hydrolyzate, Batch #1

The fermentation was completed from 150923-150925. Here, a new Gevo biocatalyst, now named LB23, was used. This biocatalyst was one produced by the Gevo Strain Development program outside of the NARA project and was leveraged for NARA work because it performed better than the most current adapted strains. Samples were collected during the fermentation, and immediate data for iBuOH by GC, cell mass by hemocytometer count, and glucose by YSI were collected. Additional analyses by the Gevo Analytical team for HPLC (methods LC12 and LC9), IC (method IC2), and GC (method GC13) were collected the subsequent week for fermentation broth sugars, byproducts, and iBuOH heavy and light phase compositions, respectively. Fermentation of two baby GIFT® vessels was completed according to a run plan where nutrients, aeration, temperature, pH and agitation we all added or controlled. This was the first time running concentrated hydrolyzate. Due to scheduling limitations, we were unable to run a native hydrolyzate (unconcentrated) control. However, previous data from the Andritz 45-min FS-20 pretreated material (Ley et al., 2015) is indicative of unconcentrated hydrolyzate. In this experiment, no pre-growth or adaptation of the isobutanol biocatalyst was done. The fermentation was carried out as a "direct pitch" fermentation using yeast cream manufactured by Gevo.

The purpose of hydrolyzate concentration is to 1) increase the effectiveness of GIFT® operation by increasing the starting fermentation sugar concentration to 130-150 g/L glucose or higher and 2) reduce the number of fermentation runs during scale up required to produce iBuOH for the 1,000 gal IPK task.

Here, the fermentation was a direct pitch. Gevo isobutanol producing yeast strain LB23 was directly inoculated into concentrated hydrolyzate with no previous adaptation. Process improvements such as faster fermentation rates, reduced lag, and perhaps higher yield are likely if a pre-adaptation in 10-20% v/v hydrolyzate is included. The results of the fermentation are shown in Figure LIF-5.10.

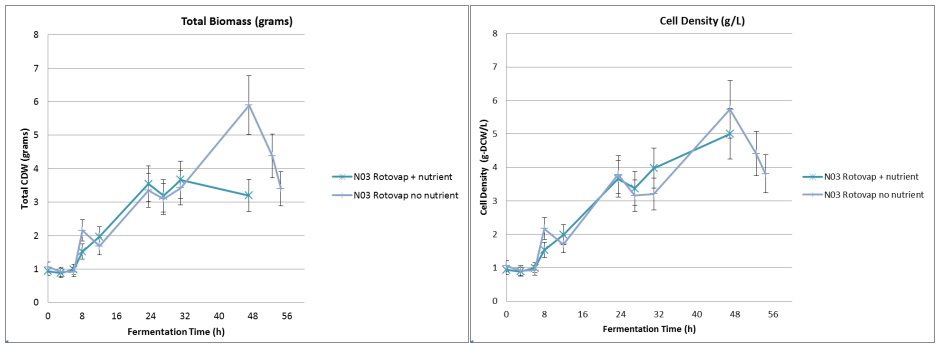

Figure LIF-5.10. Growth profiles for the concentrated hydrolyzate FS-20 ZeaChem NR-03 hydrolyzate.

Initially, 1 g/L CDW was inoculated into the fermentations. A ~8h lag was observed. At Fermentation Time of ~8h, a second dose of 1 g/L (total 2 g/L CDW) was added to the fermenters. This jump-started the fermentation as observed in growth, sugar consumption, and iBuOH production plots (Figure LIF-5.11).

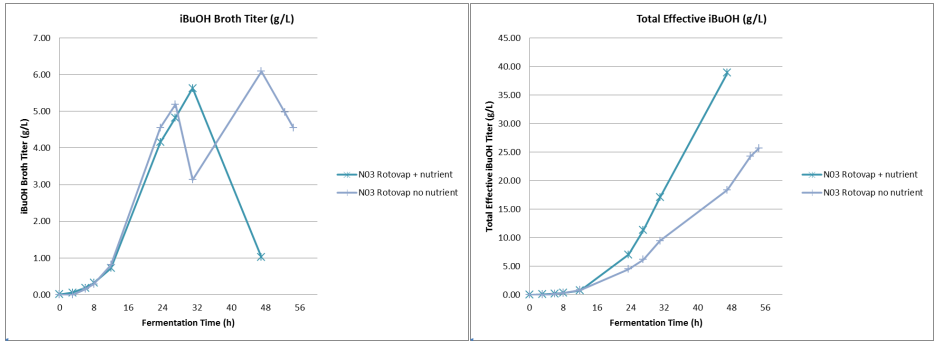

Figure LIF-5.11. iBuOH broth titer was controlled with GIFT®.

The only real difference between the two vessels plotted in Figures LIF-5.10, LIF-5.11, LIF 5.12, was addition of nutrient to the first vessel, and no nutrient to the second. The second vessel was diluted with evap. condensate from the RotoVap concentration process until the cells started producing. However, the lack of a complex nutrient had a negative impact on the fermentation performance. iBuOH broth titer in the first vessel decreased between the 33 and 48h sample point because sugar was exhausted overnight, but GIFT® continued to recover iBuOH from the broth (Figure LIF-5.11). However, note the 8-10h lag during the experiment shown in Figure LIF-5.12.

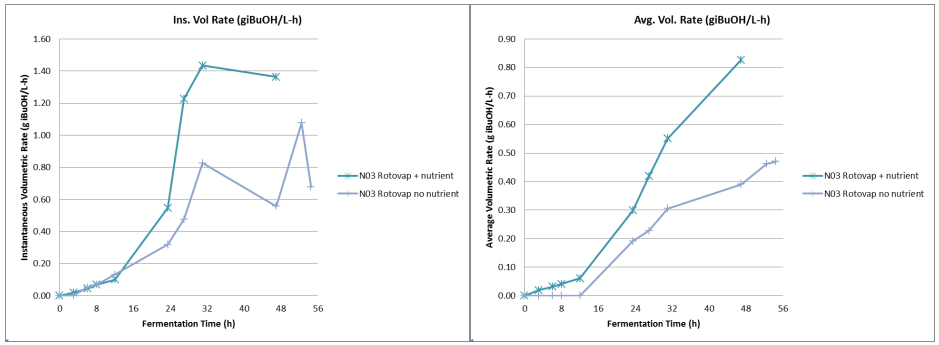

Figure LIF-5.12. Average volumetric rates (averaged over the entire fermentation time) are consistent with previous hydrolyzate fermentations.

We cannot yet explain this. Instantaneous volumetric rates (point-to-point) start low, but by 24h have increased to above 1 g/L/h. Note here again that sugar was exhausted sometime between the 33 and 48 h time point for the first vessel, as shown in Figure LIF-5.13. Acetate consumption and byproduct consumption are shown in Figures LIF-5.14 and LIF-5.15 respectively.

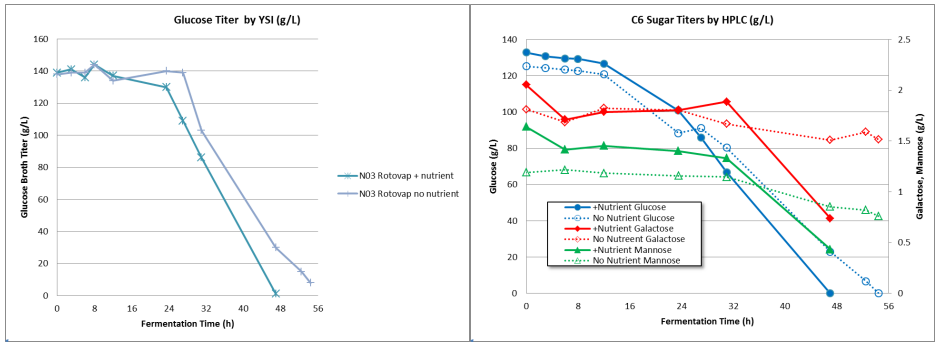

Figure LIF-5.13. Sugar concentration profiles, showing glucose by YSI measurement (left) and HPLC-12 method (right).

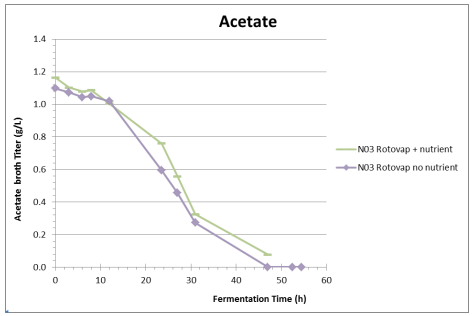

Figure LIF-5.14. Acetate was also consumed during the fermentation by the Gevo iBuOH yeast LB23.

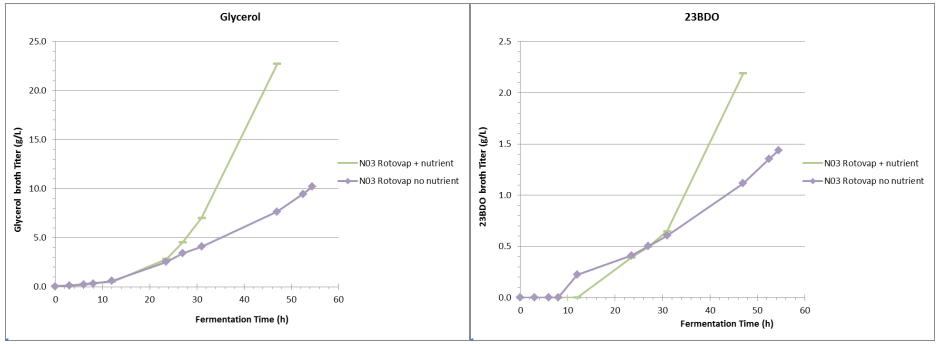

Figure LIF-5.15. An expected suite of byproducts were produced by the Gevo iBuOH yeast LB23 in these hydrolyzate samples.

The carbon balance was nearly closed for the vessel containing nutrient. However, the second vessel without nutrient showed poor carbon closure. We are continuing to analyze this data set to determine why the vessel without nutrient had poor closure. We recommend that we use nutrient in scale-up fermentations because other performance factors such as iBuOH yield and iBuOH volumetric rate were significantly enhanced compared to the conditions without this nutrient.

If we assume some loss of iBuOH and CO_2 from the nutrient supplemented fermentor that showed 92.5% carbon recovery and scale the carbon closure to 100%, the iBuOH yield falls in the standard range for LB23. This assumption is based on months of laboratory analysis and an error propagation analysis hunt that was performed at Gevo. A summary of volume and mass fractions of isobutanol collected during the 1L GIFT® fermentations is presented in Table LIF-5.1.

Table LIF-5.1. Summary of volume and mass fractions of isobutanol collected during the 1L GIFT® fermentations.

Vessel	B6	B7
Condition	w/ nutrient	No added nutrient
Total distil. Vol. (mL)	297.5	156.5
Light phase (mL)	12	12
Heavy phase (mL)	285.5	144.5
Light phase iBuOH (grams)	15.2	8.7
Heavy phase iBuOH (grams)	29	12.3

Volumes of heavy phase iBuOH were recovered due to controlling the fermentor broth titer at ~5-10 g/L and removing a higher proportion of water. Controlling the iBuOH broth titer at higher iBuOH concentrations will increase the proportion of isobutanol-rich light phase and decrease the amount of water removed.

Fermentation of concentrated v. unconcentrated hydrolyzate, Batch #2 & Batch #3

These fermentations were carried out using the isobutanol biocatalyst, LB23. The fermentation was completed from 151007-151008. Samples were collected during the fermentation and immediate data for iBuOH by GC, cell mass by hemocytometer count, and glucose by YSI were collected. Additional analysis by the Gevo Analytical team for HPLC (methods LC12 and LC9), IC (method IC2), and GC (method GC13) were collected the subsequent week for fermentation broth sugars, byproducts, and iBuOH heavy and light phase compositions, respectively. Fermentation of four baby GIFT® vessels was completed according to a run plan specified. Two conditions were tested; each in replicate fermenters. Unconcentrated hydrolysate material with nutrient supplementation was compared to concentrated hydrolysate material with nutrient supplementation. As with the previous fermentation, this fermentation was a direct pitch. Gevo isobutanol producing yeast strain LB23 was directly inoculated into concentrated hydrolyzate with no previous adaptation or growth. The results of the fermentation are as follows. All four vessels reached peak cell densities of approximately 6 g CDW/L.

Unconcentrated hydrolysate reached peak cell density within 12 hours. Concentrated hydrolysate grew slower than the un-concentrated condition and reached peak cell density by 24 hours (Figures LIF-5.16 & LIF-5.17). Un-concentrated conditions had total effective isobutanol titers of 16-19 g/L; average volumetric rate 1.2 to 1.3 g/L-h; and peak instantaneous rate of 1.8 g/L-h. Concentrated conditions had total effective isobutanol titers of 30 to 34 g/L; average volumetric rate 1.3 to 1.4 g/L-h; and peak instantaneous rate of 1.6 to 1.8 g/L-h (Figures LIF-5.18, LIF-5.19, LIF-5.20, LIF-5.21). All vessels consumed all available sugar by 27 hours. YSI indicated no free sugar remaining at 27h, and the process Mass Spec used to monitor off-gas composition indicated drastically reduced CO_2 evolution compared to peak values. pH also increased indicating carbon source scavenging (no sugar). Yield to isobutanol from glucose was within expected range (Figures LIF-5.22, LIF-5.23, LIF-5.24, LIF-5.25). Concentrations for acetate, glycerol, 2,3-butanediol and Isobutyrate are provided in Figures LIF-5.26, LIF-5.27, LIF-5.28 and LIF-5.29 respectively.

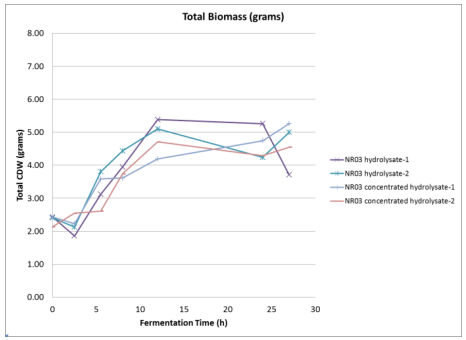

Figure LIF-5.16. Total Biomass.

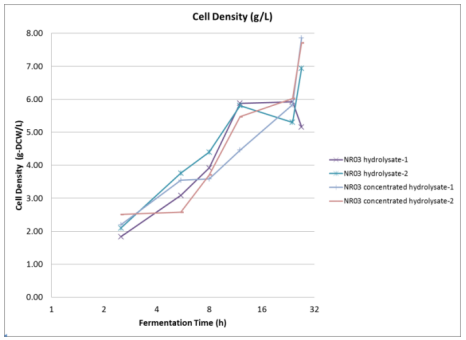

Figure LIF-5.17. Cell Density.

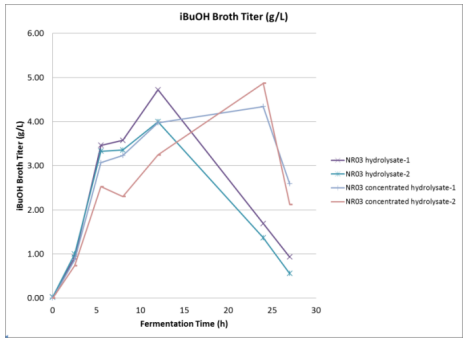

Figure LIF-5.18. Isobutanol broth titer.

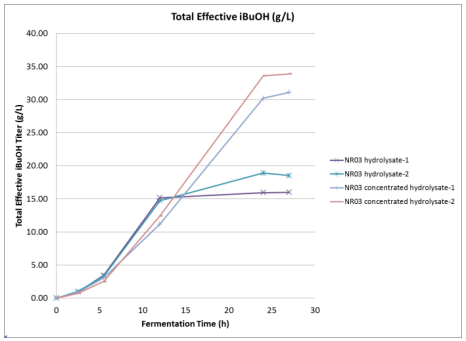

Figure LIF-5.19. Total effective isobutanol.

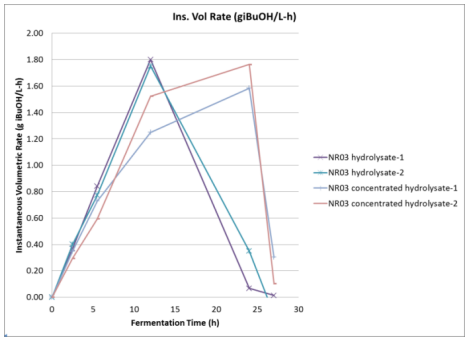

Figure LIF-5.20. Instantaneous volumetric iBuOH rate.

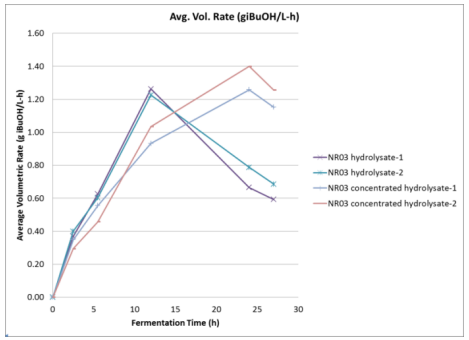

Figure LIF-5.21. Average volumetric iBuOH rate.

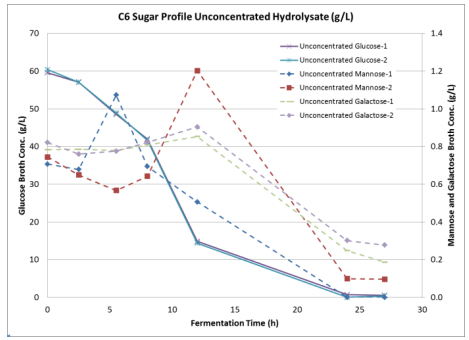

Figure LIF-5.22. Unconcentrated 6 C sugar profile.

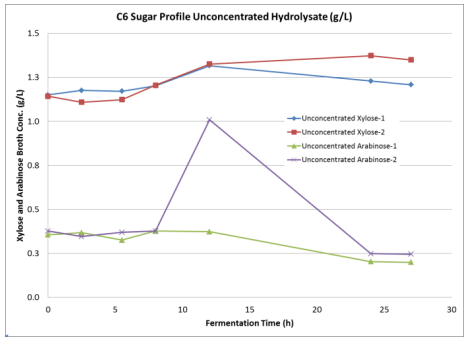

Figure LIF-5.23. Unconcentrated 5 C sugar profile.

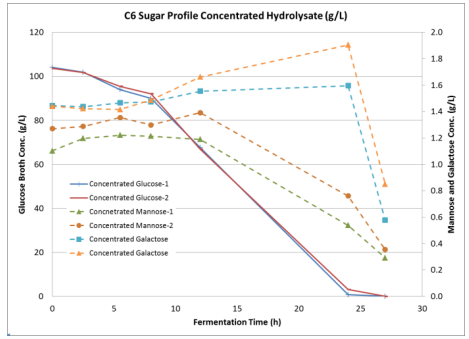

Figure LIF-5.24. Concentrated 6 C sugar profile.

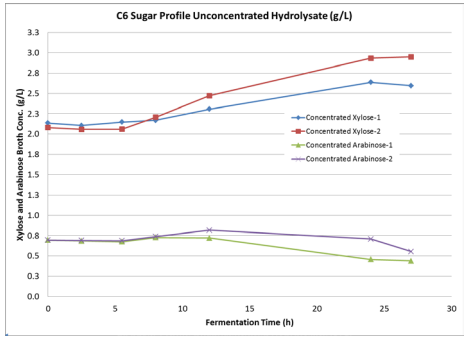

Figure LIF-5.25. Concentrated 5 C sugar profile.

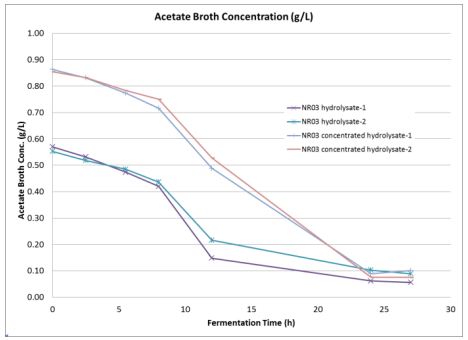

Figure LIF-5.26. Acetate broth concentration.

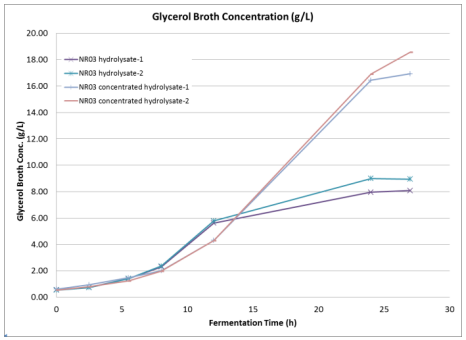

Figure LIF-5.27. Glycerol broth concentration.

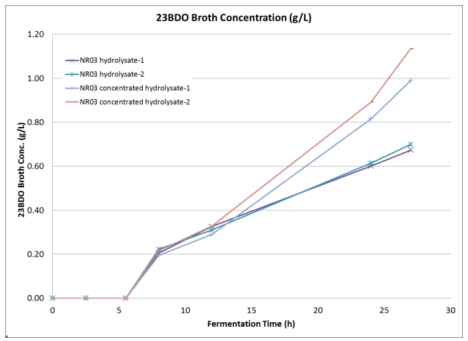

Figure LIF-5.28. 2,3-Butanediol broth concentration.

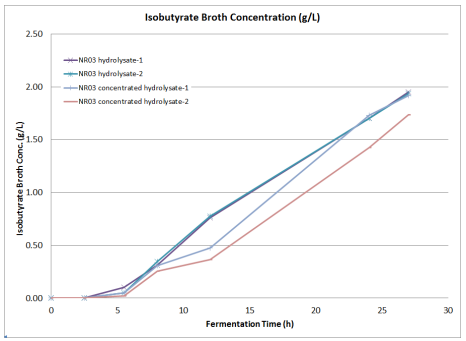

Figure LIF-5.29. Isobutyrate broth concentration.

The carbon closures based on all analytes that were tracked were in the 85%-100% range. The carbon closures of less than 100% were adjusted the balance to 100% closure by making the assumption that all missing carbon can be accounted for as isobutanol and CO_2. This assumption and error propagation analysis on isobutanol has previously been validated in labs at Gevo. Due to mechanical limitations at the 1L scale, it is not possible to trap and/or quantitate all iBuOH and CO_2 from the system.

Fermentation of Cosmo Reject Fiber Hydrolyzate Filtered vs. Autoclaved

Gevo received samples of a reject fiber stream from Cosmo Specialty Fibers, Inc. This reject stream is ordinarily a waste stream and would be used to fire boilers for the process at Cosmo. Generating a value-added product, like isobutanol and/or biojet, could prove advantageous. Further, Cosmo as indicated a willingness to help make the NARA project successful and this reject fiber stream has already been pre-treated by a method that is similar to SPORL, yet different enough to test in the lab.

The fermentation with the Cosmo fibers was completed from 151029-151030. The objective was to evaluate fermentation performance of Cosmo hydrolyzate and compare material that has been autoclaved (steam sterilized at pressure) at 121 °C for 20 minutes with the nutrient package already added (except for one component) to filter sterilized material. It was hypothesized that growth and isobutanol production will be similar regardless of sterilization conditions used. However, these two options are significantly different in a larger scale with regard to ease of execution and costs. Samples were collected during the fermentation, and immediate data for iBuOH by GC, cell mass by hemocytometer count, and glucose by YSI were collected. Additional analysis by the Gevo Analytical team for HPLC (methods LC12 and LC9), IC (method IC2), and GC (method GC13) were collected the subsequent week for fermentation broth sugars, byproducts, and iBuOH heavy and light phase compositions, respectively. Fermentation of two baby GIFT® vessels was completed according to the run plan established. Two conditions were tested; each in single fermenters. Filtered Cosmo hydrolysate material with nutrient supplementation was compared to Autoclaved Cosmo hydrolysate material with nutrient supplementation. Again, the fermentation was a direct-pitch fermentation. Gevo isobutanol producing yeast strain LB23 was directly inoculated into Cosmo hydrolyzate with no previous adaptation or growth on the material. The results of the fermentation are as follows. Both vessels reached peak cell densities of approximately 4 g CDW/L. Both conditions exhibited similar growth rates and peak cell densities (Figures LIF-5.30 and LIF-5.31).

Filtered conditions: total effective isobutanol titer of 12.8 g/L; average volumetric rate 0.82 g/L-h; and peak instantaneous rate of 1.1 g/L-h. Autoclaved conditions: total effective isobutanol titers of 12.1 g/L; average volumetric rate 0.78 g/L-h; and peak instantaneous rate of 0.9 g/L-h (Figures LIF-32, LIF-33, LIF-34, LIF-35). All vessels consumed all available sugar by 15.5 hours. YSI indicated no free sugar remaining at 15.5h, the process mass spec indicated drastically reduced CO_2 evolution compared to peak values, and pH increased indicating carbon source scavenging at that time point. All were indications that sugar was exhausted (Figures LIF-36 and LIF-37). Concentrations for acetate, glycerol, 2,3-butanediol and isobutyrate are provided in Figures LIF-5.38, LIF-5.39, LIF-5.40 and LIF-5.41 respectively. Yield to isobutanol met expected mass/mass levels. Fermentation performance in terms of yeast growth and isobutanol production was similar regardless of sterilization method used (autoclaved or filter sterilized). Batch sterilization using heat should be sufficient as long as nutrient is added after heat sterilization and total heat history is kept to a minimum.

The raw carbon closure for Cosmo hydrolysis fermentations were 85-95% closed, then adjusted to 100% closure by assuming that missing carbon is accounted for as isobutanol and CO_2. See previous sections for an explanation on this approach.

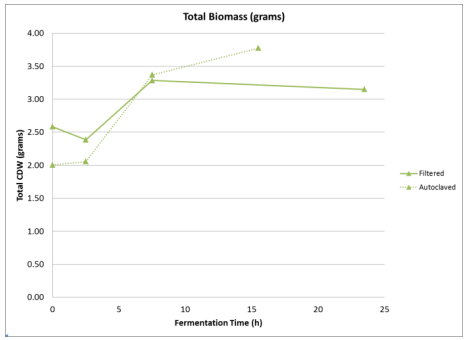

Figure LIF-5.30. Biocatalyst cell mass.

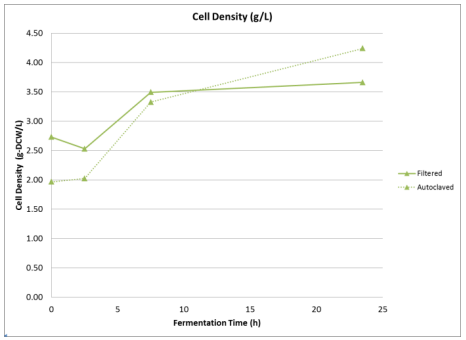

Figure LIF-5.31. Biocatalyst cell density.

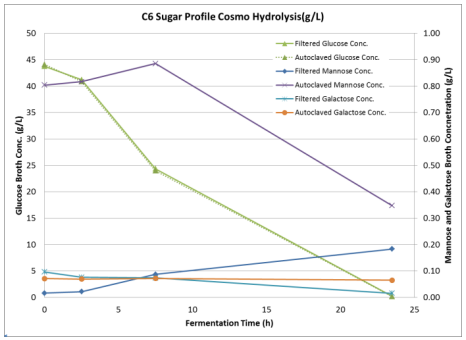

Figure LIF-5.36. 6 C sugar profile.

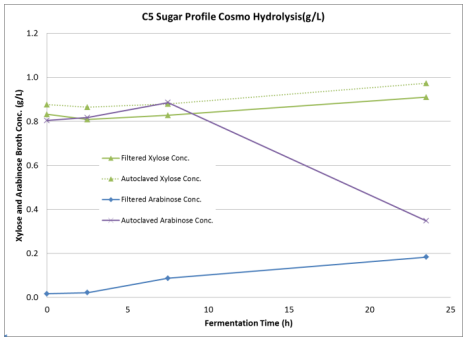

Figure LIF-5.37. 5 C sugar profile.

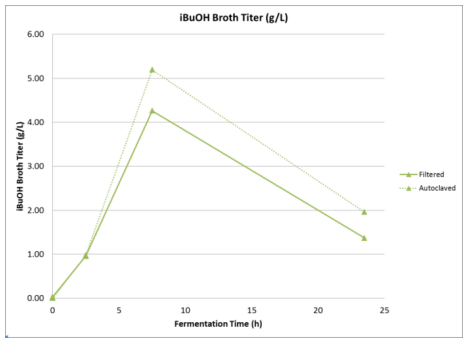

Figure LIF-5.32. Isobutanol broth concentration.

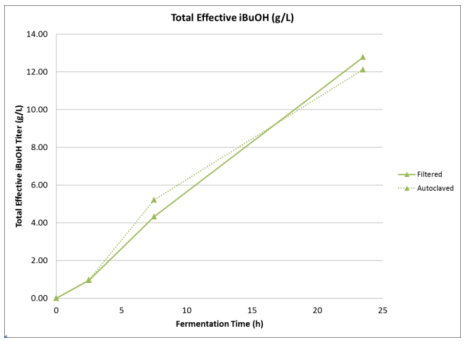

Figure LIF-5.33. Total isobutanol concentration.

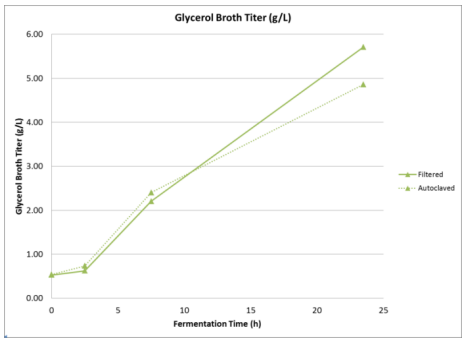

Figure LIF-5.38. Glycerol broth concentration.

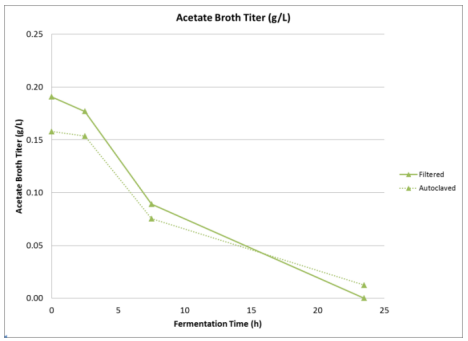

Figure LIF-5.39. Acetate broth concentration.

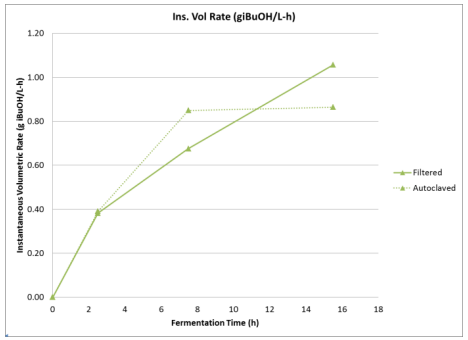

Figure LIF-5.34. Instantaneous volumetric iBuOH rate.

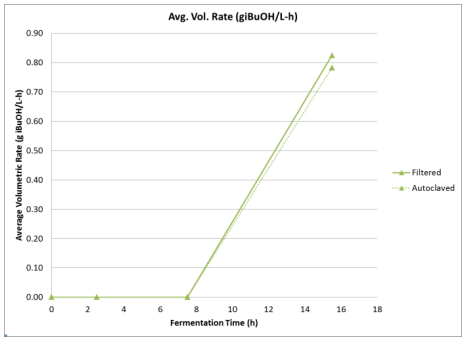

Figure LIF-5.35. Average volumetric iBuOH rate.

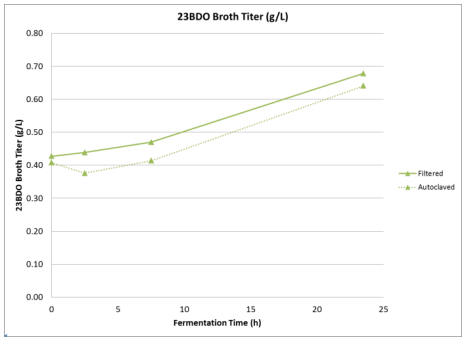

Figure LIF-5.40. 2,3-Butanediol broth concentration.

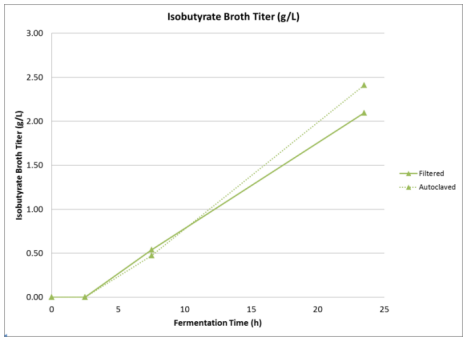

Figure LIF-5.41. Isobutyrate broth concentration.

TASK 6: ANALYSIS OF ISOBUTANOL PRODUCED TO CLOSE MASS BALANCE AND DETERMINE POTENTIAL LOW-LEVEL IMPURITIES

Analysis and impurity tracking were optimized through fermentation development to close the mass balance, decrease impurities, and increase isobutanol yield. Production of isobutanol from 1L GIFT® fermentations using Douglas-fir biomass sugars has provided relevant samples to compare to corn starch derived isobutanol for analysis. Preliminary analysis of isobutanol quality from Douglas fir biomass and corn starch is similar. Analysis of isobutanol to produce an optimized product for conversion to biojet is ongoing and will continue at each scale-up.

Isobutanol collected from FS-10 (CMW) hydrolyzate lab-scale GIFT® fermentations were analyzed by gas chromatography for impurities. Table LIF-6.1 shows very similar profiles for isobutanol produced from the mock hydrolyzate (pure sugars) medium and FS-10 CMW hydrolyzate. Consultation with the Gevo biojet conversion team did not identify any impurities that would be detrimental to the biojet conversion process. Work in this area will continue when the FS-17 feedstock using a single pretreatment method is fermented using GIFT® technology for process development work, scale-up, and production of ~1,000 gal biojet.

iBuOH Product Quality Analysis

The isobutanol heavy phase (water-rich) and light phase (iBuOH-rich) from Fermentation #1 (see Task 5: Produce isobutanol in 1L GIFT® fermentation from pretreated biomass sugars using the adapted yeast biocatalyst) were analyzed by method GC-13 for components (see Table LIF-6.2.). Additional data for these products are required to calculate mass percentage of each product. However, relative area from the GC does give an inventory of the compounds present in the iBuOH heavy and light phase products (Figure LIF-6.1). For perspective – this composition looks similar to known, acceptable light phase that has been produced at Gevo's commercial plant in Luverne, MN using corn mash. No unexpected components were observed. Note that some unknowns that represent a very small fraction of the total are ob-served. We will continue to analyze and try to identify these unknowns.

Table LIF-6.1. Impurity profile of isobutanol produced in FS-10 CMW mock and 100% v/v FS-10 CMW hydrolyzate. Materials were analyzed by gas chromatography.

	FS-10 CMW Mock Media (Weight %)	FS-10 CMW Hydrolyzate (Weight %)
Methanol	0.0	0.0
Ethanol	3.0±0.2	3.9±0.7
Acetone	0.0	0.0
Isopropanol	0.0	0.0
1-Propanol	0.1	0.1
Isobutyraldehyde	0.2	0.1±0.1
2,3-Butanedione	0.0	0.0
2-Butanone	0.0	0.0
2-Butanol	0.0	0.0
Isobutanol	76.8±0.2	75.6±1.0
1-Butanol	0.0	0.0
Acetoin (3-Hydroxy-2-butanone)	0.0	0.0
3-Methyl-1-Butanol	1.1±0.1	1.0±0.1
2-Methyl-1-Butanol	0.6	0.5±0.1
Isobutyl Acetate	0.0	0.0
Isopentyl Acetate	0.0	0.0
Isobutyl Isobutyrate	0.0	0.0
2,3,5-Trimethylpyrazine	0.0	0.0
2,3,5,6-Tetramethylpyrazine	0.0	0.0
2-Phenylethanol	0.1	0.1
Phenethyl Acetate	0.0	0.0
All Unknown Peaks	0.1	0.2±0.1
Density	82.0±0.2	81.6±0.3
Water (weight %)	18.1±0.2	18.6±0.4
Water (Vol %)	21.4±0.5	21.8±0.3

Table LIF-6.2. Low level impurities present in the light and heavy phase GIFT condensate from fermentation experiments reported in Task 5: Produce isobutanol in 1L GIFT® fermentation from pretreated biomass sugars using the adapted yeast biocatalyst.

		B7 Light Phase	B7 Heavy Phase	B6 Light Phase	B6 Heavy Phase
No.	Peakname	Rel.Area	Rel.Area	Rel.Area	Rel.Area
		%	%	%	%
1	Methanol	0	0.06	0	0.03
2	n.a.	0	0	0	0.16
3	ethanol	0.28	2.3	0.29	2.22
4	1-Propanol	0.05	0.13	0.03	0.07
5	Isobutyraldehyde	0.06	0.06	0.04	0.03
6	2,3-Butanedione	0	0.01	0	0
7	isobutanol	96.75	96.44	95.97	96.13
8	n.a.	0.01	0	0	0
9	1-butanol	0.01	0	0	0
10	n.a.	0.03	0	0	0
11	3-Hydroxy-2-Butanone	0	0	0	0.04
12	n.a.	0.02	0	0	0
13	3-Methyl-1-Butanol	1.93	0.69	2.7	0.96
14	2-Methyl-1-Butanol	0.73	0.27	0.62	0.23
15	Isobutyl Acetate	0.02	0	0.01	0
16	2-Phenylethanol	0.1	0.05	0.33	0.13
Total:		100	100	100	100

n.a. – not identified, a.k.a. unknown peak.

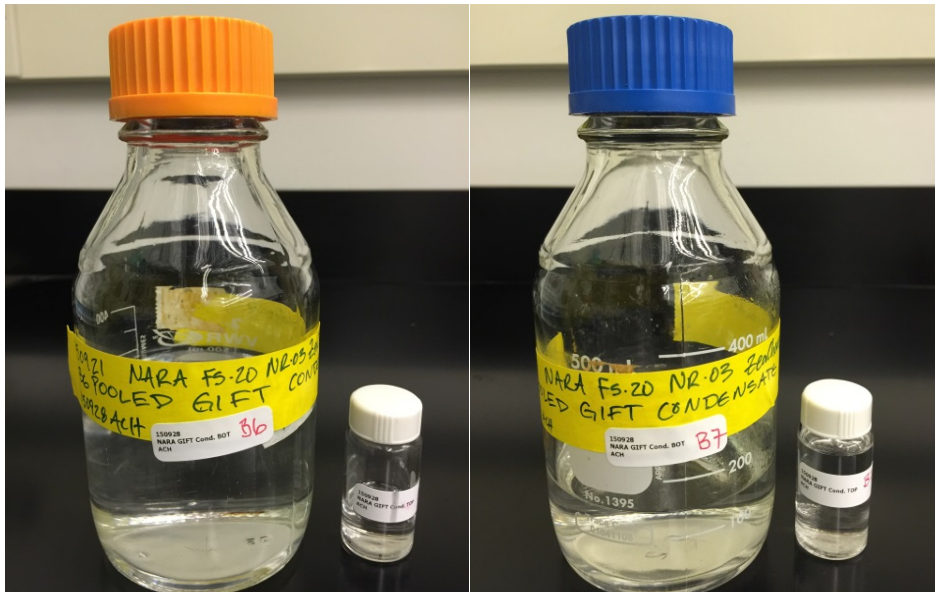

Figure LIF-6.1. Physical appearance of the light phase and heavy phase from the 150923 baby GIFT® fermentations with concentrated hydrolyzate from Batch #1. Heavy phase containing water, isobutanol, and other components is depicted on the left side of each photo. Light phase containing isobutanol, water, and other components is depicted on the right side of each photo. The left panel shows vessel B6 GIFT® condensate, which contained nutrient. The right panel shows vessel B7 GIFT® condensate, which contained no nutrient.

iBuOH Product Quality Analysis

Samples from the 1kIPK task (C-AF-1.8) were delivered to Gevo for testing and anal-ysis at various stages of the scale-up process. Additional information can be found in the final report authored by Dr. Robert Wooley on the 1kIPK task (Wooley et al, 2016). Gevo conducted tests for alcohols by GC, acids by potentiometric titration, density, and water by Karl Fisher. The objective of these tests was to determine along the way and with final product how closely the isobutanol produced meets the fuel grade specification. The fuel grade specification is shown in Figure LIF-6.2.

gevo

Product Specifications

Product Code: IBF001
Product Description: Fuel-Grade Isobutanol

Synonyms: isobutanol, isobutyl alcohol, 2-methyl-1-propanol	**Chemical Formula:** $C_4H_{10}O$
Molecular Weight: 74.12 g/mol	**CAS Number:** 78-83-1

Physical Properties	Methods	Specification	Units
Isobutanol	Annex A1	96 min	vol %
Methanol	Annex A1	0.4 max	vol %
Water by Karl Fischer (%)	ASTM E1064	1.0 max	vol %
Acid Number (as Acetic Acid)	ASTM D1613	0.007 max	mass %
Inorganic Chloride Content	ASTM D512	8 max	ppm
Solvent Washed Gum	ASTM D381	5 max	mg/100 mL
Sulfur Content	ASTM D5453	30 max	ppm
Sulfate Content	Annex A2	4 max	ppm

DRAFT

Note: Commercial processes used to manufacture isobutanol from biological feedstocks typically yield some fusel alcohols such as pentanol and other higher alcohols.

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Figure LIF-6.2. Gevo Fuel-Grade isobutanol specification listing testing methods required for each component and limits on each component.

The specification indicates a minimum of 96% isobutanol, <1% water, and several other metrics that are to be met when the isobutanol is used as a motor-fuel. However, isobutanol that meets this specification can also be used as the feedstock to convert to IPK. The isobutanol heavy phase (water-rich) and light phase (iBuOH-rich) from fermentations performed at ICM were analyzed. Further, all heavy phase was re-processed using Gevo's GIFT® system in the ICM facility in St Joseph, MO and converted to light phase. Multiple 300 gal totes of light phase were produced and sampled for analysis by Gevo. No unexpected components were observed. Note that some unknowns that represent a very small fraction of the total were observed. We will continue to analyze these unknowns. ICM's facility did not have the proper equipment (distillation) to fully dehydrate the iBuOH beyond light phase. Thus, a third party vendor (WhiteFox) was used for this final step. A representative analysis is shown below in Table LIF-6.3 for light phase that was sent to WhiteFox for further dehydration. Photos of the samples of final light phase totes are also shown below in Figure LIF-6.3.

Table LIF-6.3. Representative analysis of isobutanol light phase produced at ICM during the 1kIPK scale up task and analyzed at Gevo.

C0310-827	Area	Mass %	Vol %
Methanol	0.0117	0.02	0.02
Ethanol	0.3631	0.32	0.34
Acetone	0.0207	0.02	0.02
Isopropanol	0.0158	0.01	0.01
1-Propanol	0.1563	0.11	0.11
Isobutyraldehyde	0.0606	0.07	0.07
2,3-Butanedione		0.00	0.00
2-Butanone		0.00	0.00
2-Butanol		0.00	0.00
Isobutanol	148.9085	83.64	86.04
1-Butanol	0.0074	0.00	0.00
Acetoin (3-Hydroxy-2-butanone)		0.00	0.00
3-Methyl-1-Butanol	3.76	2.23	2.27
2-Methyl-1-Butanol	0.701	0.39	0.40
Isobutyl Acetate	0.0159	0.01	0.01
Isopentyl Acetate		0.00	0.00
Isobutyl Isobutyrate		0.00	0.00
2,3,5-Trimethylpyrazine		0.00	0.00
2,3,5,6-Tetramethylpyrazine		0.00	0.00
2-Phenylethanol	0.0187	0.01	0.01
Phenethyl Acetate		0.00	0.00
All Unknown Peaks	0.0596	0.02	0.02
Density	0.825	86.86	89.32
Water (weight %)	13.16		
Water (Vol %)	10.86		
Acid Number (weight %)	0.01		
Acid Number (Vol %)	0.01		
Chloride (ppm)			
Sulfate (ppm)			
Total Closure (%)		**100.02**	**100.18**

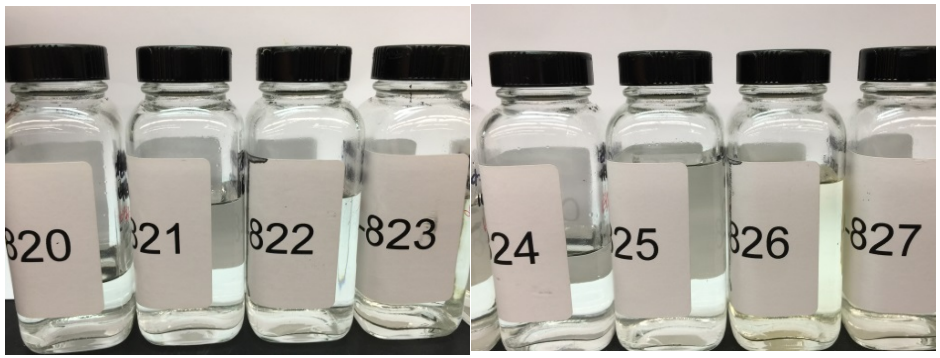

Figure LIF-6.3. Physical appearance of isobutanol product samples received from ICM and tested by Gevo

TASK 7: OPTIMIZE PROCESS PARAMETERS FOR ISOBUTANOL FERMENTATION FROM PRETREATED BIOMASS

Originally, this task was envisioned as a process scale-up task to bridge from Gevo's 1L baby GIFT® fermenters to a larger scale (20L) before scale up to demonstration scale at 40,000 L. The original task was entitled "Produce isobutanol in 20L GIFT® SSF fermentation from pretreated biomass". At the beginning of Year 4 of the NARA project, Gevo and NARA agreed that this task was no longer needed because Gevo had been successful in scaling directly from 1L baby GIFT fermentations in Gevo's lab to commercial scale at Gevo's commercial plant in Luverne, MN. The advantage to the project was multifold: 1) less pretreated feedstock was required to remain at the 1L baby GIFT scale, which allowed pretreatment teams to focus on optimization instead of production of larger scale of material 2) less material shipping and handling (all at a cost) between NARA partners was required 3) Gevo could conduct more experiments and more replicates to improve technical robustness at the 1L baby GIFT scale as compared to the 20L scale. Nevertheless, a 20L GIFT® pilot scale system was designed and built, but not used for process optimization.

Beginning in Year-4, effort was put towards defining process conditions such as hydrolyzate loading during growth and production, and nutrient package development. This process information will be applicable to scale up from the 1L GIFT® scale and could be applied to producing isobutanol in the 20L GIFT® fermentation system as well as producing 1,000 gallons of biojet. Gevo, in consultation with NARA (Dr. Robert Wooley), no longer believes a step up to the 20L scale is required. Gevo has successfully scaled from 1L to hundreds of thousands of L directly. This, scaling from 1L GIFT® to ~23,000 L demonstration scale fermentations is directly feasible. Upon receiving a large pretreated FS-17 sample (15-20 kg) which resembles the conditions expected during the 1,000 gallon biojet production process, a 20L GIFT® fermentation experiment could be conducted if deemed necessary. Process development (including nutrient package development) will continue at the 1L scale.

Nitrogen supplementation using a variety of inorganic and organic (complex) nitrogen sources as well as supplemented with vitamins and minerals were tested using the LB21 biocatalyst. Based on the previously reported isobutanol productivity results, nutrient package (NP) 2.0 supplemented with a nitrogen source during propagation/growth and fermented in hydrolyzate containing NP 2.0 will create optimal conditions for isobutanol titer and volumetric isobutanol productivity (Figure LIF-7.1).

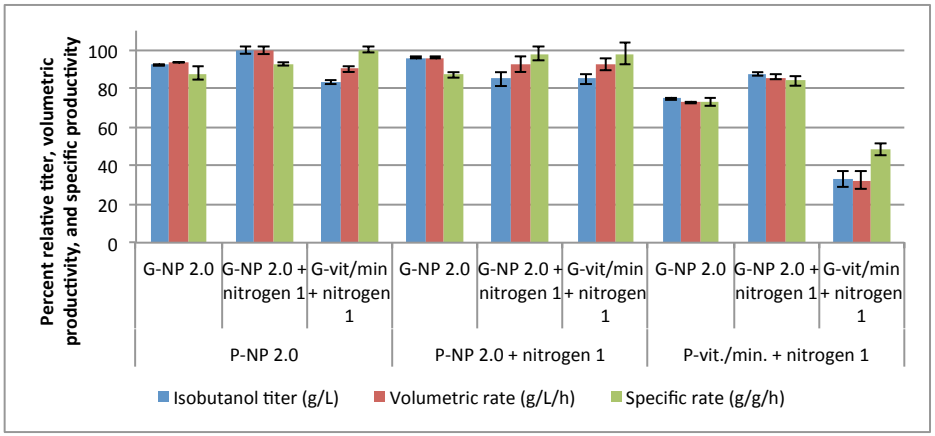

Figure LIF-7.1. LB21 isobutanol metrics during nitrogen source optimization for isobutanol production. LB21 was grown (G) under high aeration conditions for 23 hours in shake flasks in either NP 2.0, NP 2.0 + nitrogen 1, or vit/min + nitrogen 1. After 23 hours of growth, production (P) was started with LB21 by transferring 1:4 volumetrically into shake flasks containing NP 2.0, NP 2.0 + nitrogen 1, or vit/min + nitrogen 1. All permutations of growth and production were tested for the different nitrogen media. The fermentation occurred under low aeration conditions for 24 hours. All media contained the same buffering agent Growth was carried out at 33ºC. Cell density was measured using a spectrophotometer. Error bars represent the standard deviation. Abbreviations: NP 2.0, yeast nutrient package containing complex nitrogen source; nitrogen 1, inorganic nitrogen source; vit/min, yeast vitamin and mineral mix essential for growth.

In April/May 2014, three various industrially relevant operating pH ranges for growth of LB21 were also tested using 40% (v/v) FS-10 SPORL-Na^+ pretreated hydrolyzate (previously labeled unwashed mild bisulfite solids, UMBS). Growth at pH C resulted in 40% and 22% higher biomass yield compared to pH A and B, respectively. After 23 hours of growth/propagation at the pH A/B/C the cultures were transferred into 100% (v/v) FS-10 SPORL-Na^+ pretreated hydrolyzate held at the same pH. Volumetric isobutanol productivity was 80% and 40% higher at pH C compared to pH A and pH B, respectively (Figure LIF-7.2).

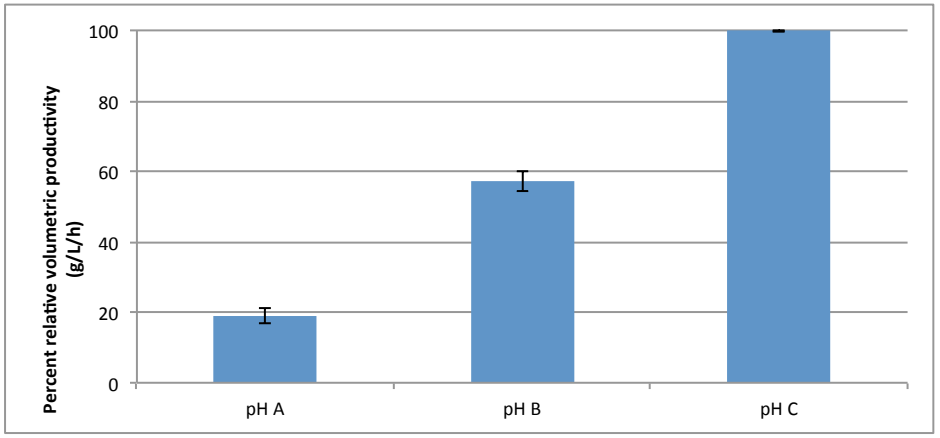

Figure LIF-7.2. LB21 volumetric isobutanol productivity during fermentation at different industrially relevant pH conditions in 85% v/v FS-10 SPORL-Na+ pretreated unwashed solids hydrolyzate. To replicate potential commercial production conditions a 1:4 volumetric transfer from the 40% v/v FS-10 SPORL-Na+ pretreated unwashed solids hydrolyzate growth flask at pH A, B, and C into 100% v/v FS-10 SPORL-Na+ pretreated unwashed solids hydrolyzate fermentation flask at pH A, B, and C was carried out to yield an 85% v/v FS-10 SPORL-Na+ pretreated unwashed solids hydrolyzate medium. All media contained nutrient package 2.0 (NP 2.0). All conditions contained a buffering agent. Growth was carried out at 33ºC. Cell density was measured using a spectrophotometer. Error bars represent the standard deviation of 3 replicates.

TASK 8: PRODUCE ≥1000 GALLONS ISOBUTANOL FROM GIFT® SSF FERMENTATIONS AT 40,000 L DEMONSTRATION SCALE. CONVERT LIGNOCELLULOSIC ISOBUTANOL TO ≥ 1000 GALLONS BIOJET FOR FURTHER TESTING

The scale-up process for this task will begin as soon as the FS-17 pretreated material arrives in sufficient quantities at Gevo in Year 4 or 5. In an effort to determine if a direct pitch of LB23 into a production vessel was feasible, a 1L GIFT® fermentation using 85% (v/v) FS-10 SPORL-Mg^{2+} pretreated hydrolyzate (32% solids) containing NP 2.0 was performed. The initial inoculation cell density of LB23 was identical to a similar LB21 1L GIFT® fermentation run but unlike the LB21 fermentation, the LB23 inoculum was not pre-conditioned to the hydrolyzate and a slight lag in growth and isobutanol production was noted. The amount of isobutanol produced by LB23 compared to LB21 in FS-10 SPORL-Mg^{2+} pretreated hydrolyzate (Figure LIF-8.1) was greater in large part because of the nearly 50% higher hexose sugar concentration in the LB23 fermentation (89 g/L vs. 172 g/L). Also, LB23 has a 17% higher theoretical isobutanol production yield compared to LB21 meaning that more hexose sugar is being converted to isobutanol compared to other metabolic co-products. Finally, under the direct pitch conditions, LB23 had a 20% higher volumetric isobutanol productivity compared to LB21.

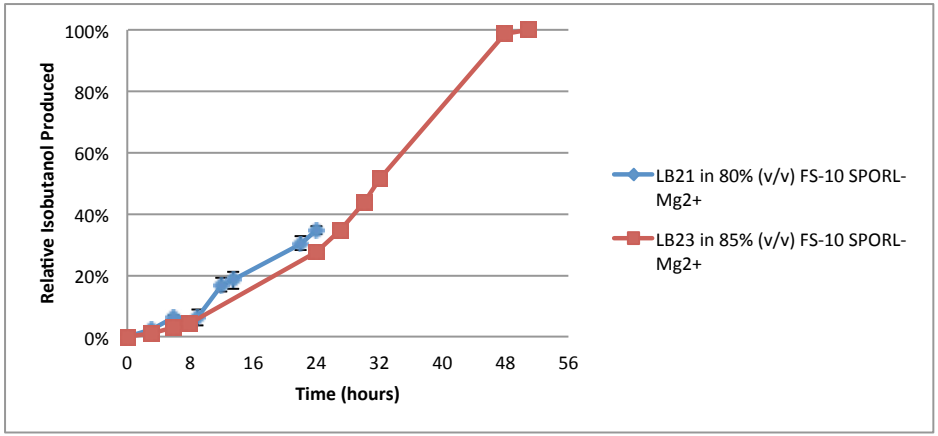

Figure LIF-8.1. Isobutanol produced by LB21 in 80% v/v FS-10 SPORL-Mg2+ pretreated and LB23 in 85% v/v FS-10 SPORL-Mg2+ pretreated hydrolyzate. Production of isobutanol was conducted in 1L fermentation vessels equipped with GIFT®, held at 33°C and pH controlled. Medium was created using the corresponding mock media as the balance of 100%. LB21 was pre-conditioned in 20% v/v FS-10 SPORL-Mg2+ pretreated hydrolyzate before inoculating 80% v/v hydrolyzate compared to LB23 which was directly pitched into 85% v/v hydrolyzate. Error bars represent the standard deviation of two replicates. LB21 in 80% FS_10 SPORL-Mg2+ fermentation was run until all consumable sugars were exhausted.

LB23 is the current best NARA and Gevo biocatalyst. Therefore, LB23 will be utilized going forward in the 1,000 gallon biojet production. Process optimization will be pursued aggressively in the time remaining in Year 4 and into Year 5 to maximize the performance of the LB23, or LB23 hydrolyzate evolved strains, yeast biocatalyst in FS-17 SPORL pretreated hydrolyzate.

Additional Tasks Not Included in Gevo's Original Scope:

Collaboration with NARA Environmentally Preferred Products Team (P. Smith, Penn State University)

Gevo has interfaced with the NARA EPP Team and in particular with Dr. Paul Smith and a MBA student team project entitled Bio-APEX at Penn State University to develop basic understanding of biojet market potential and go-to market strategies for the Pacific Northwest. A proposal to Penn State's MBA Program Capstone Course, APEX (Applied Professional EXperience), was developed and implemented Spring Semester 2012. The class project, "Bio-APEX", consists of a 5-member MBA student team under the direction of Paul Smith (Penn State), Andrew Hawkins (Gevo) and Jeff Scheib (Gevo).

Bio-APEX Project Expectations:
At the conclusion Spring Semester, 2012, the Bio-APEX team will present its recommendations and provide a written report detailing the analysis and intuition upon which the recommendations are predicated, including the following:

1. A Strategic Perspective of the Market Opportunity: This section examines the current market and market projections. The market will be segmented by geographic regions within the Pacific Northwest, as well as consumer type (i.e. Commercial, Military, Civilian) and includes market insights relating to customers, types of fuels, regulatory and macro-economic drivers, and market barriers.

2. Identification of Key Success Factors to Lead in Biojet in the Pacific Northwest: This section examines the qualities and capabilities that are required to be a leader in the biojet market today and within a 5-10 year horizon. Items may include company attributes and positioning, pace to scale, partnerships, and infrastructure.

3. Go To Market Strategy: This section provides a specific action plan to enter the biojet market including recommendations and rationale regarding geographic and customer segment strategies, channels, key market adoption enablers, and a market access pathways phase-in plan.

4. Definition of Value Proposition: This section examines key messages for a new biofuels entrant in terms of the product's features, advantages, and benefits. Specific pitch propositions may also include market based needs, overcoming price sensitivity, and overcoming the inertia of Petro-jet.

5. Supply Chain Optimization: This section addresses the key levers in the current supply chain, which hold the highest opportunity for optimization initiatives.

ASTM Completes Revision of Standard Specification Gevo's Alcohol to Jet Fuel Now Eligible to be Used for Commercial Flights

Gevo, Inc. (NASDAQ:GEVO), announced on April 12, 2016 that ASTM International has now completed its process of approving the revision of ASTM D7566 (Standard Specification for Aviation Turbine Fuel Containing Synthesized Hydrocarbons) to include alcohol to jet synthetic paraffinic kerosene (ATJ-SPK) derived from renewable isobutanol. As previously announced on March 28, 2016, the proposed revision passed on the committee ballots, but ASTM still needed to complete the Society Review, perform a final ballot tally, and publish the new specification. The ASTM process is now completed in all respects.

As a result, ASTM International has published the revision of ASTM D7566 (Standard Specification for Aviation Turbine Fuel Containing Synthesized Hydrocarbons) on its website (http://www.astm.org/Standards/D7566.htm) and Gevo's renewable alcohol to jet fuel ("ATJ") is now eligible to be used as a blending component in standard Jet A-1 for commercial airline use in the United States and in many other countries around the globe. Gevo's ATJ is eligible to be used for up to a 30% blend in conventional jet fuel for commercial flights.

NARA OUTPUTS

- *2014 :Andrew Hawkins presented at the Joint USDA AFRI CAP/AAIC Meeting in Washington, DC.*
- *2013: Grant Balzer presented at the Harvesting Clean Energy Conference in Helena, MT.*

Harvesting Clean Energy Abstract:
Fermentative Conversion of Hydrolyzed Douglas fir Biomass into Isobutanol and Biojet

The pretreatment and hydrolysis of lignocellulosic biomass to release nutrients and sugars can ultimately lead to the production of biofuels and biochemicals. Gevo has developed fermentation and process technology to convert biomass sugars to isobutanol, followed by chemical processing into renewable jet fuel. Gevo uses GIFT®, Gevo Integrated Fermentation Technology, to produce isobutanol at high productivity, titer, and yield using a biocatalyst. As part of the Northwest Advanced Renewables Alliance (NARA), Gevo's goal is to fermentatively convert hydrolyzed Douglas fir biomass into isobutanol at a specification developed by Gevo that ensures the isobutanol will be further converted into renewable biojet using existing Gevo technology. The specific tasks of Gevo's project for NARA include: (1) Characterize and evaluate representative samples of pretreated Douglas fir biomass; (2) Adapt yeast biocatalysts to pretreated biomass hydrolyzates; (3) Produce isobutanol at laboratory scale to optimize isobutanol production using the adapted yeast biocatalyst; (4) Economic assessment of wood to isobutanol, biojet; (5) Analyze isobutanol to close the mass balance and determine potential low-level impurities; (6) Produce isobutanol at demonstration scale using GIFT® fermentations and convert the lignocellulosic derived isobutanol to biojet for further testing.

- *2014: Grant Balzer presented at the Northwest Wood-Based Biofuels + Coproducts Conference in Seattle, WA*

Northwest Wood-Based Biofuels + Coproducts Conference Abstract:
Conversion of Douglas fir Biomass into Isobutanol and Biojet

The pretreatment and hydrolysis of lignocellulosic biomass to release nutrients and sugars can be utilized for the production of biofuels and biochemicals. Gevo has developed fermentation and process technology to convert biomass derived sugars into isobutanol, followed by chemical processing into renewable jet fuel. Gevo uses GIFT®, Gevo Integrated Fermentation Technology, to produce isobutanol at high productivity, titer, and yield using a biocatalyst. As part of the Northwest Advanced Renewables Alliance (NARA), Gevo's goal is to fermentatively convert hydrolyzed Douglas fir biomass into isobutanol at a specification developed by Gevo that ensures the isobutanol will be further converted into renewable biojet using existing Gevo technology. The specific tasks of Gevo's project for NARA include: (1) Characterize and evaluate representative samples of pretreated Douglas fir biomass; (2) Adapt yeast biocatalysts to pretreated biomass hydrolyzates; (3) Produce isobutanol at laboratory scale to optimize isobutanol production using the hydrolyzate adapted biocatalyst; (4) Economic assessment of wood to isobutanol, biojet; (5) Analyze isobutanol to close the mass balance and determine potential low-level impurities; (6) Produce isobutanol at demonstration scale using GIFT® fermentations and convert the lignocellulosic derived isobutanol to biojet for further testing.

- *2015: Demonstration site for hydrolysis and fermentation was established*
- *2015: Demonstration site for conversion of isobutanol to isoparaffinic kerosene was established*
- *2015: Isobutanol recovered from 2L GIFT scale fermentations on various substrates.*

Hawkins, A.C., Development and commercialization of fermentative isobutanol production. Gevo, Inc, Englewood, Colorado, United States. American Chemical Society Division of Biochemical Technology. 249th ACS National Meeting. March 2015. Presentation number BIOT 144.

ABSTRACT

Gevo is a leading renewable chemicals and advanced biofuels company. We are developing biobased alternatives to petroleum-based products using a combination of synthetic biology and chemistry. We produce isobutanol, a versatile platform chemical for the liquid fuels and petrochemicals markets. Isobutanol has broad market applications as a solvent and a gasoline blendstock that can help refiners meet their renewable fuel and clean air obligations. It can also be further processed into jet fuel and feedstocks for the production of synthetic rubber, plastics and polyesters. Vision. We envision the development and commercialization of biorefineries that can connect the ethanol industry's infrastructure and agricultural supply chain to the petrochemical industry's infrastructure of existing refineries and pipelines. We hope to see biorefineries deliver low carbon solutions, provide renewed economic prosperity to rural areas and contribute to energy independence from fossil fuels. Technology. We have pioneered a platform technology based on a proprietary fermentation method that relies on an innovative biocatalyst and the efficient separation of isobutanol. The combination of these two proprietary innovations, Gevo's Integrated Fermentation Technology® (GIFT®), was designed to enable the low cost retrofit of existing ethanol capacity for isobutanol production. This provides Gevo a faster route to market. And,

when cellulosic biomass processing technology is ready for commercialization, we plan to deploy cellulosic butanol technology. Strategy. We have developed technology for the production of a building block for biobased fuels and chemicals that can be sold directly into existing refining and petrochemical value chains to provide customers with a bio-derived alternative to fossil fuels at a price that is competitive and less volatile than petroleum.

Parker, A.C., G.J. Balzer, L.T. Robinson, M.H. Schmalisch and A.C. Hawkins. 2014. Fermentative Conversion of Hydrolyzed Douglas fir Biomass into Isobutanol. 2014 Annual NARA Meeting, Seattle, WA, September 15-17.

Date	Title
10/11/16	Gevo Produces First Cellulosic Renewable Jet Fuel Specified for Use on Commercial Airline Flights
09/08/16	Gevo Announces Pricing of $15.6 Million Public Offering of Common Stock and Warrants
09/07/16	Gevo Signs Heads of Agreement with Lufthansa for Commercial Supply of Renewable Jet Fuel
09/07/16	Gevo Announces Proposed Public Offering of Common Stock and Warrants
09/06/16	Gevo Announces Participation at the 18th Annual Rodman & Renshaw Global Investment Conference in New York City on September 12th, 2016
08/09/16	Gevo Reports Second Quarter 2016 Financial Results
07/28/16	Gevo to Host Conference Call to Report Second Quarter Financial Results on August 9, 2016
06/16/16	Gevo Signs Agreement with Musket Corporation to Supply Isobutanol for Gasoline Blending
06/10/16	Gevo Announces Pricing of $9.5 Million Public Offering of Common Stock
06/09/16	Gevo Announces Proposed Public Offering of Common Stock
06/07/16	Alaska Airlines to Fly Today on Gevo's Renewable Alcohol to Jet Fuel
06/07/16	Alaska Airlines To Fly on Gevo's Renewable Alcohol to Jet Fuel
05/31/16	Gevo Hires Cowen & Company to Explore Strategic Alternatives
05/27/16	Gevo to Attend Cowen and Company Annual Technology, Media & Telecom Conference on June 1, 2016
05/19/16	Clariant to Scale-Up Catalysts for Gevo's Ethanol-to-Olefins (ETO) Technology
05/12/16	Gevo Reports First Quarter 2016 Financial Results
05/05/16	Gevo to Host Conference Call to Report First Quarter Financial Results on May 12, 2016
04/12/16	ASTM Completes Revision of Standard Specification
03/29/16	Gevo Reports Fourth Quarter 2015 Financial Results
03/29/16	Gevo Announces Pricing of $3.5 Million Public Offering of Common Stock and Warrants
03/28/16	Gevo's Alcohol to Jet Fuel Meets Approved ASTM Standard
03/28/16	Gevo Announces Proposed Public Offering of Common Stock and Warrants
03/24/16	Gevo to Host Conference Call to Report Fourth Quarter Financial Results on March 29, 2016
03/03/16	Gevo to Participate at the Northland Capital Markets Growth Conference on March 9, 2016
02/16/16	Gevo to Present at the Advanced Bioeconomy Leadership Conference on February 19, 2016
02/02/16	Gevo Signs Licensing and Joint Development Agreements With Porta
01/19/16	Gevo Adds William H. Baum to Board of Directors
12/17/15	Gevo Provides Update on Alcohol to Jet Certification Process
12/08/15	Gevo Announces Pricing of $10 Million Public Offering of Common Stock and Warrants
12/07/15	Gevo Announces Proposed Public Offering of Common Stock and Warrants
11/09/15	Gevo Signs Licensing and Joint Development Agreements With Praj
11/05/15	Gevo Reports Third Quarter 2015 Financial Results
11/04/15	Gevo and ValvTect to Collaborate in Bringing Renewable Isobutanol Fuel Blends to Marinas
10/19/15	Gevo to Host Conference Call to Report Third Quarter Financial Results on November 5, 2015
09/28/15	Gevo Announces Sales of Isooctene to BCD Chemie, a Subsidiary of Brenntag
09/23/15	Gevo Announces Key Operational and Financial Targets for 2016 Following Settlement With Butamax
09/22/15	Gevo Names Director Ruth Dreessen as Chairman; Shai Weiss Steps Down
09/08/15	Gevo Provides Update Following Settlement With Butamax
09/03/15	Gevo Announces First Marina Sales of Isobutanol-Blended Gasoline at Harbor Marina Pumps at Lake Pomme de Terre in Missouri
09/01/15	Gevo To Present at 17th Annual Rodman & Renshaw Global Investment Conference on September 9th
08/24/15	Butamax and Gevo Enter Into Global Patent Cross-License and Settlement Agreements to Accelerate Development of Markets for Bio-Based Isobutanol and End All Litigation
08/06/15	Gevo to Present at Jefferies 2015 Industrials Conference on August 10
08/04/15	Gevo Reports Second Quarter 2015 Financial Results
07/30/15	Gevo Announces First Pump Sales of Isobutanol-Blended Gasoline at Express Lube Service Station in Texas
07/28/15	Gevo to Host Conference Call to Report Second Quarter Financial Results on August 4, 2015
07/15/15	Gevo Adds Minho Roth to Board of Directors; Ganesh Kishore, Ph.D. Steps Down
06/17/15	National Marine Manufacturers Association Endorses Use of Gevo's Isobutanol in the Marine Fuel Market
06/04/15	Gevo's Jet Fuel to be Used in First Ever Test Flight Flown on Fuel Derived From Wood Waste
06/03/15	Gevo Signs Agreement With FCStone to Originate and Supply Corn for Luverne Plant
05/20/15	Gevo to Participate on a Panel at the Cowen and Company 43rd Annual Technology, Media & Telecom Conference
05/14/15	Gevo Announces Pricing of Public Offering of Common Stock and Warrants
05/13/15	Gevo Announces Proposed Public Offering of Common Stock and Warrants
05/12/15	Gevo Reports First Quarter 2015 Financial Results
05/07/15	Alaska Airlines to be Gevo's Commercial Launch Partner for Renewable Alcohol-Based Jet Fuel
05/04/15	Gevo to Host Conference Call to Report First Quarter Financial Results on May 12, 2015
04/20/15	Gevo Announces 1-for-15 Reverse Stock Split
03/26/15	Gevo Reports Fourth Quarter 2014 Financial Results
03/24/15	Gevo Signs Strategic Alliance Memorandum of Understanding With Praj
03/09/15	Gevo Sells Renewable Jet Fuel to NASA
03/06/15	Gevo to Present at the 27th Annual ROTH Conference
03/04/15	Gevo to Host Conference Call to Report Fourth Quarter Financial Results on March 26, 2015
02/13/15	Marine Industry Consortium Confirms Positive Test Results Using Gevo's Isobutanol in Boat Engines
02/12/15	Gevo Appoints Andy Marsh, President & CEO of Plug Power Inc., to Its Board of Directors
01/29/15	Gevo Announces Pricing of Public Offering of Common Stock and Warrants
01/28/15	Gevo Announces Proposed Public Offering of Common Stock and Warrants
01/27/15	U.S. Supreme Court Rules in Gevo's Favor
01/20/15	Gevo Provides Corporate Update
01/12/15	Gevo Launches Sales of Renewable Isobutanol to Brenntag Canada
01/08/15	Luverne Plant Update - Fourth Quarter Isobutanol Production Milestone Achieved Under Side-by-Side
01/05/15	Gevo Transfers Listing to Nasdaq Capital Market
04/22/14	Lufthansa to Evaluate Gevo's Renewable Jet Fuel
12/22/14	U.S. Navy Flies Supersonic With Gevo ATJ
12/23/13	Gevo Supplies U.S. Army With Fuel for the Black Hawk Helicopter

Gevo, Inc. Press releases that may or may not impact the NARA project.

NARA OUTCOMES

- Build a public awareness that biomass waste and specifically woody biomass waste can be put to good use.
- Build awareness that isobutanol and biojet fuels can be made from renewable resources, like woody biomass.
- Educate the public that renewable fuels, like Gevo's renewable biojet, are already approved for use in commercial airplanes.
- Demonstrate technical feasibility of the conversion of woody biomass into fermentable sugars, into isobutanol, and into renewable biojet fuel.
- Move alternative jet fuel from biomass from the R&D laboratory to commercial reality.
- Established a roadmap for R&D development and tech transfer (i.e. feedstock screening, adaptation and evolution, process development and scale up)

FUTURE DEVELOPMENT

Gevo plans to continue research and development and production of biojet fuel from bio-based renewable resources. We continue to work on our pathway of starch to renewable biojet and in partnership with partners around the world, other sugar sources to biojet. We have also applied as partners on two new USDA AFRI-CAP proposals that were submitted in September. These proposals focus on 1) coproduct optimization and valorization of the Pacific Northwest woody biomass to biojet supply chain and 2) production of biojet via isobutanol from sugars derived from switchgrass and energy sorghum grown in the Midwest in the marginal, flood-prone lands along the Mississippi and Missouri rivers.

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Marrs, G., Spink, T. & Gao, A. (2016). Process design and economics for biochemical conversion of softwood lignocellulosic biomass to isoparaffinic kerosene and lignin co-products. In *NARA Final Reports.* Retrieved from https://research.libraries.wsu.edu/xmlui/handle/2376/5310

Ley, J.W., Hathaway, M., Fiala, R., Parker, A.C. & Hawkins, A.C. (2015). *Fermentative conversion of hydrolyzed douglas fir biomass into isobutanol*. Poster session presented at the NARA Annual Meeting, Spokane, WA. Retrieved at https://nararenewables.org/documents/2016/12/2015-09-15-poster-15-fermentative-conversion-of-hydrolyzed-douglas-fir-biomass-into-isobutanol.pdf