Final Technical Report

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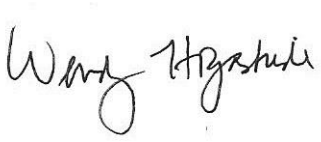

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Table of Contents

List of Acronyms, Abbreviations, Media, and Strains

DO	**Dissolved Oxygen**
EB0022	**OE9 *pta*::PLlacO1-*xylAB***
EB0024	**OE9 *yjgB*::PCP25-*xylAB adhE*::PCP25-*xylFGH***
ETOH	**Ethanol**
Fermentation Media	**2 g/L KH_2PO_4, 3 g/L $(NH_4)_2SO_4$, 3 g/L Yeast Extract, 1 g/L $MgSO_4$ $7H_2O$, 10 mg/L $MnSO_4$ $7H_2O$, 10 mg/L $FeSO_4$ $7H_2O$, 20 mg/L L-Ile, 20 mg/L L-Leu, 20 mg/L L-Val**
g	**Gram**
h	**Hour**
IBAL	**Isobutyraldehyde**
IBOH	**Isobutanol**
L	**Liter**
OD	**Optical Density**
OE9	**Easel's Isobutyraldehyde Production Strain**
SLPM	**Standard Liter Per Minute**

List of Figures

List of Tables

I. Executive Summary

Easel Biotechnologies, LLC's Bio-Oxo process has demonstrated that isobutyraldehyde can be biologically produced from corn stover hydrolysate up to 56 g/L in a 14L fermentor. This was accomplished by metabolically engineering bacterial strains to not only produce isobutyraldehyde, but to do so by co-utilizing corn stover hydrolysate sugars, glucose and xylose. Also essential to the success of the Bio-Oxo process was that it utilized gas stripping as a means of product separation, allowing for the continuous removal of isobutyraldehyde. This aided in not only reducing energy costs associated with separation, but also alleviating product toxicity, resulting in higher production.

Although we were not able to complete our economic analysis based on pilot scale fermentations, the improvements we have made from strain engineering to product separation, should result in the reduced cost of isobutyraldehyde. Still, as the project has ended prematurely, there is room for additional optimization. Improvements in productivity and sugar utilization would result in a further reduction in capital and recovery costs.

As a biological-based process, the utilization of corn stover results in reduced greenhouse gas emissions as compared to petroleum-based chemical synthesis. In addition, as a true replacement chemical "drop in" system, no downstream production units need to be changed. Jobs can also be created as farm waste needs to be collected and transported to the new production facility.

II. Introduction

Oxo chemicals are used for the synthesis of a wide array of industrial and consumer products, including plasticizers, fine chemicals, and pharmaceuticals. Currently, more than 10 million metric tons are being produced by a chemical process called hydroformylation, which requires petroleum-derived propylene and a significant expenditure of energy. The latter is necessitated by the high temperature and pressure conditions demanded by this catalyst-driven process.

Our Bio-Oxo technology is inherently an energy efficient process: our innovative, metabolically-engineered bacteria convert renewable resources to oxo chemicals in low-temperature, energy-efficient conditions. Furthermore, our Bio-Oxo technology allows for efficient in situ product recovery, taking advantage of the physical characteristics of our target product, isobutyraldehyde. Isobutyraldehyde's low boiling point allows for its collection and purification by our energetically efficient gas-stripping process. While increasing initial yield, this process also reduces the exposure of the organisms to the product, making the entire system significantly more sustainable than biological processes used in the production of other chemicals.

Throughout the DOE Innovative Manufacturing Initiative program, our goal was to develop the industrial-grade production microorganism to produce isobutyraldehyde with 2 g/L/hr productivity as well as the other unit processes including *in situ* recovery and downstream purification processes. After successfully achieving our target, our plan was

to test the commercial feasibility by constructing a demonstration pilot facility. Unfortunately, our project ended prior to this step.

III. Background

Over 10 million metric tons of aldehydes are consumed annually for the synthesis of a wide array of industrial and consumer products, including plasticizers, antifreeze products, aircraft and runway deicing products, solvents, hydraulic fluids, paints, lubricants, cosmetics, fine chemicals, as well as pharmaceuticals1. Currently, the dominant technology for C3-C15 aldehyde production is hydroformylation, also known as oxo synthesis or oxo process. This catalytic driven chemical process involves the addition of a formyl group and a hydrogen atom to an olefin (a hydrocarbon with a carbon-carbon double bond) under pressure.

The target oxo chemical in this work, isobutyraldehyde, is one of the commercially valuable aldehydes produced by oxo synthesis that is used in the production of a multitude of industrial and consumer products, including direct solvents, lube oil additives, surface coatings and adhesives, plasticizers, antiwear agents, reaction solvents, fertilizers, cosmetics , herbicides, insecticides, surfactants, flame retardants, binders, adhesives.

Current oxo chemical production processes adopt one of three catalytic processes. One process involves a cobalt hydrocarbonyl catalyst driven process that utilizes HCo(CO)4 to react liquid propylene with syngas (H2:CO=1:1) at 110~170 °C and 1500~4000 psi. This process usually produces a 2:1 to 4:1 ratio of n-butyraldehyde : isobutyraldehyde. The second process uses a trialkylphosphine-modified cobalt catalyst ([Co(CO)3P(C4H9)3]2 that promotes direct conversion of propylene to butanol and 2-ethylhexanol. With a syngas ratio of H2 : CO = 2:1 at 160 °C and 500 psig, n-butanol/2-ethylhexanol : isobutanol ratios of 10:1 - 12:1 are commonly achieved. The higher preference for n-butanol has resulted in the development of a rhodium-based catalysts process. Using a triphenylphosphine Rh hydrocarbonyl catalyst, HRh(CO)(Ph3)3, at 110 °C and 100~300 psig, approximately 8:1 to 12:1 ratio of n-butyraldehye : isobutyraldehyde is produced. As described above, these chemical oxo processes utilize high temperature and pressure conditions, which require significant energy consumption. In addition, these oxo chemical processes all require downstream separation processes to separate n-butyraldehyde from isobutyraldehyde. These fractional distillation processes are also energy intensive.

In our Bio-Oxo process, we take advantage of the C-C bond formation innate to microorganisms to produce pure isobutyraldehyde from renewable resources under mild operation conditions (30~37°C, 0 psi). Our fermentation process utilizes air from the environment to meet the O_2 demands of our engineered strain. This feeding allows for efficient *in situ* product recovery, taking advantage of the physical characteristics of isobutyraldehyde. Isobutyraldehyde's low vapor pressure and boiling point (63°C at 1 atm) allows for its collection and purification by the energetically efficient gas-stripping process. This process also has the additional advantage of reducing toxicity and inhibitory effects that would be a result of prolonged exposure of the strain to the product. To our knowledge, such a process has not been attempted previously, possibly

because of the following reasons: 1) The possibility of using valine biosynthesis pathways for chemical production was not reported in the literature until around the writing of our original proposal, 2) oxo chemicals are chemically reactive and can form products with many classes of biological molecules, and 3) oxo chemicals are toxic to microorganisms, which limits large-scale biological production. Despite these limitations, we have demonstrated oxo chemical production using our proprietary metabolically-engineered bacteria using the Bio-Oxo process.

Compared to the current oxo process, our proposed Bio-Oxo technology is not only less dependent on fossil fuels. Specifically, we have determined that our Bio-Oxo technology will decrease current isobutyraldehyde production energy costs by two-fold. This determination is based upon life cycle assessment (LCA) of butyraldehydes produced via oxo processes vs. our technology. The LCA for the oxo processes were conducted by the Stanford Research Institute (SRI) 1, while the LCA for our Bio-Oxo process was determined by Easel Biotechnologies, LLC by modifying the n-butanol LCA analysis by Argonne National Laboratory.

With the aim of further developing our platform Bio-Oxo technology, this project's objectives included:

1. Construct production strain that resists the toxins and inhibitors in the corn stover hydrolysate.
2. Engineer the isobutyraldehyde production pathway so that the production strain can obtain high yield (60% of theoretical yield) using corn stover hydrolysate.
3. Optimize Bio-oxo process so that isobutyraldehyde production from corn stover hydrolysate can obtain 1 g/L/hr productivity in lab scale (5L) and pilot scale (30L) fermentors.
4. Reduce energy consumption of the oxo chemical production process by at least 50% (from 56.0 MJ/Kg to 28.0 MJ/Kg).

The research efforts of Easel Biotechnologies, LLC have been guided by Drs. Kwang Myung Cho, Yixin Huo, and Wendy Higashide. Dr. Cho is one of the most experienced experts in industrial biotechnology. Dr. Cho has more than 20 years of research experience in the production of biofuels and biochemicals. Previously, he served in various project leader and director positions at CJ Corporation, one of the largest fermentation companies in the world (Amino acid: World #2, Nucleotides: World #1 Manufacturer). Dr. Huo has published 9 papers as first author or leading senior author in high profile international scientific journals and had several book chapters. Dr. Huo's status as a leading expert in bioengineering and biofuel production is also evidenced by the fact that he has been invited to review papers submitted to several major journals in the field. Dr. Higashide was one of the key inventors who developed the first direct production of isobutyraldehyde from CO_2 and sunlight using cyanobacterium Synechococcus sp. PCC 7942. She has also developed a cellulose degrading Clostridium strain to produce isobutanol directly from cellulose, which was highlighted by the White House blog. In addition, Dr. James C. Liao, currently the President of Academia Sinica (Taiwan), has served as a consultant for Easel. Dr. Liao is a pioneer in Metabolic Engineering and Synthetic Biology, having developed the synthetic pathways for higher alcohol biosynthesis that form the foundation of the proposed work. Dr. Liao received

numerous awards, including the Merck Award for Metabolic Engineering (2006), Food, Pharmaceutical, and Bioengineering Division award of American Institute of Chemical Engineers (AIChE) (2006), Charles Thom Award of the Society for Industrial Microbiology (2008), Marvin Johnson Award of American Chemical Society (2009), Alpha Chi Sigma Award of AIChE (2009), James E. Bailey Award of Society for Biological Engineering (2009), and the Presidential Green Chemistry Challenge Award (2010).

IV. Results and Discussion

The following summarizes our research findings during our active project period, 12/22/2014—08/31/2017. The results are presented according to Subtask.

1. Subtask 1.1: Isobutyraldehyde Strain Improvement.

For our initial steps for strain improvement, our metabolic engineering strategies included: 1) improving isobutyraldehyde tolerance; 2) increasing production strain stability; 3) eliminating byproduct formation by knocking out genes involved in competing pathways; and 4) improving the corn stover hydrolysate consumption rate.

The details for each approach are described in detail below. This includes original hypotheses, approaches used, problems encountered, and assessment of their impact on the project. The following is a summary of the milestones for strain improvement:

Milestone 1.1.1:	By the end of Q1, finish knocking out the competing pathways for the production of acetate and succinate.
Milestone 1.1.2:	By the end of Q2, finish knocking out the competing pathways for the production of L-Valine, L-Leucine, and D-pantothenate.
Milestone 1.1.3:	By the end of Q3, evolve the engineered strain to tolerate 10 g/L isobutyraldehyde.
Milestone 1.1.4:	By the end of Q4, increase the corn stover monosaccharides consumption rate to higher than 3 g/L/hr.

a. Milestone 1.1.1: <u>Finish knocking out the competing pathways for the production of acetate and succinate.</u>

Deadline: Q1 (SOPO), Y1Q2 (Budget Period)
Status: 100% Completed

[NOTE: Milestone 1.1.1 was edited in Revised SOPO 12/23/15]

Competing pathways may result in the loss of isobutyraldehyde precursors (eg phosphoenolpyruvate, pyruvate), leading to a decrease in the amount of isobutyraldehyde produced. Thus, elimination of these pathways may result in the increase in isobutyraldehyde titer. The genes encoding enzymes of these competing pathways were knocked out in OE9. To prevent the conversion of phosphoenolpyruvate (PEP) to succinate, *frdBC* was knocked out. To prevent the conversion of pyruvate to acetate (through acetyl-CoA), *pta* was deleted. OE9 Δ*pta*

and OE9 Δ*frdBC* expressing the isobutyraldehyde pathway genes were examined in the 5L fermentor. The results were compared to a previous run with the parental wildtype (**Figure 1**).

OE9 Δ*pta* resulted in improved isobutyraldehyde yield as compared to the parental strain OE9 with an increase of 55% to 65% of the maximum theoretical yield (**Figure 1**).

We confirmed that this Δ*pta* mutation reduced acetate production significantly (data not shown). While the parental strain continues to produce acetate up to 12.55 g/L acetate (at 92hr), OE9 Δ*pta* maintains ~ 4-5 g/L acetate.

While we have decreased the amount of acetate produced during the fermentation, 4-5 g/L acetate is still significant. It has been reported that acetate inhibits cell growth when its concentration exceeds 5 g/L (Chong *et al*, 2013, PLOS ONE, 8:e77422). Unfortunately, it has been previously reported that additional mutations in the other genes involved in acetate production (*eg ack* and *poxB*) have not been able to further reduce acetate production (Baez *et al*, 2011, Appl Microbiol Biotechnol, 90:1681).

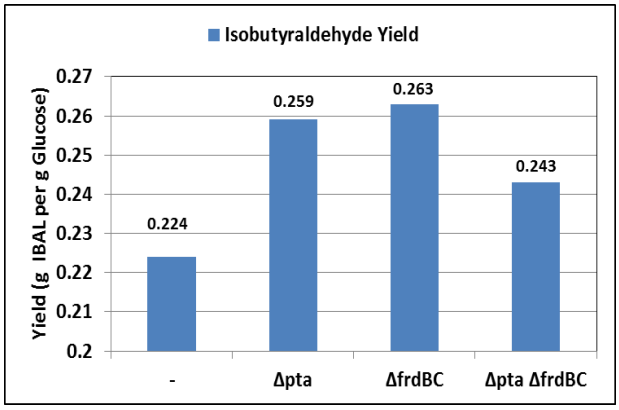


Figure 1. Effect of Δ*pta*, Δ*frdBC*, and Δ*pta*Δ*frdBC* mutations on isobutyraldehyde yield. Isobutyraldehyde yield (g isobutyraldehyde per g glucose) of 5L-scale fermentations of strains OE9, OE9 Δ*pta*, OE9 Δ*frdBC*, and OE9 Δ*pta* Δ*frdBC* expressing *alsS ilvCD* and kivd over ~90hr fermentations.

We also tested OE9 Δ*frdBC* in a 5L-scale fermentor. Similar to OE9 Δ*pta*, even though it did not demonstrated a significant increase in isobutyraldehyde titer (data not shown), it demonstrated improved isobutyraldehyde yield as compared to the parental strain (**Figure 1**). To confirm the decrease in succinate for OE9 Δ*frdBC*, supernatant samples from 5L fermentation runs were analyzed on HPLC and compared to the parental strain. The results were inconclusive.

A strain containing knockouts in both *pta* and *frdBC* was constructed. Although OE9 Δ*pta* Δ*frdBC* demonstrated an increase in yield versus the parental strain (OE9), it did not perform as well as the single mutants, OE9 Δ*pta* and OE9 Δ*frdBC* (**Figure 1**).

In summary, we have constructed and tested OE9 Δ*pta*, OE9 Δ*frdBC*, and OE9 Δ*pta* Δ*frdBC*. We found that the single mutants, OE9 Δ*pta* and OE9 Δ*frdBC*, produced isobutyraldehyde more efficiently than both the parental strain OE9, and the strain with both mutations, OE9 Δ*pta* Δ*frdBC*. Moving forward, we will continue with both OE9 Δ*pta* and OE9 Δ*frdBC* until additional data confirms that one strain is superior to the other.

b. Milestone 1.1.2: <u>Knock Out Competing Pathways- L-valine, L-leucine, D-pantothenate</u>
Deadline: Q2 (SOPO), Y1Q3 (Budget Period)
Status: 100% Completed

Before knocking out the competing pathways, L-valine, L-leucine, and D-pantothenate, we first determined the initial concentrations of each byproduct. HPLC analysis of 2L fermentation production by OE9 *alsSilvCD kivd* demonstrated that L-valine, L-leucine, and D-pantothenate do not accumulate to appreciable levels (**Figure 2 and 3**).

Figure 2 demonstrates that an insignificant amount of L-leucine and L-valine are produced over a 105-hr, 2L isobutyraldehyde fermentation. Specifically, 1.57 g/L L-leucine and 0.45 g/L L-valine were produced by our production strain expressing the isobutyraldehyde pathway genes. During this same time period, a significant amount of isobutyraldehyde, 91 g/L, was produced.

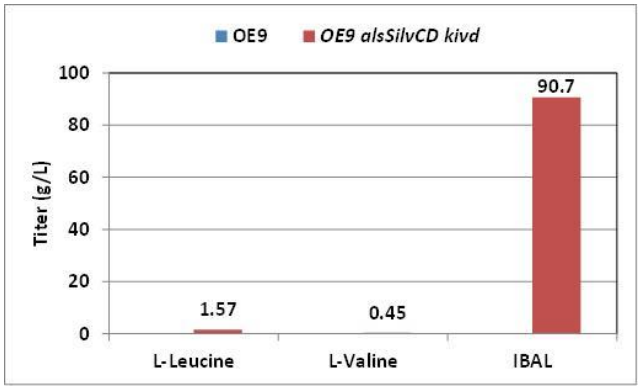


Figure 2. HPLC analysis of L-leucine and L-valine production. HPLC analysis of L-leucine, L-valine, and isobutyraldehyde (IBAL) production after 105 hours by OE9 and OE9 *alsSilvCD kivd* in 2L fermentations.

The HPLC was also used to analyze the amount of D-pantothenate that accumulates during our fermentation process. As shown in **Figure 3**, our HPLC analysis demonstrates that D-pantothenate was not produced by OE9 *alsSilvCD kivD* at all timepoints tested -18h, 29h, 77h, and 105h of a 2L fermentation. The detection level of our analysis was confirmed with 0.55 g/L and 5.5 g/L D-pantothenate standards (far-left in graph).

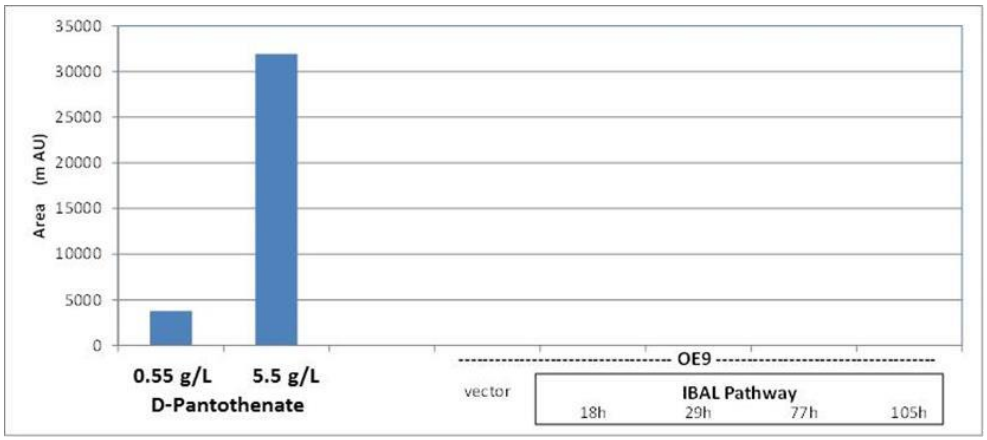


Figure 3. HPLC analysis of D-pantothenate production.
HPLC analysis of D-pantothenate production (mAU) during a 2L fermentation of OE9 *alsSilvCD kivd* at 18h, 29h, 77h, and 105h. D-pantothenate standards (0.5 and 5.5 g/L) are shown on the far-left of the graph.

In summary, HPLC analysis has demonstrated no appreciably accumulation of L-leucine, L-valine, and D-pantothenate. Knocking out the genes in these pathways will not be necessary to improve isobutyraldehyde titer.

c. Milestone 1.1.3: <u>Evolve Strain to Tolerate 10 g/L Isobutyraldehyde</u>
Deadline: Q3 (SOPO), Y1Q4 (Budget Period)
Status: ON HOLD

Initially, we had planned to employ a serial transfer method to evolve our production strain OE9 to tolerate up to 10g/L isobutyraldehyde. Specifically, the strains were to be subcultured daily in increasing concentrations of isobutyraldehyde. This protocol has been used successfully to improve the isobutanol tolerance of *E. coli* strains (Atsumi *et al*, 2010, Mol Syst Biol, 6:449).

After preliminary experiments, we have discovered that this method will not be successful. We have found that the mutations we were observing were not the result of altered physical properties of the cell (*eg* the cell's membrane). Instead, the mutations had the unexpected effect of significantly increasing the conversion of isobutyraldehyde to the less toxic isobutanol. *E. coli* can tolerant ~6 g/L of isobutanol, but only 0.6-1.5 g/L of isobutyraldehyde (data not shown).

We had expected that OE9 would convert some of the isobutyraldehyde we had added to isobutanol because, although it has deletions in several alcohol dehydrogenases (*eg yqhD*, *yiaY*, *adhE*, and *yjgB*), it still has some remaining ADH activity. However, we did not anticipate that it would be significant enough to affect the experiment.

Thus, in order to continue this experiment, it would be necessary to either eliminate all of the remaining ADHs or find another approach. Unfortunately, at the completion

of Milestone 1.3.4 (see below), despite our best efforts, we were not able to eliminate all of the ADHs.

d. Milestone 1.1.4: Increase Corn Stover Consumption
Deadline: Q4 (SOPO), Y2Q1 (Budget Period)
Status: **70% Completed (at end of Q4 Deadline); 100% Complete**

The corn stover hydrolysate that we will be using for the duration of our project contains approximately 69 g/L glucose, 42 g/L xylose, and 3 g/L arabinose (note exact concentrations are batch dependent). Due to elaborate regulation in *E. coli*, sugars are consumed sequentially rather than simultaneously as desired. First glucose is entirely consumed, then arabinose, and only then, xylose.

This milestone has proven to be more complicated than initially anticipated. Because of this complexity, background information and previously reported data is still included in this progress report for guidance.

Background and Previous Data (see "Data" below)
It has been reported that *E. coli* strains can be engineered to simultaneously consume multiple sugars by mutating *E. coli* genes *ptsG*, *mgsA*, or *mlc*, or by overexpressing *Zymomonas mobilis glf* (Chiang *et al*, 2013, J Agr Food Chem, 61:7583; Nakashima and Tamura, 2012, J Biosci Bioeng, 114:38). *ptsG* encodes a component of *E. coli*'s primary glucose transporter, which also has a role in inhibiting the transport of other sugars, such as arabinose and xylose (Chiang *et al*, 2013, J Agr Food Chem, 61:7583). *mgsA* encodes methylglyoxal synthase, which converts dihydroxyacetone phosphate to methylglyoxal. MgsA's mode of action in sugar regulation is currently unknown (Chiang *et al*, 2013, J Agr Food Chem, 61:7583). In *E. coli*, *mlc* encodes a global transcription regulator that can both negatively or positively regulate the gene expression of itself and other genes. One of Mlc's targets is *ptsG*. Mlc negatively regulates *ptsG* expression. It has been found that a single base pair change in the promoter region of *mlc* increases the expression of *mlc* 17-fold (Nakashima *et al*, 2012, J Biosci Bioeng, 114:38). This *mlc** mutation increased the expression of mlc, thus resulting in an increase in the negative regulation of *ptsG*. *Z. mobilis glf*, which transports glucose by facilitated diffusion in *Z. mobilis*, has been shown to enhance glucose transport in *E. coli*. Overexpression of *glf* in *E. coli* was found to improve the glucose utilization deficiency created by the *ptsG* deletion.

Our approach has been to examine the mutations described above in our production strain, OE9. In our Y1Q2 report, we reported the impact of the deletions of *ptsG* and *mgsA*, overexpression of *Z. mobilis glf*, and the *mlc** mutation. To briefly summarize, we found that overexpression in *mgsA* had little impact on sugar utilization. The Δ*ptsG* mutation did improve xylose utilization, but also severely reduced glucose utilization. Overexpression of *glf* did little to restore glucose utilization in this strain. The *mlc** mutation provided more promising results. Previously, we found while glucose utilization is also inhibited in OE9 *mlc**, it is to a lesser degree than OE9 Δ*ptsG*. More importantly, we found that over 24hr in small scale (10 ml), OE9 *mlc** produced similar titers of isobutyraldehyde as compared to the parental strain (*ie* 4.07 g/L vs 4.88 g/L isobutyraldehyde, respectively).

We tested OE9 *mlc** in a 5L fermentor. Initially, OE9 *mlc** produced similar levels of isobutyraldehyde as OE9. However, after 24h, OE9 *mlc** did not produce isobutyraldehyde as well as OE9. At the end of the fermentation (~90hrs), OE9 produced 71 g/L, while OE9 *mlc** produced significantly less with 49 g/L isobutyraldehyde. We then compared the sugar utilization of both strains. As observed in the small scale data, OE9 *mlc** co-utilized both glucose and xylose early in the fermentation. In the 5L fermentor, OE9 *mlc** utilized 23 g/L glucose and 4 g/L xylose in the first 24hr of fermentation. However, xylose utilization ceased after 24 hours of fermentation. In addition to the cessation of xylose utilization, the glucose utilization rate was significantly lower for OE9 *mlc** than OE9. Over ~90hrs, OE9 *mlc** utilized 151 g/L glucose, while OE9 utilized 278 g/L glucose over a similar time period.

We have found that OE9 *mlc** is only able to co-utilize xylose and glucose early in the fermentation (*ie* 0-24hr). After this time period, glucose utilization continues, but at a lower rate than OE9. This decrease in sugar utilization may be the reason why isobutyraldehyde production is lower for OE9 *mlc**.

Both Δ*ptsG* and *mlc** mutations were only partially effective in improving the co-utilization of glucose and xylose in our production strain, OE9. Their roles in the metabolism of other sugars, in addition to xylose, complicate the effect of their mutations in our experiments. Because of this complication, we decided to pursue a different approach. We decided to examine the overexpression of the xylose transport genes (*eg xylE, xylFGH*) and xylose metabolism genes that convert xylose to D-xylulose 5-phosphate (*eg xylA xylB*). Surprisingly, there is little data on the overexpression of these genes in E. coli for the purpose of engineering the co-utilization of glucose and xylose. Only recently, did Kim *et al* (2015, Metabolic Engineering, 30:141–148) demonstrate co-utilization of glucose and xylose with overexpression of these metabolic xylose genes.

Data

We first tested plasmid overexpression of *xylAB* in our production strains. Data has demonstrated that expression of ~10 copies of *xylAB* significantly improved xylose utilization. Interestingly, higher expression (~50 copies) of *xylAB* resulted in more variable data and a decrease in xylose utilization. This suggests that higher expression of *xylAB* is toxic to the cell. We also examined the effects of overexpressing one of the xylose transporters, *xylE*. Both low and high copy express resulted in no significant improvement in xylose utilization. Moreover, expression of both *xylE* (50 copies) and *xylAB* (10 copies) resulted in a reduction of xylose utilization as compared to expression of *xylAB* (10 copies) alone. Thus, we found no advantage in expressing *xylE*.

In reporting period Y2Q1 we integrated the *xylA* and *xylB* xylose metabolism genes into the chromosome of our production strain and tested their ability to utilize xylose in the presence of glucose (**Figure 4**). The genes were integrated into the location of the pta gene (resulting in a deletion of the *pta* gene). Two different promoters were tested – PllacO1 and POXB20.

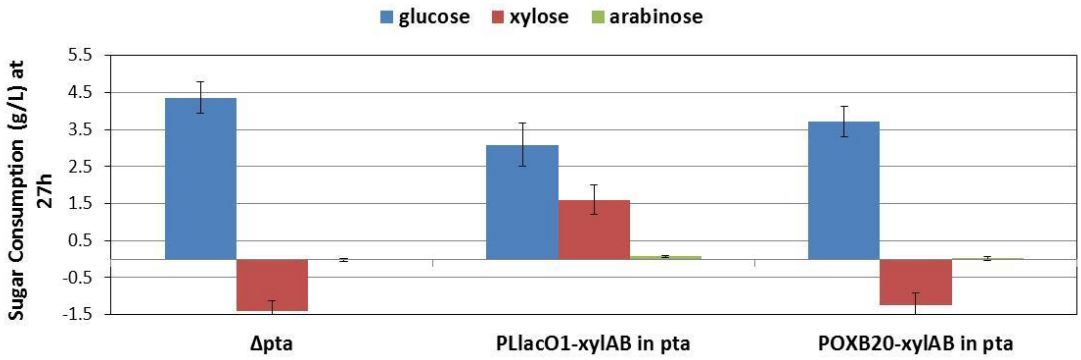


Figure 4. Analysis of glucose, xylose, and L-arabinose utilization in strains. Glucose, xylose, and L-arabinose utilization (g/L) over 27 hours in 2ml cultures by: 1) Δ*pta* (background strain), 2) PLlacO1-*xylAB* in the *pta* site, and 3) POXB20-*xylAB* in the *pta* site.

As shown in **Figure 4,** PLlacO-*xylAB* resulted in significantly more xylose utilization than the background strain (Δ*pta*). This was not the case for the integration strain with the POXB20 promoter. This demonstrates that integration of *xylAB* is sufficient to allow a strain to utilize xylose in the presence of glucose. In addition, the PLlacO1 promoter resulted in significantly more xylose utilization than POXB20.

2. Subtask 1.2.: Fermentation Process Development (5L).

For the optimal production of isobutyraldehyde using engineered *E. coli* strain in our Bio-Oxo fermentation process, we optimized media composition and process operation conditions.

Our Bio-Oxo process is based on a typical fed-batch operation. The feeding of substrate and nutrients is a key operational variable to achieve the best performance. Therefore, we have optimized the methods of feeding and the media used. In addition, our isobutyraldehyde product was eliminated continuously by air-stripping. The air flow rate was tightly controlled to obtain the maximum stripping efficiency over the fed-batch fermentation.

Milestone 1.2.1: By the end of Q2, obtain the optimized industrial media and operation conditions for 5L-scale fermentation.
Milestone 1.2.2: By the end of Q3, reach 0.8 g/L/hr productivity using purified corn stover monosaccharides as raw material.
Milestone 1.2.3: By the end of Q4, reach 0.3 g/L/hr productivity using corn stover hydrolysate as raw material.

a. Milestone 1.2.1: <u>Optimize Industrial Media and Operational Conditions</u>

Deadline: Q2 (SOPO), Y1Q3 (Budget Period)
Status: 100% Completed

For the optimal production of isobutyraldehyde using engineered *E. coli* strain in our Bio-Oxo fermentation process, we optimized the media composition and process operation conditions.
A minimum media composition was devised based on literature and experiments to support high cell density fermentation. Glucose and xylose was used separate as the carbon source. Ammonium hydroxide was used to control pH at 6.8 and also acted as a nitrogen source. See **Table 1** for media composition.

Media Component	Weight/liter	Unit	Molarity	Unit
$(NH_4)_2HPO_4$	4	g	30	mM
$MgSO_4$-$7H_2O$	1.2	g	5	mM
Ferric Citrate	60	mg	245	nM
$CuCl_2 \cdot ٢H_2O$	1.5	mg	9	nM
$CoCl_2 \cdot ٦H_2O$	2.5	mg	11	nM
$MnCl_2 \cdot ٤H_2O$	15	mg	76	nM
$Zn(CH_3COO)_2 \cdot ٢H_2O$	8	mg	36	nM
H_3BO_3	3	mg	49	nM
$Na_2MoO_4 \cdot ٢H_2O$	2.5	mg	10	nM
Glucose	27.5	g	153	mM
Citric Acid	1.7	g	9	mM
KH_2PO_4	13.3	g	98	mM
Thiamine	4.5	mg	13	nM
EDTA	8.4	mg	23	nM
Feed				
Glucose	700	g	3.9	M
$MgSO_4$-$7H_2O$	19.7	g	80	mM
pH control 6.8				
14% NH_4OH				

Table 1. Minimal media composition for isobutyraldehyde fermentation.

Two different feeding strategies were investigated. One strategy maintained a constant carbon level using a feeding program set to manually add feed once an hour. The second strategy maintained a low carbon source level by utilizing a cascade control based on the dissolved oxygen level. The feed and agitation were controlled using a cascade program based the dissolve oxygen concentration. Increased agitation resulted in an increase in oxygen availability via the cascade control. As the oxygen uptake rate increased, the carbon source was added at a higher rate.

Figure 5 shows the growth and glucose concentration curves of the two different feeding strategies. Cascade-controlled feeding resulted in a significant increase in cell mass (OD600). The glucose level drops and then is maintained at a low concentration. On the right, the glucose drops and then regains a concentration around 30 g/L. The cascade control is able to produce an OD over 95, while the constant feeding program only reaches an OD of 21. An OD of 95 is over dry cell weight of 54 g/L. Xylose was also tested as a carbon source, it was able to sustain a dry cell weight of 20 g/L.

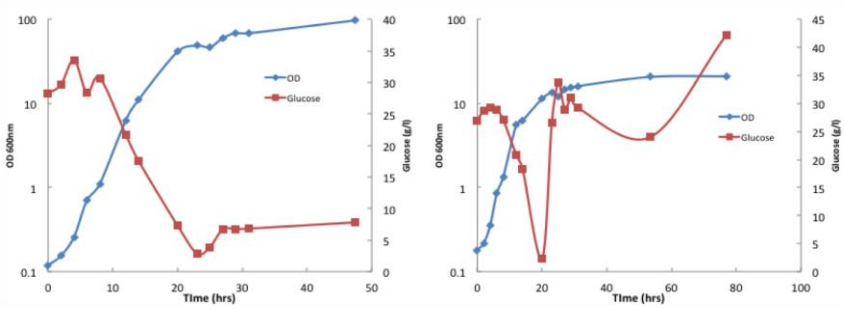


Figure 5. Comparison of cascade-controlled glucose feeding vs. constant glucose feeding. Growth and glucose concentration of OE9 pGGL2bb2c7 (*kivd-alsS-ilvD-ilvC*) grown in the minimal media described in Table 1 with either a cascade-controlled (DO-based) glucose feeding (left) or a constant glucose feeding (right).

To test the minimal media (**Table 1**) for isobutyraldehyde production, strain OE9 pGGL2bb2c7 (*kivd-alsS-ilvDC*) was grown using the cascade feed strategy. There was initial growth and then a lag in growth until T=40h (T=0 is the start of fermentation), after which the cells started growing and producing isobutyraldehyde (data not shown). It obtained a dry cell mass of 22 g/L and a final titer of 50 g/L isobutyraldehyde.

b. Milestone 1.2.2: <u>Obtain 0.8 g/L/h Productivity from Purified Corn Stover Monosaccharides</u>
Deadline: Q3 (SOPO), Y1Q4 (Budget Period)
Status: 100% Completed

At the end of Y1Q4, our production strain OE9 pSA69 pEB5 produced 0.98 g/L/h isobutyraldehyde over 113h (**Figure 6**). The periodic productivity varied from 0.45 g/L/h to 1.83 g/L/h, depending on the stage of fermentation.

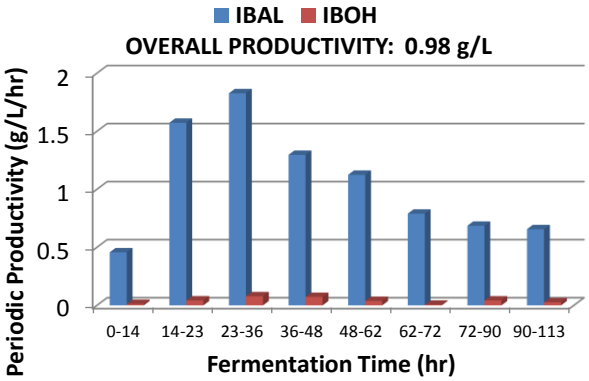


Figure 6. Periodic isobutyraldehyde productivity of OE9 pSA69 pEB5. Periodic and overall isobutyraldehyde productivity (g/L/h) of OE9 pSA69 pEB5 over 113 h 5L-fermention run using purified corn stover monosaccharides.

c. Milestone 1.2.3: <u>Obtain 0.3 g/L/h Productivity from Corn Stover Hydrolysate</u>
Deadline: Q4 (SOPO), Y2Q1 (Budget Period)
Status: 100% Completed

At the end of Y1Q4, our production strain OE9 Δ*dkgB* pSA69 pEB5 produced up to 0.38 g/L/h (**Figure 7**).

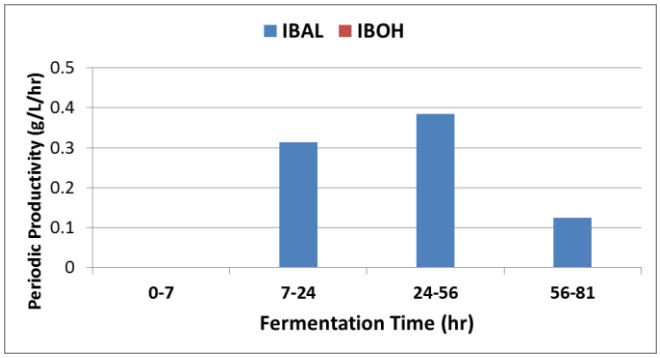


Figure 7. Periodic isobutyraldehyde productivity using corn stover hydrolysate. Periodic isobutyraldehyde productivity (g/L/h) of OE9 Δ*dkgB* pSA69 pEB5 over 81h 5L-fermention using corn stover hydrolysate.

3. Subtask 1.3: Optimize the pathway gene expression.

This subtask focused on the engineering of the isobutyraldehyde production pathway, not the host strain. Our goal was to obtain the best production pathway for isobutyraldehyde production. Specifically, our approach was to: 1) analyze key enzymes and identify limiting steps of isobutyraldehyde production pathway; 2) evolve the key enzymes' activities of isobutyraldehyde production pathway; 3) deregulate and overexpress engineered key enzymes; and 4) eliminate residual alcohol dehydrogenase (ADH) activities.

Milestone 1.3.1: By the end of Q1, identify the key pathway enzymes whose activities need to be significantly improved.
Milestone 1.3.2: By the end of Q2, increase the expressions and activities of the key pathway enzymes (identified in Q1) by at least 3 folds.
Milestone 1.3.3: By the end of Q3, eliminate the feed-back regulations of the key pathway enzymes.
Milestone 1.3.4: By the end of Q4, eliminate residual alcohol dehydrogenase (ADH) activities.

a. Milestone 1.3.1: <u>Identify Key Pathway Enzymes</u>

Deadline: Q1 (SOPO), Y1Q2 (Budget Period)
Status: 100% Completed

Our isobutyraldehyde pathway consists of the overexpression of *alsS* (*B. subtilis*), *ilvC* (*E. coli*), *ilvD* (*E. coli*), and *kivd* (*L. lactis*). To optimize our pathway, we determined the activity of these individual enzymes over the course of a 5L-fermentation.

An HPLC examination of a time course using a 5L fermentor over 115h confirmed that there was no accumulation of pyruvate, nor 2-ketoisovalerate. This would occur if there are insufficient activities of AlsS and Kivd, respectively. The precursors for *ilvC* (2-acetolactate) and *ilvD* (2,3-dihydroxy-isovalerate) are difficult to determine by our analytical methods (GC/HPLC), thus we examined a time course of their activities by in vitro enzyme assays.

Our production strain, OE9, overexpressing *alsS, ilvC, ilvD, kivd* was examined in a 5L vessel. Samples were taken periodically, usually every 10-14 hours. At each time point, the bacterial pellets were washed and then stored at -80˚C. After the end of the fermentation, the pellets were lysed and the enzymes were assayed.

For Milestone 1.3.1, we had previously reported that IlvD was the limiting enzyme in our fermentation process. We had determined this by conducting enzyme assays of AlsS, IlvC, IlvD, and Kivd for each time point for a 2L fermentation run. From those assays, we found that only IlvD demonstrated a decline in activity that corresponded with the decline in isobutyraldehyde production. This data was first reported in the Y1Q2 Quarterly Report submitted on 4/30/15.

During the commencement of this current milestone, Milestone 2.1.2, the IlvD enzyme assay was repeated. Surprisingly, our new results (**Figure 8**) contradicted our Milestone 1.3.1 results - the decline in isobutyraldehyde productivity corresponds with an *increase* in IlvD activity as determined by the enzyme assay data. In fact, IlvD activity increases steadily up to 75 hours of fermentation. This suggests that IlvD activity is not related to the early decline in productivity that we have observed. This data contradicts the data we reported in our Y1Q2 Quarterly Report. To determine, which data was correct, additional fermentation runs were examined. Independent experiments confirmed that IlvD activity is not correlated with isobutyraldehyde production (**Figure 8 and data not shown**).

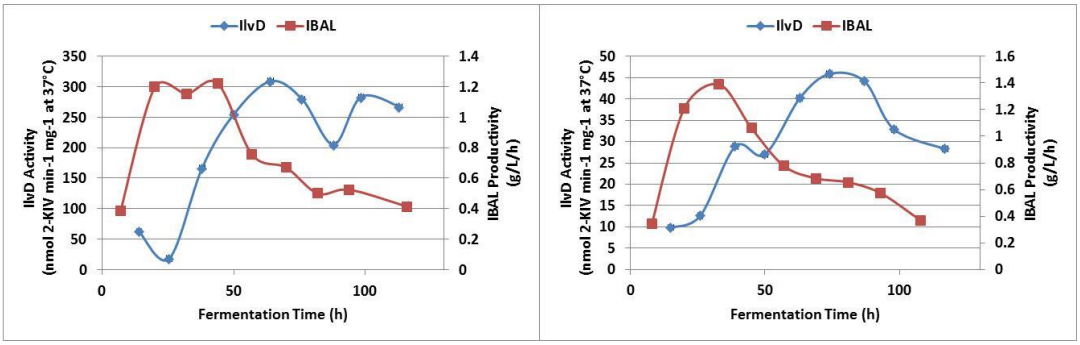


Figure 8. IlvD activity of OE9 *alsSilvCD kivd* during course of 2L fermentation.
Graphs showing the relationship between IlvD activity (measured by production of 2-kiv per min per mg of soluble protein at 37°C) and isobutyraldehyde production at different time points for 2 independent 2L fermentations of OE9 expressing *alsSilvCD kivd*.

Because of this conflict in data, we re-examined the activities of enzymes AlsS, IlvC, and Kivd for 3 independent 2L fermentations runs. From these enzyme assay data performed on three independent 2L fermentation runs, we have come to the conclusion that a decline in enzyme activity is not likely responsible for the decline in isobutyraldehyde production.

b. Milestone 1.3.2: <u>Increase Expression and Activities of Key Pathway Enzymes</u>
Deadline: Q2 (SOPO), Y1Q3 (Budget Period)
Status: 100% Completed

For production, we use a fed batch culture scheme, where the cells spend a majority of the culture time in a late-exponential to stationary phase. In order to better understand this phase, we used a 6-histidine epitope tag over the time course to visualize the relative protein concentration in western blots. Secondly, in order to enhance the transcription of thd production genes in the stationary phase, we looked for promoters that are regulated by sigma factor S (*rpoS*). *rpoS* is transcribed in late exponential phase, and RpoS is the primary regulator of stationary phase genes and general stress response genes. On the operational side, we tested oxygen limiting conditions in serum bottles and small flasks in an attempt to mimic the conditions in the fermenter.

Monitor protein concentration

In order to see the relative protein concentration of the crucial proteins for isobutyraldehyde production, a 6-histidine epitope tag was attached to every protein using the golden gate method. We were able to confirm the quantities AlsS, KivD, IlvC, and IlvD by Coomassie staining and/or western blot.

Stationary phase promoters

During our fermentations, most of the cells' time is spent in the stationary phase, where the cell mass is maintained with an equilibrium between death and growth of cells. The sigma factor S (*rpoS*) is responsible for gene regulation during this phase. Therefore, in order to increase production during this phase, we cloned 8 potential promoters regulated by *rpoS* (**Table 2**).

ID	Name	Description
S1	*csiD*	carbon starvation protein
S2	*osmY*	periplasmic protein
S3	*dps*	Fe-binding and storage protein; stress-inducible DNA-binding protein
S4	*otsB*	trehalose-6-phosphate phosphatase, biosynthetic
S5	*fic*	stationary-phase adenosine monophosphate-protein transferase domain protein
S6	*treA*	periplasmic trehalase
S7	*aldB*	aldehyde dehydrogenase B
S8	*xthA*	exonuclease III

Table 2. Candidate stationary promoters for stationary phase expression of isobutyraldehyde pathway genes.

These promoters were then tested with operon structures containing: 1) either *kivD* or *alsS* as the first or second gene, 2) *ilvD* as the third gene, 3) and *ilvC* last gene in a synthetic operon. These operons were examined with two different origins of replication, p15A (~10 copies/cell) and colE1 (~50 copies/cell). The constructs were tested in a 96- well plate format, using quintuplicates of 4 independent colonies picked from the same transformation plate; the plates were grown in a complex media at 37°C for 28 hours in a plate shaker. **Figure 9** shows the isobutyraldehyde production of these constructs.

The first s1 through s8 set has *alsS* as its first gene and the second s1 through s8 set has *kivD* as its first gene. Overall, the colE1 origin of replication seems to produce more isobutyraldehyde, which is likely due to colE1's higher copy number. The S3 promoter seems to be the best promoter as its production is high in almost every

perturbation, despite its lower copy number plasmid and *alsS* as its first gene. S3 is the promoter of *dps*, which codes for a Fe-binding, storage, and stress-inducible DNA-binding protein.

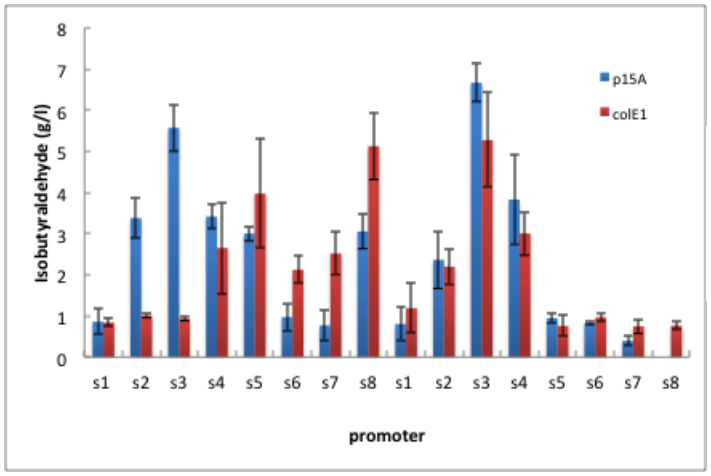


Figure 9. Isobutyraldehyde production of isobutyraldehyde pathway genes using stationary promoters.

Oxygen limiting condition

Oxygen is often the limiting condition in the fermenter runs, so we attempted to duplicate oxygen limiting conditions in a shaker flask. Serum bottles were used with butyl stoppers so oxygen couldn't enter the bottle once sealed. By adding different amounts of media into the bottle, oxygen becomes the limiting growth factor. We used 5, 10, 15, 20 and 25 ml of industrial media with a starting glucose concentration of 27.5 grams of glucose to grow OE9 strains containing the origin of replication p15A and the *dps* stationary promoter in a shaking incubator at 37°C. Samples were taken at 36.5 and 60 h. **Figure 10** shows the results of these time points.

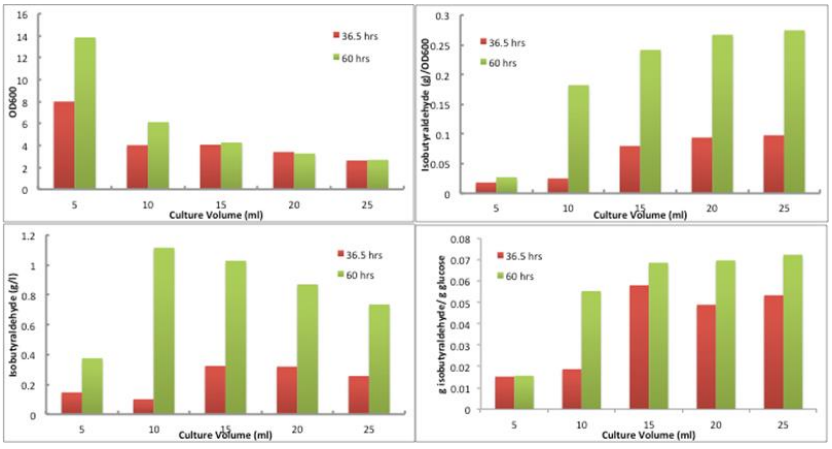


Figure 10. Effect of oxygen limitation on production strain. Growth (OD600), isobutyraldehyde titer (g/L), gram isobutyraldehyde per growth (g/OD600), and gram isobutyraldehyde per gram glucose (g/g) for cultures grown under

In the top left panel of **Figure 10**, the optical density at 600nm is shown for each of the volumes used. The culture volume of 5 ml produced the highest cell mass, but it only produced 0.38 g/L isobutyraldehyde. The bottom left panel shows isobutyraldehyde production, the highest production per liter of 1.1 g/L belonging to the 10 ml culture; the production per liter tapered off as the volume increased after 10 ml. Both the top right panel, that shows isobutyraldehyde production per OD, and the bottom right panel that shows grams of isobutyraldehyde produced per gram of glucose consumed contain trends of leveling off after 15 ml culture volume. This

experiment indicates that at very low culture volumes, oxygen isn't that limiting as the cells are mostly using glucose to create cell mass; it is in larger volumes, where oxygen becomes more limited, that the cells look for other ways to unload their NADH pools which is accomplished by producing isobutyraldehyde.

c. Milestone 1.3.3: <u>Eliminate Feed-back Regulation of Key Pathway Enzymes</u>
Deadline: Q3 (SOPO), Y1Q4 (Budget Period)
Status: 100% Completed

In Milestone 1.3.1, all the isobutyraldehyde pathway enzymes were found not to have declining activity that corresponded with the decline in production. Thus, it was not necessary to eliminate the feed-back regulation of any of these enzymes.

d. Milestone 1.3.4: <u>Eliminate Alcohol Dehydrogenases (ADHs)</u>
Deadline: Q4 (SOPO), Y2Q1 (Budget Period)
Status: 95% Completed (at Y1Q4); 100% (Δ*dkgB* experiment repeated in Y2Q2)

E. coli is plagued with many highly active alcohol dehydrogenases (ADHs) that convert aldehydes, such as isobutyraldehyde, to alcohols. In addition, these enzymes are able to convert acetyl-CoA into ethanol, an undesired byproduct in our fermentation. Unfortunately, not all of these ADHs have been identified.

This subtask is dedicated to identifying and eliminating the remaining alcohol dehydrogenase activity in OE9. As mentioned in the previous quarterly report, we have identified ~70 ADH candidates by ADOMETA/NCBI searches. These candidates were cloned, expressed, purified, and assayed for alcohol dehydrogenase activity.

From the assays and the literature, we have identified 9 isobutyraldehyde ADHs: *adhP*, *dkgA*, *dkgB*, *yahK*, *ybbO* (identified by our experiments); *eutG*, *fucO*, *gldA*, *yghA* (from the literature). **Table 3** details our current progress for each mutant.

Deletions in our production strain, OE9, were created for each of the nine candidates. All were constructed, except *dkgA*, which was found to be a lethal knockout. The eight were then tested in small scale (10ml) isobutyraldehyde fermentations. Of the eight, Δ*dkgB* and Δ*eutG* demonstrated some improvement in isobutyraldehyde production and were further tested in large scale fermentations (5L fermentor, 2L working volume). In the 5L fermentors, OE9 Δ*dkgB* demonstrated improved IBAL/glucose (g/g) yield.

	Gene	OE9 Knock Out	IBAL Fermentation 10ml	IBAL Fermentation 5L
1	*adhP*	Constructed	-	
2	*dkgA*	LETHAL		
3	*dkgB*	Constructed	+/-	+
4	*eutG*	Constructed	+/-	-
5	*fucO*	Constructed	-	
6	*gldA*	Constructed	-	
7	*yahK*	Constructed	-	
8	*ybbO*	Constructed	-	
9	*yghA*	Constructed	-	

+/- *IBAL titer or yield improvement, but not statistically signficant*

\- *no change*

Table 3. Summary of ADH mutations created in OE9. Summary of progress of ADH mutations created in OE9 with the results of their IBAL fermentations in small scale (10ml) and large scale (2L).

In **Figure 11**, 2L fermentations of OE9 pSA69 pEB5 and OE9 Δ*dkgB* pSA69 pEB5 are compared. OE9 Δ*dkgB* demonstrates similar isobutyraldehyde titer as compared to the parental strain. However, the amount of isobutanol, ethanol, and cell density (OD600) is lower for the Δ*dkgB* mutant. Related, the amount of glucose utilized was less for the Δ*dkgB* mutant, resulting in a higher isobutyraldehyde yield. OE9 Δ*dkgB* had a 0.256 g/g yield over 114h vs 0.223 g/g over 105hr for OE9 with the *dkgB* mutation.

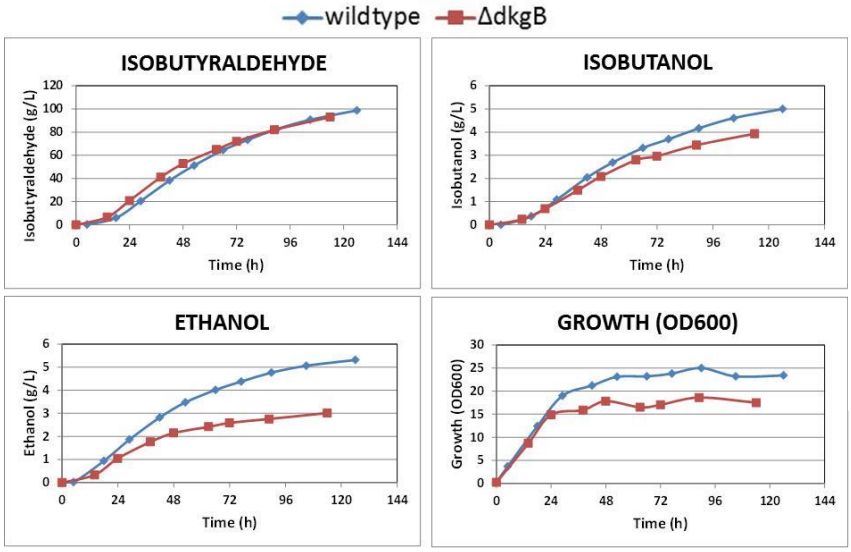


Figure 11. Summary of 2L fermentation of production strains. Isobutyraldehyde, isobutanol, and ethanol production and growth (OD600nm) of "wildtype" (OE9 pSA69 pEB5) and "ΔdkgB" (OE9 Δ*dkgB* pSA69 pEB5) in 5L-fermentations (working volume = 2.0L).

In summary, after identifying and testing ~70 ADH candidates, only one, Δ*dkgB*, was found to decrease isobutanol and ethanol titers with improved isobutyraldehyde yield in a 5L-fermentation. However, when we repeated this examination in additional 5L fermentation runs, we were not able to confirm a significant difference between the wildtype and Δ*dkgB* mutant (March 2016).

Note that we have completed our plan and examined ~70 ADH candidates, and still we have not eliminated isobutanol and/or ethanol production. We were aware of the difficulties of this subtask at the beginning as we knew that it would be difficult to identify all the genes with this common activity.

4. Subtask 2.1: Obtain the Robust Isobutyraldehyde Production Strain and Optimize the Pathway Gene Expression.

For Subtask 2.1, our plan was to: 1) genetically engineer our production strain for long-term strain stability; 2) improve the consumption rate of corn stover hydrolysate; 3) evaluate economic effects of using different fermentation processes on isobutyraldehyde production; 4) use protein engineering tools to improve IlvD activity; and 5) and select the best integrated process.

Milestone 2.1.1: By the end of Q5, integrate isobutyraldehyde pathway genes into production strain.
Milestone 2.1.2: By the end of Q6, increase IlvD activity at later fermentation timepoints (>T=60h) by 2-fold determine the cause of declining isobutyraldehyde production.
Milestone 2.1.3: By the end of Q7, increase the corn stover monosaccharides consumption rate to at least 5 g/L/hr.

a. Milestone 2.1.1: <u>Integrate Isobutyraldehyde Pathway Genes into the Production Strain</u>

Deadline: Q5 (SOPO), Y2Q2 (Budget Period)
Status: 100% Complete

[Note: Milestone 2.1.1 was edited in Revised SOPO 11/14/16]

By integrating the isobutyraldehyde pathway gene into the chromosome, there is no need for selectable markers to maintain the expression plasmids. The major advantage is that this has the potential of creating a more stable production strain. However, one negative consequence of integration is that there are fewer copies of the genes to produce the enzymes needed for production.

We have successfully integrated the genes, but isobutyraldehyde production was lower than expected (**Figure 12**). We believe that this is a result of suboptimal expression of the pathway genes, which is directly related to the reduction in gene copies. We have technically achieved the milestone, but we will continue to work on integrating new constructs into the chromosome.

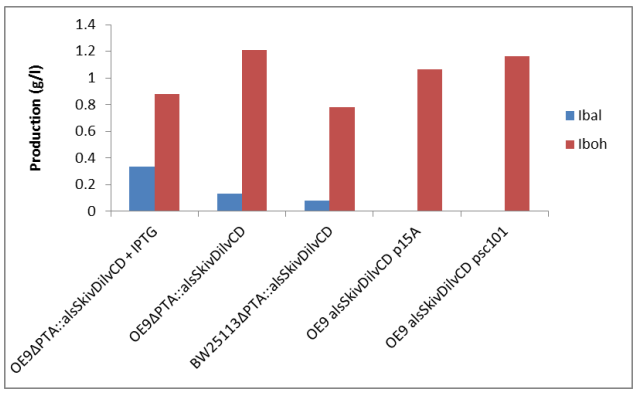


Figure 12. Production with integrated isobutyraldehyde pathway. Isobutyraldehyde pathway genes integrated in the *pta* gene locus for OE9 and BW25113.

b. Milestone 2.1.2: <u>Determine the cause of declining isobutyraldehyde production.</u>
Deadline: Q6 (SOPO), Y2Q3 (Budget Period)
Status: 100%

In Milestone 1.3.1 we have ruled out enzyme activity as a factor for declining production. In this milestone we examined other possibilities. Other experiments have hinted at product toxicity issues associated with our fermentation. Thus, we decided to thoroughly analyze our production strain's growth during our fermentation process.

Our current method for monitoring culture growth consists of measuring optical density (OD) at 600 nm with a spectrophotometer. This rapid and convenient method is a commonly used and accepted practice. However, we decided to confirm cell viability by cell plating, which is a direct indicator of cell growth.

Figure 13 shows the relationship of isobutyraldehyde titer compared to two methods for measuring cell growth: OD600 nm measurements and plating for viable cells. Similar to what we normally observe in fermentations, the OD peaked then very slowly declined. This differs from isobutyraldehyde productivity, which declines fairly quickly and early. Because we normally only monitor growth with OD data, we did not initially suspect strain viability to be the main issue for our decline in production.

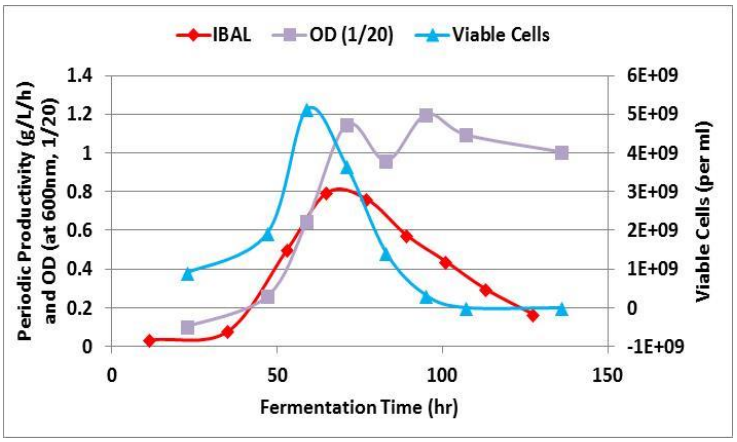


Figure 13. Comparison of viable cell count and optical density of 2L fermentation.
Isobutyraldehyde productivity (g/L/h), optical density at 600 nm, and viable cell count for a 2L fermentation. OD was divided by 20 for to allow for clearer graphical comparison.

On the other hand, cell plating demonstrated a curve that is similar to isobutyraldehyde productivity. This data contradicts OD growth measurements and instead demonstrates that the strain population is dying significantly earlier than we had previously thought.

Moreover, the isobutyraldehyde productivity is following the same pattern as the curve for viable cell count, increasing after it increases, and then declining after it declines. Note that the decline in viable cell also slightly precedes the decline in production curve. The slight delay in the graph is due to how the two parameters are measured; viability is measured at an exact time, while production/productivity is a measurement of isobutyraldehyde collected over a period of time in the traps (and

current fermentor concentration). This highly suggests that productivity is related to early cell population death that we had not confirmed previously.

c. Milestone 2.1.3: <u>Increase the Corn Stover Monosaccharides Consumption Rate to 5 g/L/hr</u>
Deadline: Q7 (SOPO), Y2Q4 (Budget Period)
Status: 100% Complete

The corn stover hydrolysate that we will be using for the duration of this project contains approximately 1 : 0.61 : 0.5 glucose:xylose:arabinose by weight (note exact concentrations and ratios are batch-dependent). Due to elaborate regulation in *E. coli*, sugars are consumed sequentially rather than simultaneously as desired. For this milestone, we are focusing on engineering our production strain to utilize these sugars at a rate of 5 g/L/h.

Through the concerted actions of strain engineering and optimization of our fermentation process, we currently utilize 5.6 g/L/h corn stover monosaccharides in a 2L fermentation. This exceeds our milestone target of 5 g/L/h. In **Figure 14,** our engineered strain EB0022 demonstrates a peak glucose and xylose consumption of 5.6 g/L/h in Lars V1 media (Milestone 1.2.1) supplemented with 8% Luria Broth Media when grown at 33°C, at 3.0 SLPM, pH 6.8, with DO maintained at 5%.

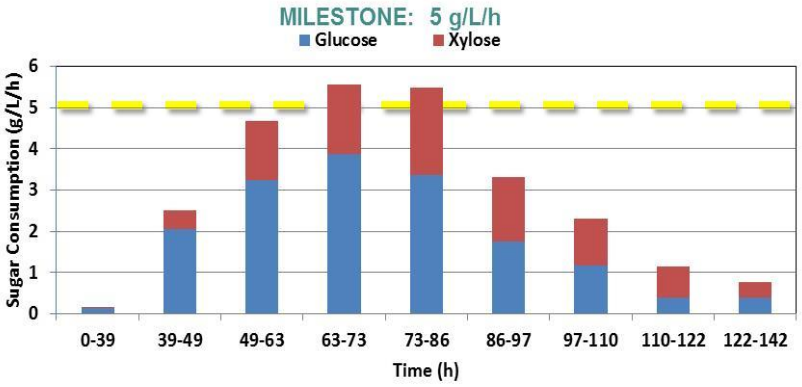


Figure 14. Sugar consumption in 2L fermentation.
Glucose and xylose consumption of 2L Fermentation of EB0022 expressing *alsSilvCD kivd* in Lars V1 media + 8% LB at 33°C, 3 SLPM, pH 6.8, constant 5% DO.

In order to meet this milestone, we continued our efforts strain engineering and optimizing media and fermentation conditions. Our production strain, EB0022, was constructed by the integration of expression cassette, PLlacO1-*xylAB*, into the chromosome of our production strain, OE9. The cassette was integrated into the DNA sequence of *pta*, a gene encoding phosphate acetyltransferase, whose action reduces the availability of our pathway intermediate, pyruvate.

During media optimization, we found that while wildtype *E. coli* grew well in Lars V1, our production strains required additional supplementation. Significant improvement in sugar consumption was the result of the addition of 8% LB at the beginning of the fermentation (no further addition throughout the run). For reference, the LB (1x) we utilized in this experiment contains 10g/L Tryptone, 5g/L Yeast extract, and 10g/L NaCl.

In addition, we found that increasing the gas flow of the fermentation from 2 to 3 SLPM aided in improving sugar consumption. We believe this was the result of improving the stripping of isobutyraldehyde from the media, which improved the strain's health, resulting in better growth (observed with higher OD600) and sugar consumption.

5. Subtask 2.2: Separation Process Development (5L)

Our separation task is much simpler than many other fermentation processes including amino acids, organic acids, ethanol, n-butanol, and isobutanol. After isobutyraldehyde is directly recovered from the fermentation unit, it can then be condensed into liquid form, which can still contain relatively high amounts of water. With higher productivity in the fermentation module, we can achieve more than 7% (wt/vol) of isobutyraldehyde concentration in the condensate.

We developed the whole process flow sheet for producing isobutyraldehyde via fermentation. We conducted process simulation, economic evaluation, and experimental phase equilibrium determinations. In addition, we completed lab and bench-scale tests for proving and scaling continuous separation processes.

Milestone 2.2.1: By the end of Q6, finish the process simulation, economic evaluation, and experimental phase equilibrium determination.
Milestone 2.2.2: By the end of Q8, complete the lab and bench-scale tests for proving and scaling continuous separation processes.

a. Milestone 2.2.1: Finish the Process Simulation, Economic Evaluation, and Experimental Phase Equilibrium Determination

Deadline: Q6 (SOPO), Y2Q3 (Budget Period)
Status: 100% Complete

We analyzed three different separations processes using Chemcad. Specifically, we examined distillation (Simulation #1), gas stripping (Simulation #2), and flashing (Simulation #3). The specific parameters of each simulation are listed in detail in the Y2Q3 Quarterly Report (April 01, 2016 to June 30, 2016; pg 10-15).

From the simulations, we concluded that gas stripping is the best method of separation for our Bio-oxo process. First, gas stripping utilizes less energy than distillation. Gas stripping requires 10 Mj/kg isobutyraldehyde for heating and 7 Mj/kg isobutyraldehyde for cooling (see Figure 11 of Y2Q3 Report). This is significantly lower than that required for distillation (26 Mj/kg heating, 14 Mj/kg cooling). Flashing has comparable energy demands as gas stripping, but the drawback with this operation is that the fermentation is under a vacuum for an extended period of time, which means the oxygen availability is very low. This could potentially harm cell grow and production. Still, this method is relatively energy efficient.

Due to isobutyraldehyde's unique physical properties, such as low Henry's law constant and limited water solubility, stripping is a very attractive recovery method for isobutyraldehyde separation. It would only be necessary to remove a dilute stream from the fermenter, which contains mostly water. The water can be separated by condensation at reasonable temperatures, with minimal loss of isobutyraldehyde. The enriched isobutyraldehyde stream can then be condensed at lower temperatures to reach a concentration above the solubility limit to create an organic phase with only 2 wt. % water.

In addition to the separation process of stripping, air is also necessary for our production strains to produce isobutyraldehyde. Sugars like glucose need oxygen to balance the reaction to make isobutyraldehyde:

$$Glucose + 4\,ADP + 4\,PO_4^{2-} + 2H^+ + O_2 \rightarrow Isobutyraldehyde + 2\,CO_2 + 4\,ATP + 6H_2O + 2H^+_{Cross}$$

Air or oxygen must be supplied to the fermenter, and subsequently released from the fermenter. Isobutyraldehyde, because of its high volatility and low Henry's law constant, will always be in the off-gas with CO_2 and other gases. Even without stripping as a recovery method, the off-gas has to be treated to remove isobutyraldehyde.

Thus, in terms of operational costs, the expense of air compressors and agitation would be more justified by serving two purposes: to maintain the redox balance necessary for continued isobutyraldehyde production and gas stripping for the separation process.

The recovery efficiency can be increased with a higher isobutyraldehyde concentration in the fermenter. That would mean higher productivity if the air flow rate is maintained at the same rate. Higher productivity would allow for a smaller fermenter and smaller air compressor, which would result in cost savings in many categories, such as capital expenditures (CAPEX), utilities, and media expense. Longer fermentations with sustained productivity would also help in cost savings in CAPEX, operational expenditures (OPEX), and media.

b. Milestone 2.2.2: <u>Complete the Lab and Bench-Scale Tests for Proving and Scaling Continuation Separation Processes</u>
Deadline: Q8 (SOPO), Y3Q1 (Budget Period)
Status: 100% Complete

The final setup of the bench scale continuous separation unit is shown in **Figure 15**. The off-gas from the fermenter contains our product and is saturated with water vapor. It enters two different gas analyzers, the first for oxygen (O_2), carbon dioxide (CO_2), and the relative gas humidity, and the second for hydrocarbons. This will provide us with data on oxygen utilization, isobutyraldehyde production, and carbon dioxide production in real time.

After passing through the sensors, the gas continues into the first condenser, where 85% of the water is removed to a dew point of 0°C. The condenser is a double coiled stainless steel tubing with 0°C ethylene glycol as the cooling medium. We will not use a temperature lower than 0°C because it results in freezing of the water vapor inside the condenser, potentially creating a potential blockage.

Between the first and second condenser is a gas dryer, which will prevent the blockage of ice in the second condenser. This dryer is a stainless steel pipe containing desiccant (Drierite) to absorb the remaining water. The off-gas is dried to a dew point of -73°C according to the manufactures' specification. This is lower than what we can accurately measure with our humidity sensor.

The dried gas enters the second condenser where the isobutyraldehyde is condensed out. The second condenser consist of a 2 feet long, 1.25 inch diameter stainless steel pipe filled with disc made out of stainless steel mesh to increase the surface area for condensation. It is designed to tolerate water contamination. It will still operate even though water enters and freezes. The outside of the stainless steel pipe is cooled with silicone oil set at -55°C. Between each unit, the relative humidity, temperature, and pressure of the gas is measured.

After the final condenser, a hydrocarbon sensor is used to identify the amount of uncondensed isobutyraldehyde. Both condensers are heavily insulated to increase cooling efficiency and to prevent outside ice buildup.

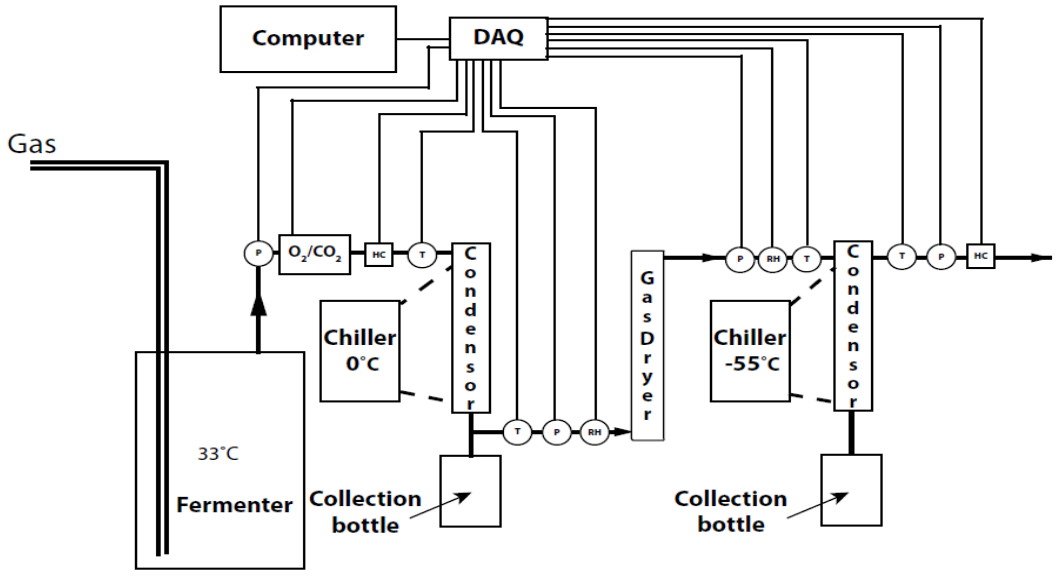


Figure 15. Schematic of the continuous recovery unit setup.
Small circles indicate sensors measurements of pressure (P), temperature (T), relative humidity (RH), and hydrocarbon (HC). All the sensors are wired to a data acquisition board (DAQ). The data was logged using Labview.

Testing water removal from the fermenter off-gas.

To test the water removal capacity of the condenser and gas dryer, water-saturated off-gas was run through the condenser using the standard operational fermenter

conditions. The off-gas entering the first condenser contains 30 g of water/m^3 on average. After the passing through the first condenser, the gas contains, on average, 4.4 g water/m^3 on average. This is a reduction of 85% of the water content (**Figure 16**). After 125 minutes, the dryer unit was introduced and the water content declines to ~ 0 g water/m^3. This is approaching the measurement limit of the relative humidity sensor. This experiment demonstrates that we can remove almost all of the water resulting in condensate of almost pure isobutyraldehyde.

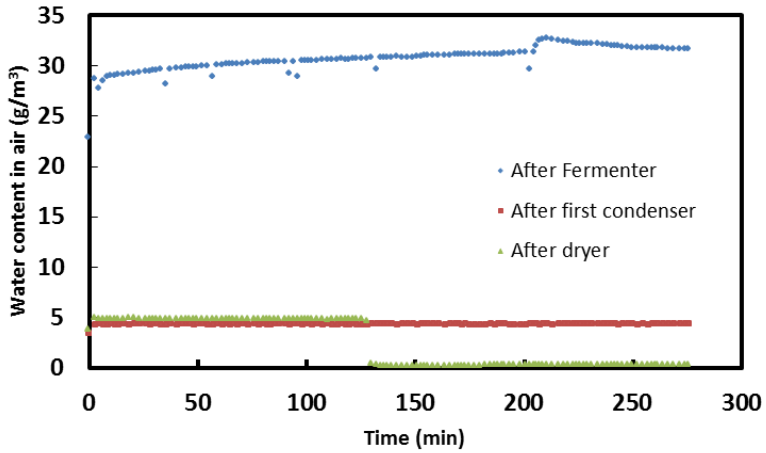


Figure 16. Testing of the water removal from the fermenter off-gas.

Testing of hydrocarbon sensors.

To measure the productivity of isobutyraldehyde in real time, we installed two hydrocarbon sensors to evaluate the production and recovery effort. The two sensors were connected in series directly after the fermenter gas outlet. Gas stripping of a fermenter was initiated at different isobutyraldehyde concentrations in the vessel to observe the response of the sensors (**Figure 17**). The data demonstrates a linear relationship between the isobutyraldehyde concentration in the vessel and the isobutyraldehyde in the gas stream. This relationship is expected from Henry's law. Both sensors demonstrated the same pattern and have a similar response in arbitrary units. The units were calibrated using know amounts of isobutyraldehyde in the gas sample.

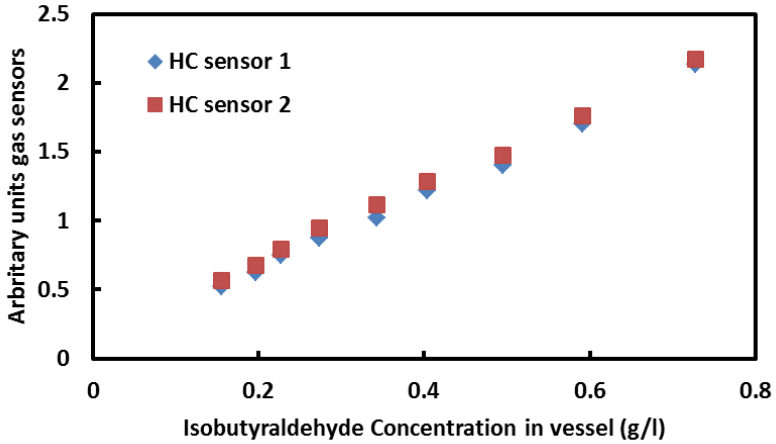


Figure 17. Testing hydrocarbon sensors' ability to measure isobutyraldehyde productivity.
Graph demonstrating the relationship between the isobutyraldehyde concentration in the vessel and the measured isobutyraldehyde concentration in the gas phase.

The sensor was then tested with addition of isobutyraldehyde to the fermenter for a final concentration of 1 g/L after 110 minutes. The isobutyraldehyde concentration in the gas phase was monitored (**Figure 18**). An almost immediate response in the gas phase can be observed, followed by a gradual decrease as the isobutyraldehyde in the fermenter is stripped off. We observed that both sensors behaved in a similar manner. A concentration of 0.017g/L isobutyraldehyde air can be seen initially, which represents a productivity of 1 g/L/h of isobutyraldehyde.

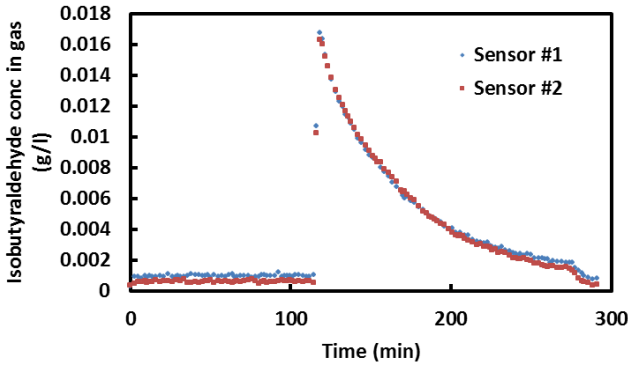


Figure 18. Testing hydrocarbon sensor's sensitivity. Isobutyraldehyde concentration in the gas phase after the addition of 1g/L isobutyraldehyde to the fermenter.

Testing of the integrated system.

In order to test the continuous recovery of the isobutyraldehyde system, we placed the first hydrocarbon sensor after the fermenter and a second sensor after the second condenser. We added isobutyraldehyde to a final concentration of 1 g/L into a fermentation vessel running at standard fermentation conditions (*eg* temperature 33°C, agitation 300 rpm, 1 VVM of gas flow). As shown in **Figure 19**, isobutyraldehyde was added at 30 minutes, which was detected by the first condenser with a sharp peak. Nothing is detected by the second sensor until 75 minutes after the addition. There is a small increase in the signal indicating that not all isobutyraldehyde is removed. After 190 minutes, an additional 2 g/L isobutyraldehyde was added to the system. Immediately, the first sensor registers a spike. At 225 min, the VVM was increased to 2.5. A faster decrease in the stripping can be seen. There is no increase in the signal after the 2nd condenser. Based on the data from both sensors, 90% of the isobutyraldehyde was recovered. A potential improvement can be made by decreasing the temperature in the second condenser and increasing the air flow.

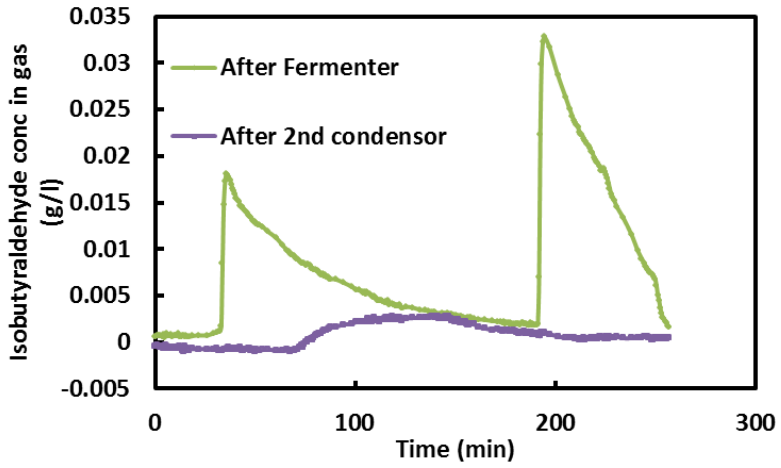


Figure 19. Testing of the isobutyraldehyde recovery unit. The hydrocarbon sensors were placed after the fermenter and after the second condensor. The fermenter was spiked with known amounts of isobutyraldehyde.

6. Subtask 2.3: Integrated Process Development (5L).

Our Bio-Oxo fermentation system uses a gas (air)-stripping process. We constructed the integrated process of fermentation and separation on a 5L bench-top scale. All the unit processes developed was used for integrated process construction, including fermentor type, proper condensation unit and downstream purification unit for concentrated isobutyraldehyde recovery. After construction of this integrated process, operational conditions were optimized with the data obtained from this process. From this data, isobutyraldehyde production cost was determined.

Milestone 2.3.1: By the end of Q6, finish the best-case economic model.
Milestone 2.3.2: By the end of Q7, reach 1.2 g/L/hr productivity using purified corn stover monosaccharides as raw material.
Milestone 2.3.3: By the end of Q8, reach 0.5 g/L/hr productivity using corn stover hydrolysate as raw material.

a. Milestone 2.3.1: <u>Finish the Best-Case Economic Model</u>

Deadline: Q6 (SOPO), Y2Q3 (Budget Period)
Status: 100% Complete

We have completed the best-case economic model for our Bio-oxo process, employing the economics of cellulosic ethanol plants as a guide. The details of our analysis are covered in detail in our Y2Q3 Report (pg 15-25).

Briefly, we aim to produce/replace 10% of the US market for isobutyraldehyde, ~30,000 ton/yr. To produce 30,000 ton/y isobutyraldehyde, we will need 695 ton/day of corn stover (assuming 20% moisture content of the corn stover, 90% recovery of fermentable sugars, 20% overall loss in production). The corn stover will be processed and stored in a central location and then transported to the biorefinery. We estimate it will take 35 railcars per 7-day week, or 49 railcars for a 5-day week. We will need two 6600 m^3 domes/silos to store 1050 tons of corn stover – enough to

support 72 hours of operation. A flowchart summary of our model's pretreatment, enzyme hydrolysis, fermentation, separation, and storage is shown in **Figure 20**. The details of each are described in Figures 18-21 of our Y2Q3 Report.

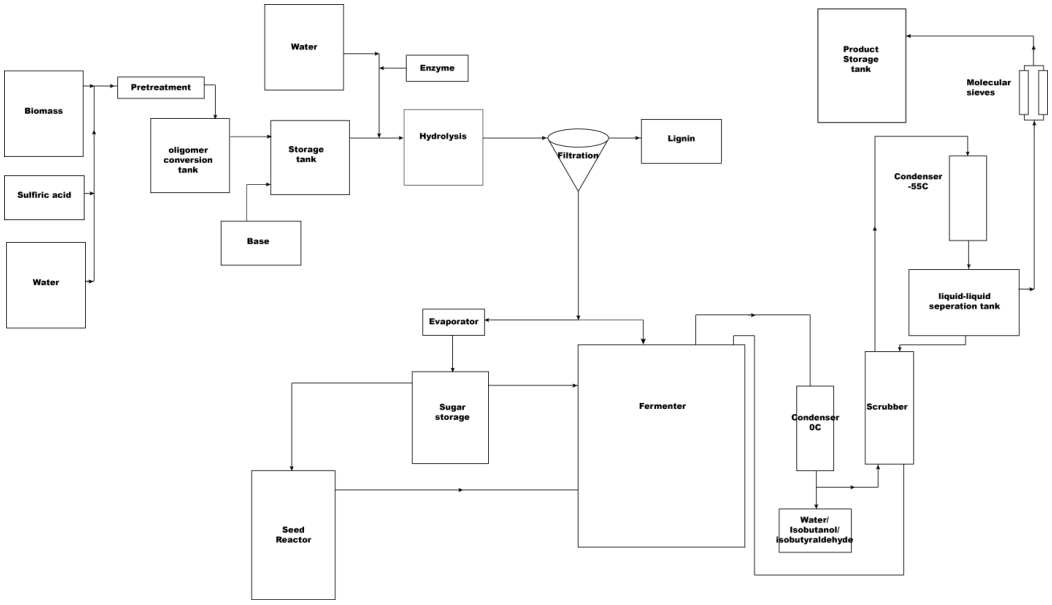


Figure 20. Summary of our process including pretreatment, enzyme hydrolysis, fermentation, separation, and storage.

We estimate that our total capital investment will be $158,760,000, which includes the cost for feed handling, pretreatment and conditioning, hydrolysis, seeding, fermentation, recovery/separation, storage, total capital cost, install cost, indirect cost, fixed capital investment, and working capital (Figure 22 of Y2Y3 Report). Our fixed operating cost of $3,828,500 includes $2,015,000 in salaries (plant manager, plant engineers, supervisors, and workers) and $1,813,500 in overhead indirect costs.

To calculate the manufacturing cost per kg isobutyraldehyde, we estimated the price of all major raw materials, utilities, fixed costs, and capital depreciation costs (**Table 4**).

Currently, the total cost for isobutyraldehyde production using our Bio-oxo process is $3.27/kg isobutyraldehyde.

Manufacturing Cost	$/kg
Corn Stover + Handling	0.56
Sulfuric Acid	0.01
Enzyme	0.28
Ammonia	0.04
Salts	1.42
Fermentation Utilities	0.21
Utilities	0.15
Fixed Costs	0.13
Capital Depreciation	0.46

Table 4. Breakdown of all manufacturing costs for the Bio-oxo process.

TOTAL	3.27

b. Milestone 2.3.2: <u>Reach 1.2 g/L/hr Productivity Using Purified Corn Stover Monosaccharides as Raw Material</u>
Deadline: Q7 (SOPO), Y1Q4 (Budget Period)
Status: 100% Complete

A

In rich media, EB0024 (OE9 *yjgB*::PCP25-*xylAB adhE*::PCP25-*xylFGH*) expressing *alsSilvCD kivd*, produced up to 1.3 g/L/h isobutyraldehyde in a 2L fermentation. This exceeds our target of 1.2 g/L/hr. **Figure 21** demonstrates the periodic productivity of this 115 h fermentation run.

B

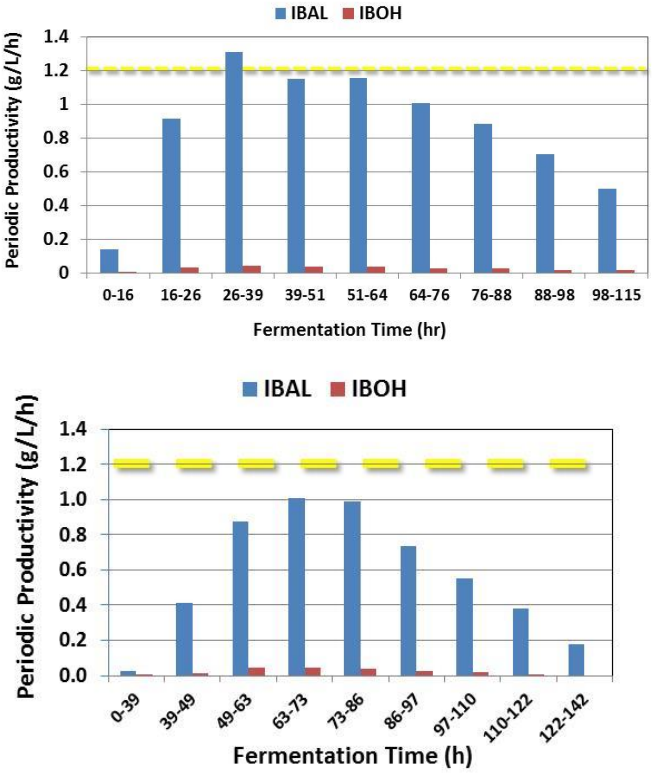


Figure 21. Reaching 1.2 g/L/hr producitivity using purified corn stover monosaccharides as raw material. Isobutyraldehyde periodic productivity of (A) EB0024 expressing *alsSilvCD kivd* in a 2L fermentation in rich media and (B) EB0022 expressing *alsSilvCD kivd* in a 2L fermentation in Lars V1 media with 5% AA media. The yellow dashed line indicates the milestone of 1.2 g/L/h isobutyraldehyde.

Although we have met our milestone, we are continuing to improve our engineered strain and operational conditions to reduce the cost of our Bio-oxo process. Because our operational costs are significantly impacted by media price, it is advantageous to maintain production using cost-effective components.

EB0022 produced up to 1.0 g/L/h isobutyraldehyde in a 2L fermentation using semi-defined media (**Figure 21**). Lars V1 was supplemented with 5% AA seed media, which at 1x contains 5% of M9 salts, 1% yeast extract, 1.5 mM $MgSO_4$, 48mM Na_2PO_4, 22mM KH_2PO_4, 9 mM NaCl, and 19 mM NH_4Cl.

c. Milestone 2.3.3: <u>Reach 0.5 g/L/hr Productivity Using Corn Stover Hydrolysate as Raw Material</u>

Deadline: Q8 (SOPO), Y3Q1 (Budget Period)
Status: 100% Complete

Corn stover hydrolysate fermentation media optimization

We examined isobutyraldehyde production in four different media – two rich and two defined/semi-defined. The two rich media were: 1) M9 salt media with yeast extract (M9-Ex) and 2) Fermentation media (FM). The two defined or semi-defined media were: 1) LarsV1 and LarsV2. FM (Acronym Table), LarsV1 (Milestone 1.2.1 in Y1Q3 report), and LarsV2 (Milestone 2.1.3 in Y2Y3 report) have been described elsewhere. Each of these media were examined with either purified monosaccharide sugars (Glu/Xyl) or corn stover hydrolysate (CS).

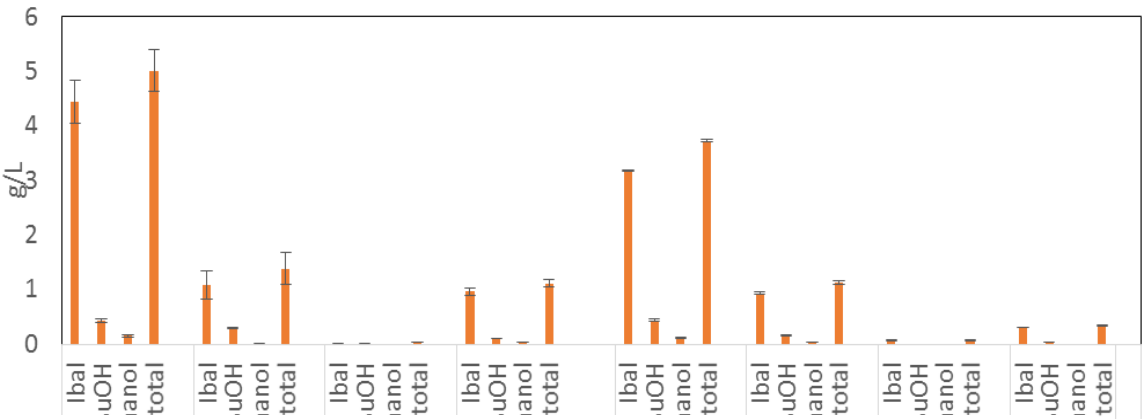
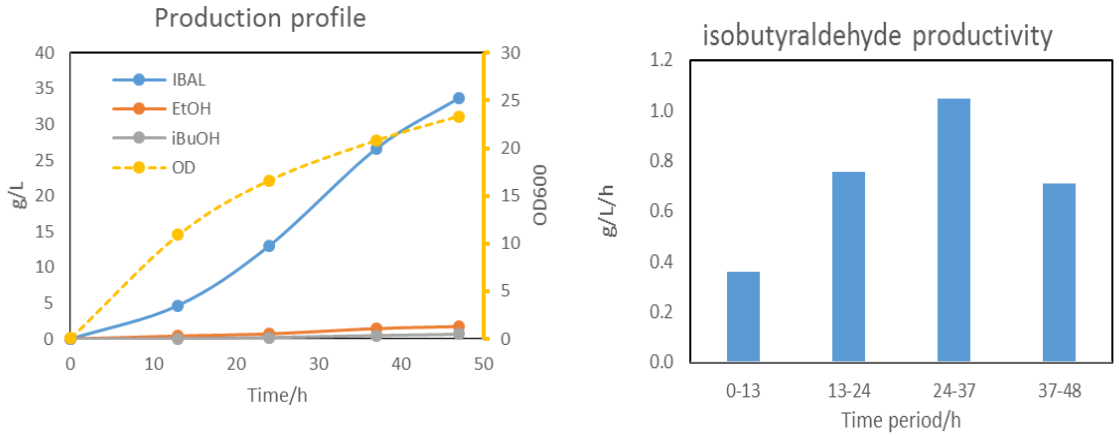


Figure 22. Isobutyraldehyde production in different corn stover media.
EB0022 expressing *alsS ilvCD kivd* were grown in 10ml of media with either purified corn stover monosaccharides ("Glu/Xyl") or NREL pretreated corn stover ("CS"). Isobutyraldehyde production was sampled after 24 hours. M9 EX = M9 salts with yeast extract; FM = Fermentation media (see pg 2); Lars V1 = defined media (see Milestone 1.2.1 in Y1Q3 report); Lars V2 = Lars V1 + 8% LB (Milestone 2.1.3 in Y2Y3 report).

In the 10ml fermentation, EB0022 demonstrated the best isobutyraldehyde production with corn stover hydrolysate in the M9-CS-Ex rich medium (**Figure 22**). This production is ~80% of that produced in the same media with the purified sugars (M9-Glu/Xyl-Ex). This medium was selected for scale up in a 5L benchtop fermentor.

Isobutyraldehyde production from corn stover hydrolysate in a 5L benchtop fermentor

EB0022 was subsequently tested in a 5L benchtop fermenter (2L working volume) with corn stover hydrolysate medium supplemented with yeast extract (**Figure 23**). Over 48 hours, 33.4 g/L isobutyraldehyde was produced. Cell mass accumulated mainly within the initial 13 hours. After this initial period of growth (T > 13h), isobutyraldehyde was produced at rates higher than 0.5 g/L/h at 3.0 SLPM, pH 6.8, with DO maintained at 5%.



(A) (B)

Figure 23. Isobutyraldehyde production from corn stover in a 5L benchtop fermentor. (A) isobutyraldehyde (iBAL), ethanol (EtOH), isobutanol (iBuOH) titer and optical density (OD). (B) iBAL productivity. EB0022 transformed with *alsS ilvCD kivd* was cultured continuously in a 5L benchtop fermentor with corn stover hydrolysate, M9 salts, and 10 g/L yeast extract.

7. Subtask 3.1: Determine the best aspect ratio and oxygen transfer coefficient (k_La) for our Bio-oxo process.

We conducted additional experiments in the 5L fermentor (1.3 – 3.8 L working volume) to determine the physical scale-up variables of our process. Specifically, we determined the best aspect ratio (1.0, 1.25, or 1.5) and the oxygen transfer coefficient (k_La).

Milestone 3.1.1: By the end of April 2017, determine the best aspect ratio (1.0, 1.25, or 1.5) for isobutyraldehyde titer and productivity in the 5L fermentor.

Milestone 3.1.2: By the end of May 2017, determine the oxygen transfer coefficient (k_La) for our process in a 5L fermentor.

a. Milestone 3.1.1: <u>Determine the Best Aspect Ratio (1.0, 1.25, or 1.5) for Isobutyraldehyde Titer and Productivity in the 5L Fermentor.</u>

Deadline: April 30, 2017
Status: 100% Completed

Our overall goal for Subtask 3.1 is to facilitate the scale up process as we transition from our current 2L (working volume) fermentor to 10L and 30L fermentors. In this process, it is essential to reduce productivity declines which can occur with each increase in scale. Towards this goal, it is possible to fix a key parameter and ensure that it remains unaltered throughout scale-up. Two parameters often chosen to

remain firmly fixed are the fermentor height-diameter aspect ratio and oxygen transfer coefficient (KLa).

In Subtask 3.1.1, we specifically focused on fermentor aspect ratio. Aspect ratio refers to the height versus the width of the fermentation culture in the fermentor (**Figure 24**). Thus, assuming the same fermentor is used in all experiments (width is constant), a higher aspect ratio corresponds to a larger volume.

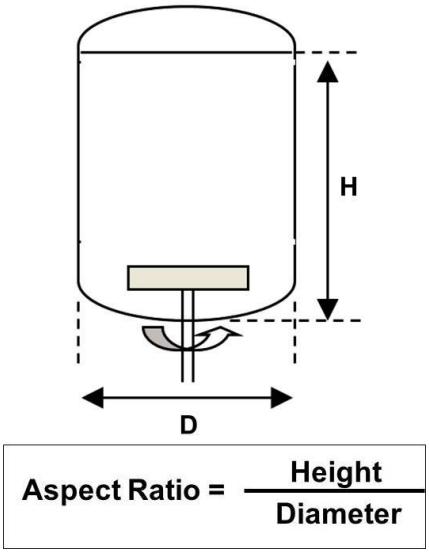


Figure 24. Explanation of fermentor aspect ratio.
Fermentor aspect ratio refers to the height to diameter ratio of a culture in a fermentation system. Different volumes will result in a different aspect ratio.

Specifically, we tested different aspect ratios to determine which results in the highest isobutyraldehyde titer/yield. For all fermentation data presented previously, we maintained a vessel volume of ~ 2.0L. This corresponds to an aspect ratio of 0.9:1. For simplicity, we refer to this as aspect ratio 1:1. To determine if other aspect ratios would improve production, we tested fermentations at 3.0L (aspect ratio ~ 1.25:1) and 3.5L (aspect ratio ~ 1.5:1).

During this reporting period, we completed our examination of the effect of aspect ratio on our fermentation process. Specifically, we conducted 2 independent fermentations at 1:1, 3 fermentation runs at 1.25:1, and 3 fermentation runs at 1.5:1. Only the aspect ratio differed between these runs. As shown in **Figure 25**, the production of isobutyraldehyde was approximately the same among for all aspect ratios tested. In addition, the titers of the byproducts, isobutanol and ethanol, do not appear to be affected by aspect ratio.

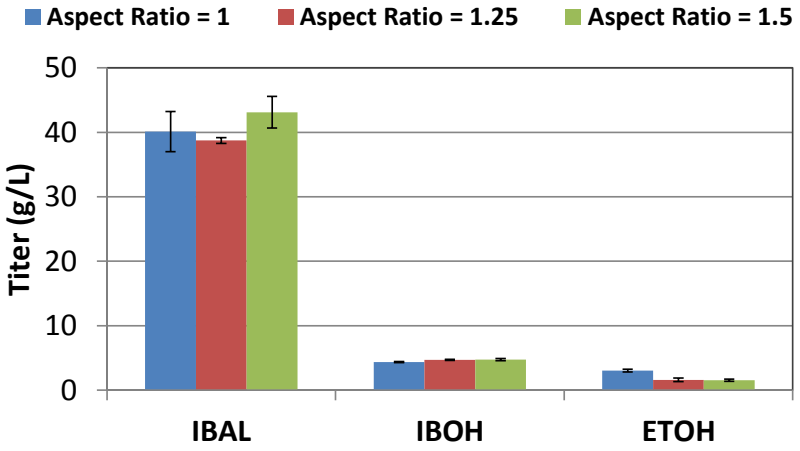


Figure 25. Isobutyraldehyde production at different aspect ratios.
Isobutyraldehyde (IBAL), isobutanol (IBOH; byproduct), and ethanol (ETOH) production of INT5 based on aspect ratio in 2L fermentations over ~100 hours. We examined production at aspect ratio = 1 (2.0L), aspect ratio = 1.25 (3.0L), and aspect ratio = 1.5 (3.5L).

b. Milestone 3.1.2: <u>Determine the Oxygen Transfer Coefficient (k_La) for Our Process in a 5L Fermentor.</u>
Deadline: May 31, 2017
Status: 100% Completed

Oxygen transfer coefficient is one parameter which can help in the scale up of bioreactors. If you can supply the same amount of oxygen to the cells in a larger vessel, as in the lab scale reactor, the chances of a successful scale up are higher. The oxygen concentration will change in larger vessels due to the higher pressure at the bottom of the tank. The change in pressure will affect the oxygen solubility and the impeller will impact the bubble size. To determine the oxygen transfer coefficient for our Bio-Oxo production in a 5L fermenter, we measured the oxygen and carbon dioxide concentrations in the off-gas from the fermenter. For this experiment, the standard fermentation conditions were utilized (**Table 5)**.

Table 5. Conditions used in fermentation runs.

Parameter	Value	Unit
Temperature	33	°C
Set DO	5	%
Starting VVM	1.5	Vessel volume per min
Starting agitation	300	Rpm

The strain used was the integrated strain INT5 and the media used was the defined Lars V1 media containing glucose and xylose. We investigated different conditions and parameters which can affect the oxygen transfer coefficient. The oxygen transfer coefficient (K_La) can be calculated from the following formula.

$$OTR = K_L a\ (C^*_{O_2} - C_{O_2})$$

C^*_{O2} is concentration of oxygen in the liquid at saturation, *ie* 100% dissolved oxygen (DO), in our case the saturated oxygen concentration at 33 °C is (7.16 mg O_2/L). CO_2 is the measured concentration in the liquid; we set the fermenter to control the agitation so the dissolved oxygen is always 5% of saturation that gives us a concentration of 0.358 mg O_2/L. The difference between the two concentrations is the driving force for the oxygen transfer.

In addition to monitor the oxygen and carbon dioxide concentration, temperature, pressure, and the hydrocarbon concentration in the off gas was also measured. The air flow was turn up from 1 VVM to 1.5 VVM at 38 h and subsequently lower back to 1 VVM at 85 h. Two independent runs on different fermenters were conducted.

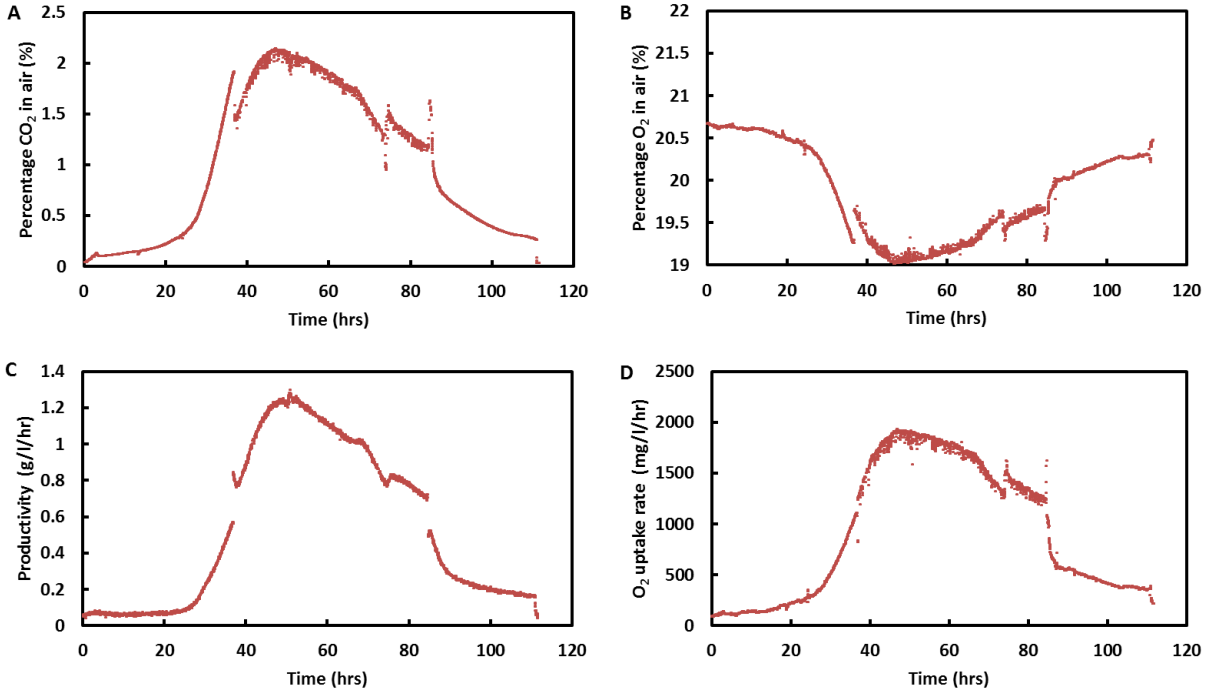


Figure 26. Percentage of CO_2 and O_2 in the air over the time course of a fermentation (Run 1). A) Percentage of CO_2 in the air (v/v) over the time course of fermentation. B) Percentage of O_2 in the air (v/v) over the time course of fermentation. C) Productivity of hydrocarbon (g/L/h) over the time course of fermentation. D) Oxygen uptake rate (OUR) (mg/L/h) over the time course of fermentation.

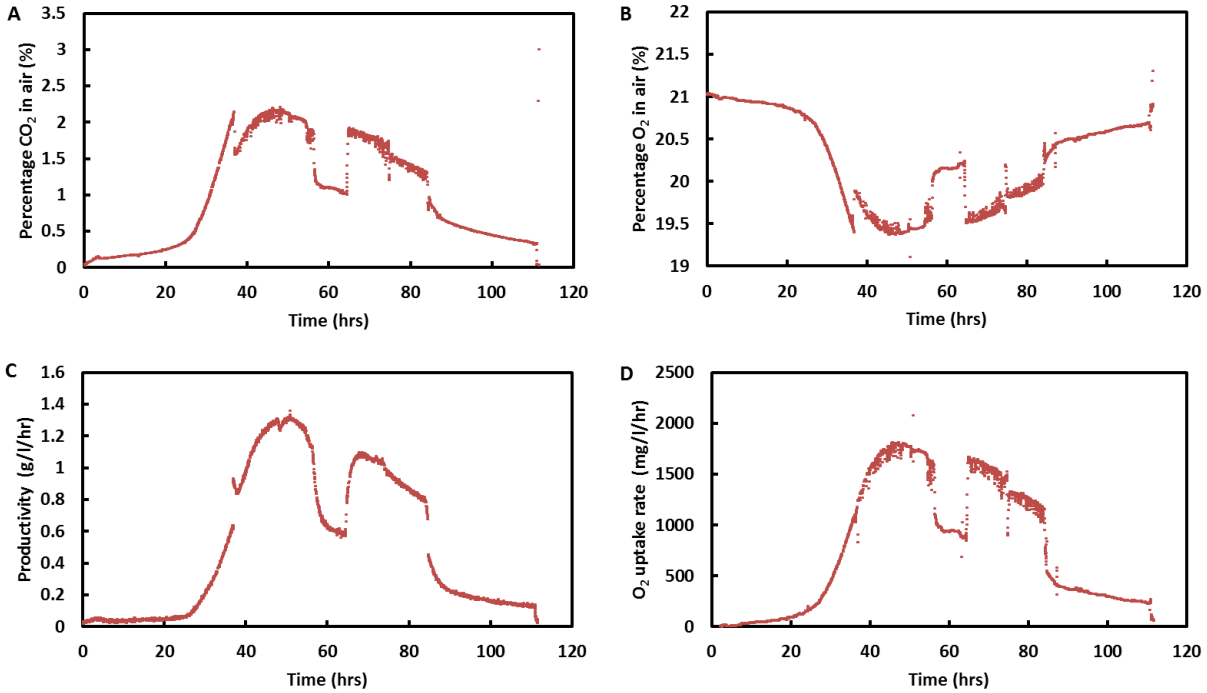


Figure 27. Percentage of CO2 and O2 in the air over the time course of a fermentation (Run 2). A) Percentage of CO_2 in the air (v/v) over the time course of fermentation. B) Percentage of O_2 in the air (v/v) over the time course of fermentation. C) Productivity of hydrocarbon (g/L/h) over the time course of fermentation. D) Oxygen uptake rate (OUR) (mg/L/h) over the time course of fermentation.

In the panel A of **Figure 26** and **27** the concentration profile of carbon dioxide can be seen. As the cell starting to grow after 25 h, the carbon dioxide increase exponentially and reaches a plateau around 2% of the total volume, then the concentration gradually decreases. The same pattern can be seen in the oxygen consumption, see panel B, but inversed. From the oxygen consumption we can calculate the oxygen uptake rate. The oxygen uptake rate is the consumption of oxygen per liter broth and hour. Panel C in **Figure 26** and **27** shows the oxygen uptake rate (OTR) for the experiment, it follows the same pattern as the carbon dioxide production. At the highest level, the uptake rate is around 2000 mg O_2/L/h, and a K_La range of 280-310 L/s. Panel C in both **Figure 26** and **27** shows the productivity of isobutyraldehyde, its highest production is when the cells consume the most oxygen. For every isobutyraldehyde molecule, two carbon dioxides are created and ½ oxygen molecule is consumed to remove the NADH excess in the cell. The dip seen in **Figure 27** for carbon dioxide and isobutyraldehyde production at 55 h through 67 h cannot be explained.

Changing the set DO to 20% lower the oxygen uptake rate slightly from around 1900-2000 mg/L/h to around 1700-1800 mg/L/h (see **Figure 28**, top right panel), which gave a K_La a range of 290-320 L/s, which are in line with the range of 280-310 L/s for the 5% DO.

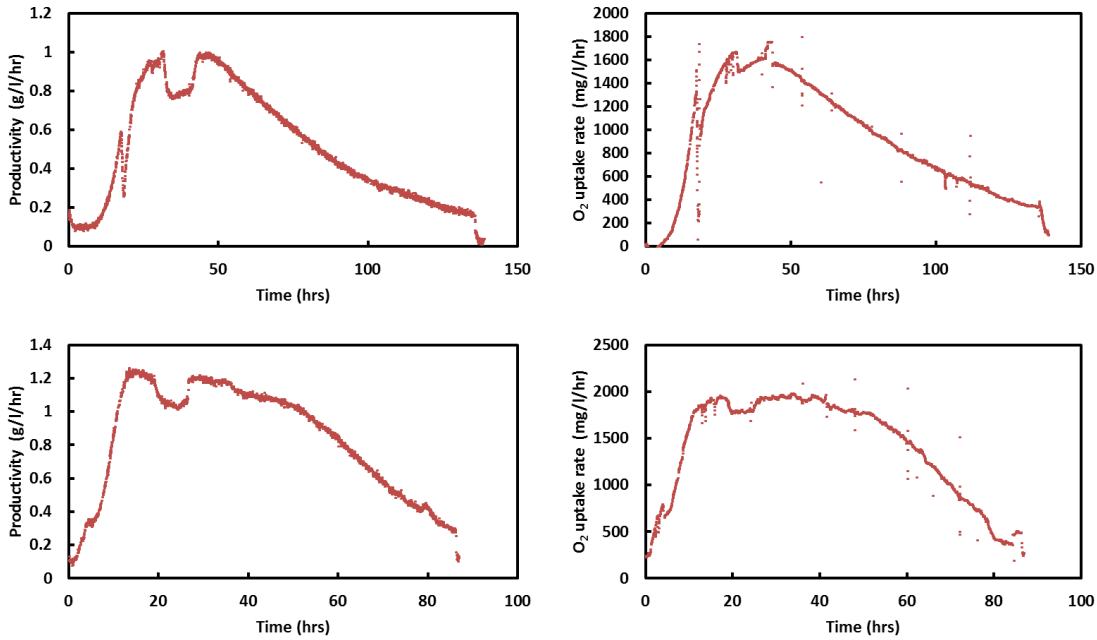


Figure 28. The oxygen transfer coefficient was also tested in rich media.
Top panel represent productivity and oxygen uptake rate for fermentation runs with dissolved oxygen set to 20%, lower panel represent productivity and oxygen uptake rate for fermentation runs in complex media.

The oxygen transfer coefficient was also tested in rich media, **Figure 28** lower panel, productivity and oxygen uptake rate did not change dramatically compare to the defined media using same parameter for the fermentation. The dissolved oxygen was set to 5% DO, the oxygen uptake rate ranged from 1900-2000 mg/L/h which gave a K_La a range of 285-300 L/s. In conclusion, the K_La ranged between 285 to 310 1/s during all conditions tested indicating that for this specific setup a K_La of 300 L/s is a good value to use in order to duplicate this conditions and parameters in a larger fermenter.

8. Subtask 3.2: Complete Setup and Conduct Fermentation (14L).

In order to confirm that our Bio-Oxo process/operational parameters are applicable to the scale up process, we applied our parameters from our 5L fermentor data to those for 14L fermentor. This data will be important in confirming the scalability of our process to the 30L scale.

Milestone 3.2.1: By the end of May 2017, complete the setup of the 14L fermentor.
Milestone 3.2.2: By the end of August 2017, obtain 0.6 g/L/h productivity and 40 g/L isobutyraldehyde titer using corn stover hydrolysate as raw material in the 14L fermentor.

a. Milestone 3.2.1: <u>Complete the setup of the 14L fermentor.</u>

Deadline: May 31, 2017
Status: 100% Complete

The assembly of the 14L fermenter and the testing of the associated auxiliary components were completed in May. The new system was tested by duplicating the 5L fermentor conditions (**Figure 29**). The defined media was supplemented with both glucose and xylose. The air flow rate was initially set to 1 VVM and after 10 hours was changed to 1.5 VVM to increase product removal. The peak productivity increased to almost 1.6 g/l/hr. This is ~33% higher than the 1.2 g/L/h that was obtained in the 5L fermenter. The fermentation was stopped after 55 h as the sugar feed ran out. A total production titer of 48 g/L isobutyraldehyde was achieved.

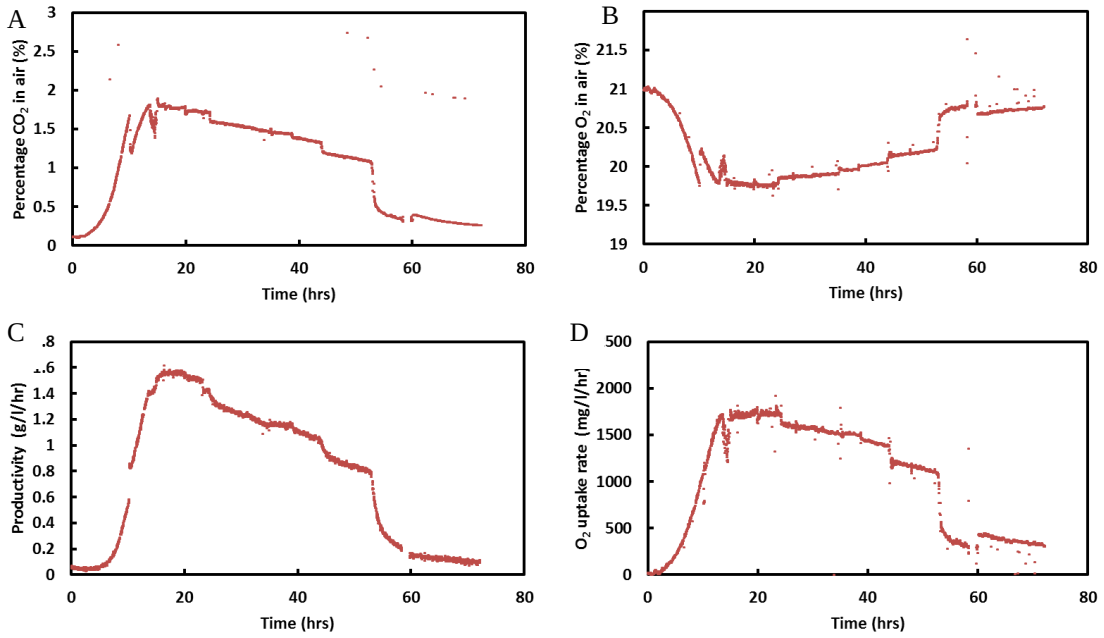


Figure 29. Testing the assembly of the 14L and auxiliary components.
A) Percentage of CO_2 in the air (v/v) over the time course of fermentation. B) Percentage of O_2 in the air (v/v) over the time course of fermentation. C) Productivity of hydrocarbon (g/L/h) over the time course of fermentation. D) Oxygen uptake rate (OUR) (mg/L/h) over the time course of fermentation.

b. Milestone 3.2.2: <u>Obtain 0.6 g/L/h productivity and 40 g/L isobutyraldehyde titer using corn stover hydrolysate as raw material in the 14L fermentor.</u>
Deadline: August 31, 2017
Status: 100% Complete

In order to reach milestone 3.2.2, we conducted a few runs using defined Lars V1 media and glucose and xylose as the carbon source acting as a synthetic corn stover hydrolysate. The air flow was set to 1.5 VVM at the start and was not changed. Minor adjustments were made to the recovery unit to ensure that all the products and water were condensed and collected. The run was stopped at 72 h after the pressure increased in the condenser due to potential ice buildup. This run resulted in a production titer of 52 g/L isobutyraldehyde with a sustained productivity of over 1 g/L/h for ~ 40 h (**Figure 30**).

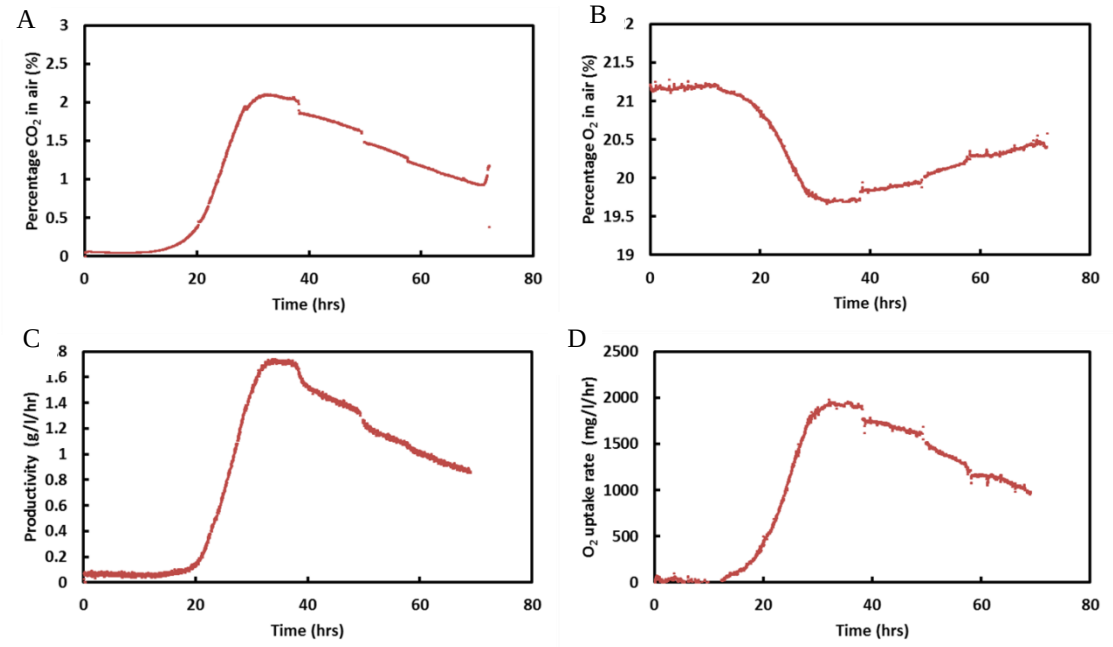


Figure 30. Demonstration of exceeding milestone of 0.6 g/L/h productivity using glucose and xylose as raw material in the 14L fermentor.
A) Percentage of CO_2 in the air (v/v) over the time course of fermentation. B) Percentage of O_2 in the air (v/v) over the time course of fermentation. C) Productivity of hydrocarbon (g/L/h) over the time course of fermentation. D) Oxygen uptake rate (OUR) (mg/L/h) over the time course of fermentation.

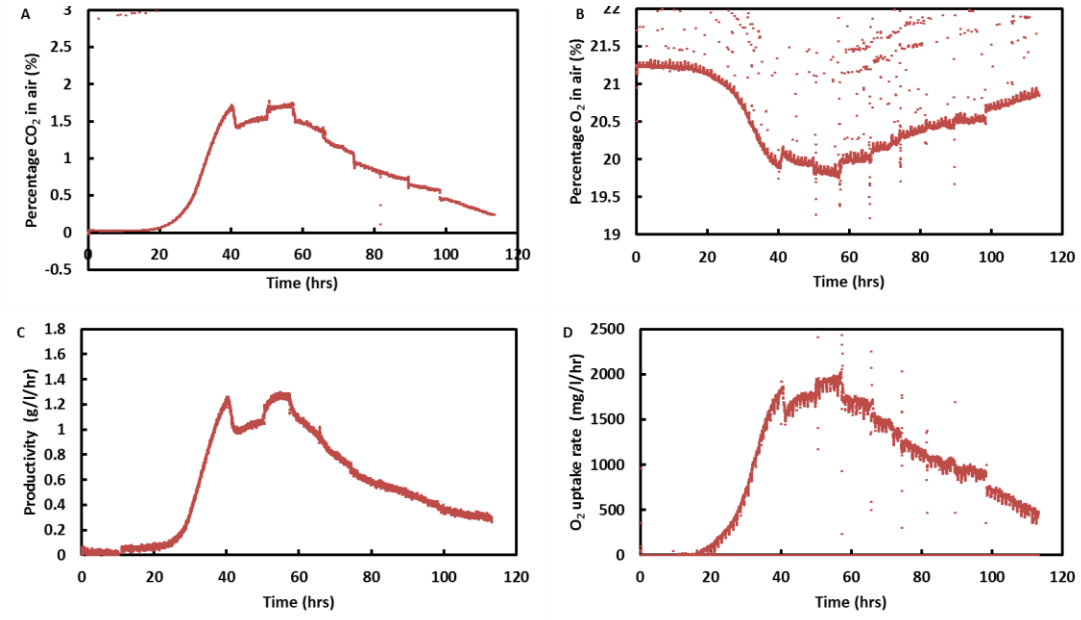


Figure 31. Demonstration of exceeding milestone of 0.6 g/L/h productivity using corn stover hydrolysate as raw material in the 14L fermentor.
A) Percentage of CO_2 in the air (v/v) over the time course of fermentation. B) Percentage of O_2 in the air (v/v) over the time course of fermentation. C) Productivity of hydrocarbon (g/L/h) over the time course of fermentation. D) Oxygen uptake rate (OUR) (mg/L/h) over the time course of fermentation.

When corn stover hydrolysate was used as the carbon substrate (versus the synthetic corn stover), the peak productivity declined from 1.6 to 1.3 g/L/h. Still, we met the milestone of 0.6 g/L/h at ~50 h. Both the oxygen uptake rate and the CO_2 percentage are slightly lower indicating less production per liter media (**Figure 31**). The overall production titer was 63 g/L, which included 56 g/L isobutyraldehyde and 7 g/L isobutanol (**Figure 32**). This surpasses the milestone by 40 g/L.

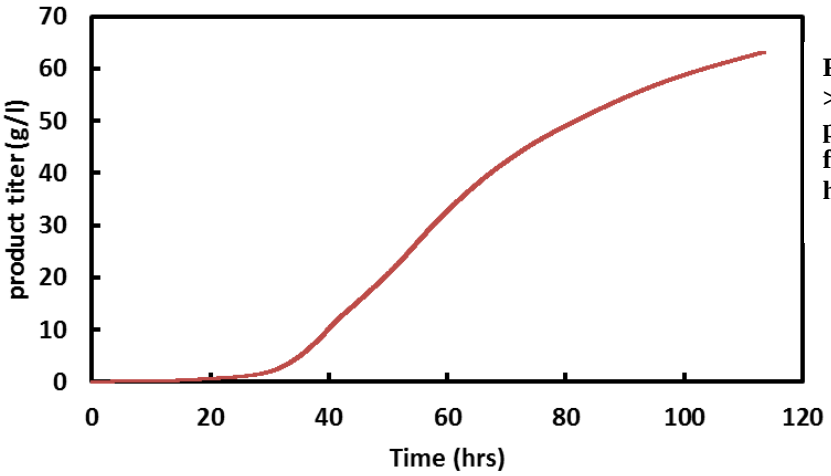


Figure 32. Demonstration of >40 g/L isobutyraldehyde production titer in 14L fermentor using corn stover hydrolysate.

One potential difference that may explain the difference in production between fermentations that utilized corn stover hydrolysate versus the synthetic mixture is the concentration of sugars in the feed. Although the synthetic feed contained 400 g/L glucose, the corn stover hydrolysate was more dilute with a concentration range of 230 - 270 g/L. The later condition resulted in the addition of significantly more water. This resulted in the effective dilution of cells. And because of the volumetric restraints of the fermentor, it was necessary to remove larger volumes of the culture more frequently to prevent exceeding capacity.

Comparison of Accomplishments with the Goals and Objectives of the Project:

1. Construct production strain that resists the toxins and inhibitors in the corn stover hydrolysate.

Prior to our work on this project, we had cautiously anticipated that the toxins and inhibitors in the corn stover hydrolysate would be significantly detrimental to our Bio-Oxo process. However, we have found that the quality of the corn stover hydrolysate to be better than expected. For example, from the NREL corn stover we received in 2017, the toxicity levels were low at 0.14 g/L HMF, 0.009 g/L furfural, and no detectable acetate.

2. Engineer the isobutyraldehyde production pathway so that the production strain can obtain high yield (60% of theoretical yield) using corn stover hydrolysate.

Easel's engineered strain demonstrates isobutyraldehyde yields that are greater than 60% of the maximum theoretical yield. Despite this success, we believe that additional optimizations can be made in the fermentation and pretreatment processes that would result in higher yields.

3. Optimize Bio-Oxo process so that isobutyraldehyde production from corn stover hydrolysate can obtain 1 g/L/h productivity in lab scale (5L) and pilot scale (30L) fermentors.

Unfortunately, the project was stopped prior to the pilot scale isobutyraldehyde fermentations (30L). However, we have been able to obtain peak productivities of over 1 g/L/h isobutyraldehyde in the 14L fermentor (10L working volume). We believe that our process was scalable to a 30L fermentor.

4. Reduce energy consumption of the oxo chemical production process by at least 50% (from 56.0 MJ/Kg to 28.0 MJ/Kg).

Our preliminary simulation results indicate that gas-stripping, the separation method used in our process, could reduce energy consumption to less than 20 MJ/Kg. With higher productivities and improvements in the recycling streams, the energy consumption could be further reduced.

V. Benefits Assessment

We maintain our previous estimate of a 48% reduction in energy use with our Bio-oxo process versus the current chemical synthesis process. Deployment would be three years after completion of the project. We believe that we could replace 10% of the isobutyraldehyde market, which would result in an energy savings of 2.6 TBtu and a CO2 reduction of 11 MIb/y. One of the first groups that could be impacted favorably by our Bio-oxo process would be farmers as they would potentially observe an increased in their corn stover sales.

VI. Commercialization

Prior to the end of our project, our plan was to attend conferences, such as the Advance Bioeconomy Leadership Conference Next (ABLCNext) and the Biobased Chemicals: Commercialization & Partnering Conference. At these conferences, we had hoped to generate leads and seed working relationships with both commercialization partners and potential investors. Unfortunately, our project had ended prior to testing of our technology at the demonstration plant scale.

VII. Accomplishments

Inventions/Patent Applications, licensing agreements:

HIGASHIDE, Wendy M.; CHO, Kwang Myung; RABIZADEH, Shahrooz; Microbial synthesis of aldehydes and corresponding alcohols. Patent 9,206,443 issued 12/08/2015.

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EDITED SOPO (rev. 11/14/16) Milestone Summary Table:

Milestone Summary Table						
Recipient Name:	**Easel Biotechnologies, LLC**					
Project Title:	**Bio-Oxo Technology**					
Task #	**Task or Subtask (if applicable) Title**	**Milestone Type (Milestone or Go/No-Go Decision Point)**	**Milestone Number* (Go/No-Go Decision Point Number)**	**Milestone Description (Go/No-Go Decision Criteria)**	**Milestone Verification Process (What, How, Who, Where)**	**Anticipated Quarter (Quarters from Start of the Project)**
1	Improve Isobutyraldehyde Productivity by Strain Engineering and Develop Fermentation and Separation Process.	Go/No-go	Go/No-Go BP1	**Go/No-Go BP1:** Obtain isobutyraldehyde production strain with 1 g/L/hr productivity and 30 g/L titer using purified corn stover monosaccharides by the end of Budget Period 1. Obtain isobutyraldehyde production strain with 0.3 g/L/hr productivity and 10 g/L titer using corn stover hydrolysate by the end of Budget Period 1.	Productivity, titer, yield evaluated using 5L fermentation	Q4
1.1	Isobutyraldehyde Strain Improvement	Milestone	Milestone 1.1.1, 1.1.2, 1.1.3 and 1.1.4	**Milestone 1.1.1:** By the end of Q1, finish knocking out the competing pathways for the production of acetate, and succinate. **Milestone 1.1.2:** By the end of Q2, finish knocking out the competing pathways for the production of L-Valine, L-Leucine, and D-pantothenate. **Milestone 1.1.3:** By the end of Q3, evolve the engineered strain to tolerate 10 g/L isobutyraldehyde. **Milestone 1.1.4:** By the end of Q4, increase the corn stover monosaccharides consumption rate to higher than 3 g/L/hr.	GC and metabolomics assays	Q1; Q2; Q3; and Q4
1.2	Fermentation Process Development (5L).	Milestone	Milestones 1.2.1, 1.2.2 and 1.2.3	**Milestone 1.2.1:** By the end of Q2, obtain the optimized industrial media and operation conditions for 5L-scale fermentation. **Milestone 1.2.2:** By the end of Q3, reach 0.8 g/L/hr productivity using purified corn stover monosaccharides as raw material.	5L fermentation tests	Q2; Q3: and Q4

				Milestone 1.2.3: By the end of Q4, reach 0.3 g/L/hr productivity using corn stover hydrolysate as raw material.		
1.3	Optimize the pathway gene expression.	Milestone	Milestones 1.3.1, 1.3.2, 1.3.3 and 1.3.4	**Milestone 1.3.1:** By the end of Q1, identify the key pathway enzymes whose activities need to be significantly improved. **Milestone 1.3.2:** By the end of Q2, increase the expressions and activities of the key pathway enzymes (identified in Q1) by at least 3 folds. **Milestone 1.3.3:** By the end of Q3, eliminate the feed-back regulations of the key pathway enzymes. **Milestone 1.3.4:** By the end of Q4, eliminate residual alcohol dehydrogenase (ADH) activities.	Direct comparison of various methods	Q1; Q2: Q3; and Q4
2	Strain and Pathway Optimization and Development of Integrated Processes.	Go/No-go	Go/No-Go BP2	**Go/No-Go BP2:** Obtain isobutyraldehyde production strain with 1.3 g/L/hr productivity and 60 g/L titer using purified corn stover monosaccharides by the end of Budget Period 2. Obtain isobutyraldehyde production strain with 0.5 g/L/hr productivity and 30 g/L titer using corn stover hydrolysate by the end of Budget Period 2.	Productivity, titer, yield evaluated using 5L fermentation	Q8
2.1	Obtain the Robust Isobutyraldehyde Production Strain and Optimize the Pathway Gene Expression.	Milestone	Milestones 2.1.1, 2.1.2, 2.1.3 and 2.1.4	**Milestone 2.1.1:** By the end of Q5, integrate isobutyraldehyde pathway genes into production strain. **Milestone 2.1.2:** By the end of Q6, determine the cause of declining isobutyraldehyde production. **Milestone 2.1.3:** By the end of Q7, increase the corn stover monosaccharides consumption rate to at least 5 g/L/hr.	Metabolomics assays	Q5; Q6; Q7; and Q8
2.2	Separation Process Development (5L).	Milestone	Milestones 2.2.1 and 2.2.2	**Milestone 2.2.1:** By the end of Q6, finish the process simulation, economic evaluation, and experimental phase equilibrium determination. **Milestone 2.2.2:** By the end of Q8, complete the lab, and bench-scale tests for proving and scaling continuous separation processes.	Whole-process fermentation test	Q6; and Q8
2.3	Integrated Process Development (5L).	Milestone	Milestones 2.3.1, 2.3.2,	**Milestone 2.3.1:** By the end of Q6, finish the best-case economic model.	Whole-process fermentation test	Q6; Q7; and Q8

			and 2.3.3	**Milestone 2.3.2:** By the end of Q7, reach 1.2 g/L/hr productivity using purified corn stover monosaccharides as raw material. **Milestone 2.3.3:** By the end of Q8, reach 0.5 g/L/hr productivity using corn stover hydrolysate as raw material.		
3	Demonstrate the Integrated Production Process in the 14L Fermentor	Hold		There is a HOLD on Task 4 and all associated costs, pending successful completion of Milestone 3.2.2.	Productivity and titer evaluated using 14L fermentation	August 2017
3.1	Determine the aspect ratio and k_La for our Bio-oxo process.	Milestone	Milestones 3.1.1 and 3.1.2	**Milestone 3.1.1:** By the end of April 2017, determine the best aspect ratio (1.0, 1.25, or 1.5) for isobutyraldehyde titer and productivity in the 5L fermentor. **Milestone 3.1.2:** By the end of May 2017, determine the oxygen transfer coefficient (kLa) for our process in a 5L fermentor.	Whole-process fermentation test	April 2017; May 2017
3.2	Complete Setup and Conduct Fermentation (14L).	Milestone	Milestone 3.2.1 and 3.2.2	**Milestone 3.2.1:** By the end of May 2017, complete the setup of the 14L fermentor. **Milestone 3.2.2:** By the end of August 2017, obtain 0.6 g/L/hr productivity and 40 g/L isobutyraldehyde titer using corn stover hydrolysate as raw material in the 14L fermentor.	Whole-process fermentation test	May 2017; August 2017

4.1	Using Final Production Strain to Conduct Pilot Scale Fermentation.	Milestone	Milestones4.1.1, 4.1.2, 4.1.3 and 4.1.4	**Milestone 4.1.1:** By the end of January 2018, finish the construction of pilot scale facility. **Milestone 4.1.2:** By the end of January 2018, obtain the final integrated strain. **Milestone 4.1.3:** By the end of March 2018, obtain 0.8 g/L/hr productivity and 40 g/L titer using corn stover hydrolysate as raw material. **Milestone 4.1.4:** Obtain 1 g/L/hr productivity, 60% of theoretical yield, and 50 g/L titer using corn stover hydrolysate as raw material by the end of June 2018.	Fermentation tests using 30L pilot scale fermentor	January 2018; March 2018; June 2018
4.2	Technoeconomic analysis.	Milestone	Milestone 4.2.1, 4.2.2 and 4.2.3	**Milestone 4.2.1:** By the end of January 2018, finish the production cost estimation. **Milestone 4.2.2:** By the end of March 2018, finish the detailed LCA analysis. **Milestone 4.2.3:** By the end of June 2018, finish the analysis for the greenhouse gas reduction potential.	In silico analysis	January 2018; March 2018; June 2018
4	Demonstrate the Integrated Production Process in Pilot Scale (30L).	Milestone	Final Objectives	**Milestone 4.1:** Utilize corn stover hydrolysate to obtain 1 g/L/hr productivity, 60% of theoretical yield, and 50 g/L titer of isobutyraldehyde. **Milestone 4.2:** Complete the economic model, cost estimate, LCA report, and commercialization plan for our Bio-Oxo process.	Productivity, titer, yield evaluated using 30L pilot scale fermentation	June 2018