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Scale Up of Malonic Acid Fermentation Process

Cooperative Research and Development Final Report

CRADA Number: CRD-16-612

NREL Technical Contact: Dan Schell

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Cooperative Research and Development Final Report

In accordance with Requirements set forth in the terms of the CRADA agreement, this document is the final CRADA report, including a list of Subject Inventions, to be forwarded to the DOE Office of Science and Technical Information as part of the commitment to the public to demonstrate results of federally funded research.

Parties to the Agreement: Lygos Inc.

CRADA number: CRD-16-612

CRADA Title: Scale up of malonic acid fermentation process

Joint Work Statement Funding Table showing DOE commitment:

Estimated Costs	NREL Shared Resources a/k/a Government In-Kind
Year 1	$ 375,000.00
TOTALS	$ 375,000.00

Abstract of CRADA Work:

The goal of this work is to use the large fermentation vessels in the National Renewable Energy Laboratory's (NREL) Integrated Biorefinery Research Facility (IBRF) to scale-up Lygos' biological-based process for producing malonic acid and to generate performance data. Initially, work at the 1 L scale validated successful transfer of Lygos' fermentation protocols to NREL using a glucose substrate. Outside of the scope of the CRADA with NREL, Lygos tested their process on lignocellulosic sugars produced by NREL at Lawrence Berkeley National Laboratory's (LBNL) Advanced Biofuels Process Development Unit (ABPDU). NREL produced these cellulosic sugar solutions from corn stover using a separate cellulose/hemicellulose process configuration. Finally, NREL performed fermentations using glucose in large fermentors (1,500- and 9,000-L vessels) to intermediate product and to demonstrate successful performance of Lygos' technology at larger scales.

"This report contains Protected CRADA Information, which was produced on, 7/10/16 – 7/15/16, 9/5/16 – 9/12/16, 1/16/17 – 1/27/17, 2/27/17 – 3/13/17 under CRADA No. 16-612 and is not to be further disclosed for a period of five (5) years from the date it was produced except as expressly provided for in the CRADA."

Summary of Research Results:

The work at NREL was broken up into three distinct efforts (tasks) as described below. The purpose of Task 1 was to demonstrate performance in small fermentor meeting Lygo's target performance metrics using a glucose feedstock. In Task 2, lignocellulose-derived sugars from pretreatment and enzymatic hydrolysis of corn stover were produced for bench scale fermentations work at LBNL's ABPDU. The purpose of the Task 3 was to scale-up Lygo's technology in 1,500-L and 9,000-L ferementors using a glucose feedstock.

Task 1

The purpose of this work was to perform 1 L scale fermentations in triplicate to produce the malonic acid intermediate product according to the latest Lygo's fermentation protocol and yeast and unique pH control

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strategy. The run began the week of July 10, 2016 and was completed the following week. During the run, online fermentation measurements of weight, temperature, pH, dissolved oxygen, off gas analysis (O_2 and CO_2 monitoring), feed profile, optical density and biomass dry cell weight were acquired. All samples (whole broth and HCl treated) from the run were shipped to Lygos on August 9, 2016.

The 1 L fermentation were conducted in triplicate (designated F1, F2, and F3) following Lygos' protocols with the following exceptions: sterile tap water was used in place of purified water for the bulk of the media and the oxygen mass transfer coefficient (k_La) was lower than expected and lower than Lygos' expectation. Concentrated stock media solutions were made with purified dionized water and all other water additions were filter-sterilized tap water. The k_La of the system was measured just prior inoculation using operating conditions of 1.0 vessel volume per min (vvm) air and agitation rate of 1000 rpm. The measured k_La was 28 mM/L/h in all three fermentors.

Plots of fermentor operating conditions (temperature, pH, agitation rate, and vessel volume) are shown in Figures 1, 3, and 5 for fermentors F1, F2, and F3, respectively. Corresponding plots of dissolved oxygen (DO), oxygen uptake rate (OUR), carbon dioxide evolution rate (CER), optical density (OD), and the glucose feed cycle rate (number of feed cycles initiated per hour) are shown in Figures 2, 4, and 6. Fermentations generally ran as expected, but showed a longer than expected lag before the dissolved oxygen (DO) dropped to a low enough value to trigger the glucose feed. The cause of this behavior is unclear. One vessel (F3) initiated feed at around 24 hours. The other two vessels were further delayed and initiated feeding at 51 and 47 hours for F1 and F2, respectively. Based on information provided by Lygos, it was expected that glucose feeding should begin near 12-15 hours after inoculation.

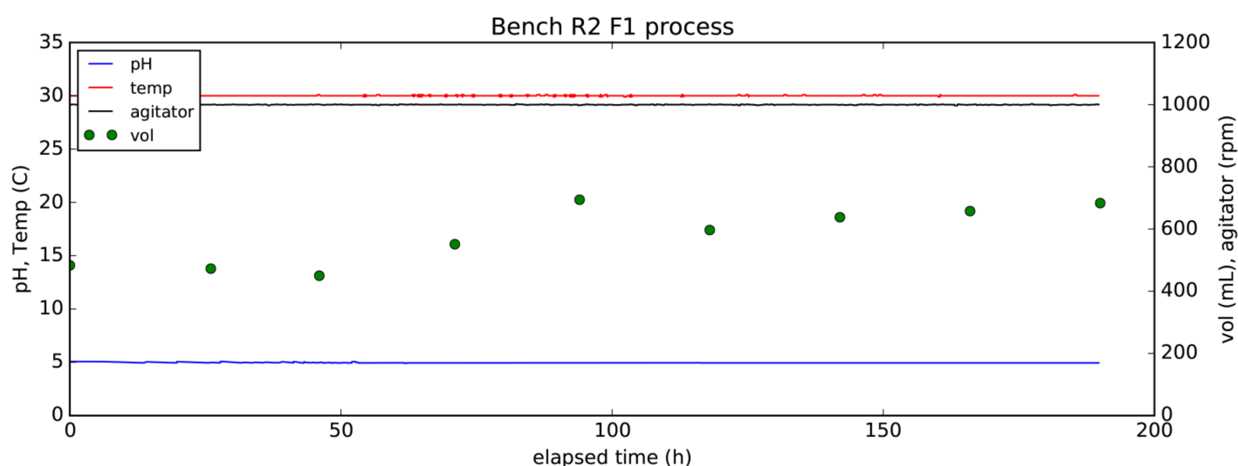

Figure 1. Fermentor F1 operating conditions and broth volume.

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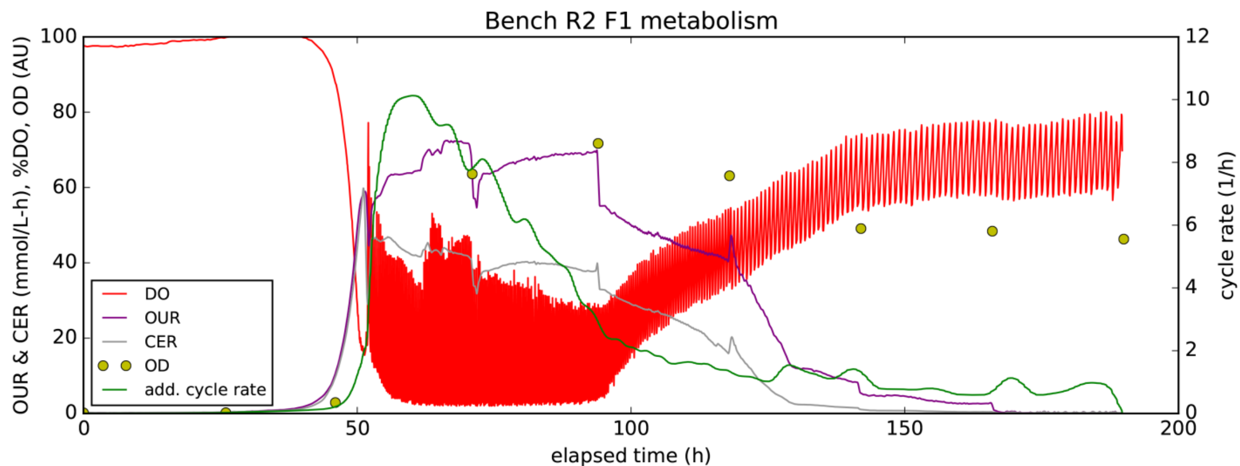

Figure 2. Fermentor F1 DO, CER, DO, OD, and glucose feed cycle rate.

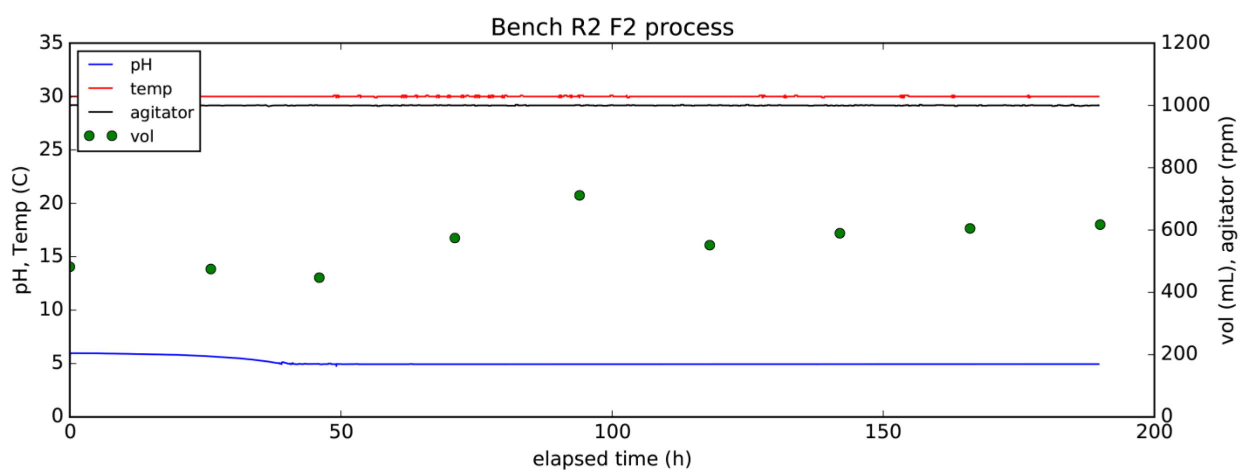

Figure 3. Fermentor F2 operating conditions and broth volume.

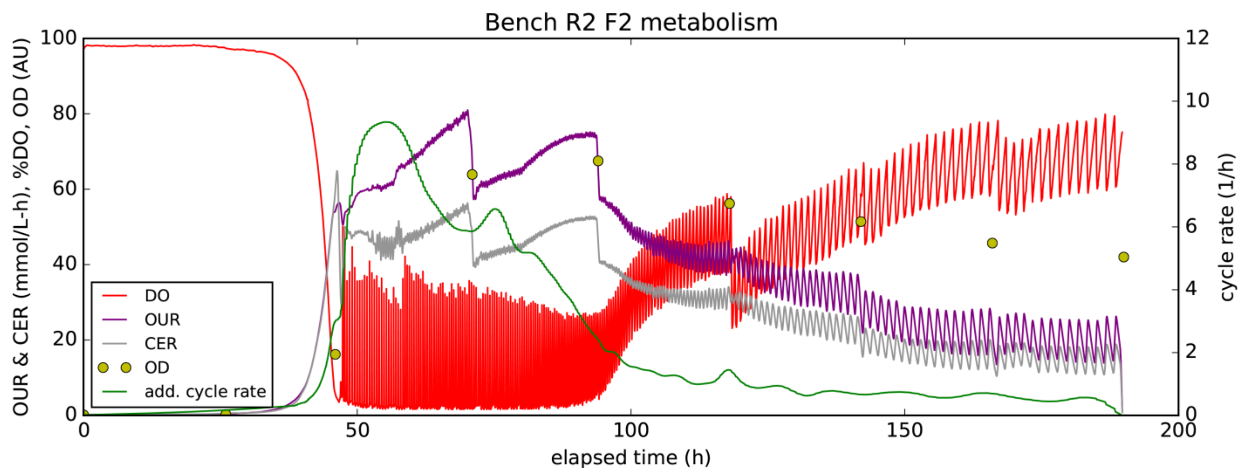

Figure 4. Fermentor F2 DO, CER, DO, OD, and glucose feed cycle rate.

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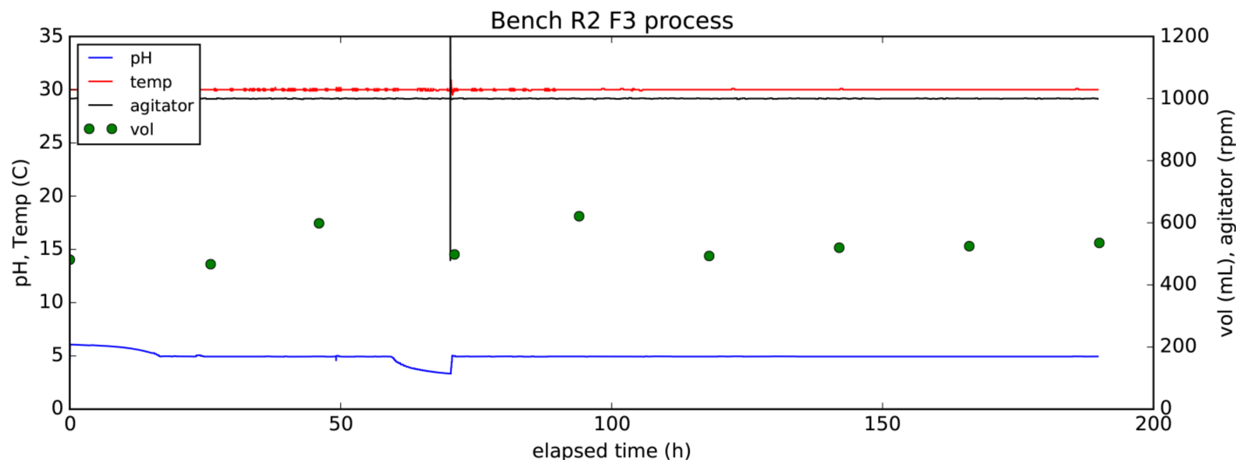

Figure 5. Fermentor F3 operating conditions and broth volume.

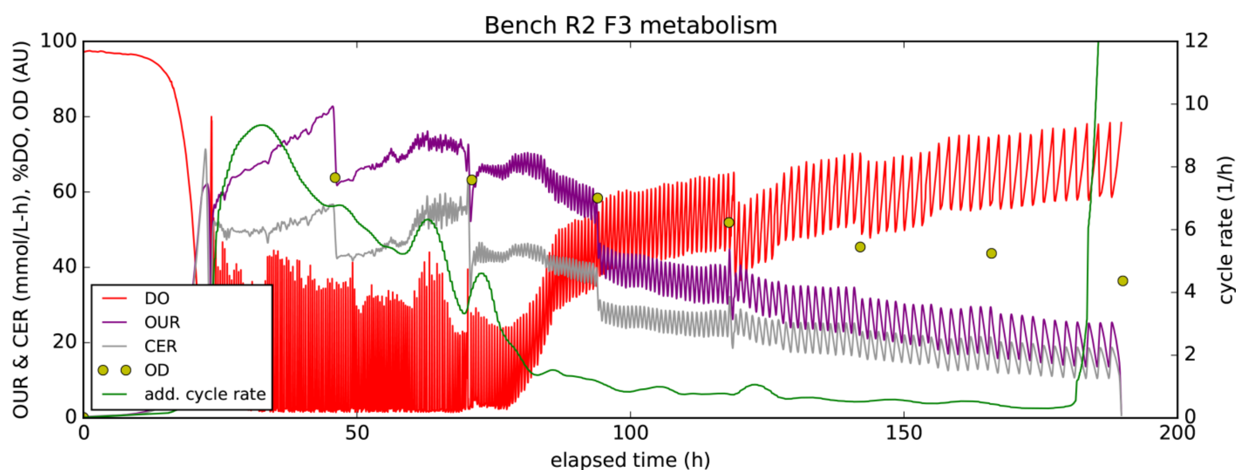

Figure 6. Fermentor F3 DO, CER, DO, OD, and glucose feed cycle rate.

Generally, the pH control process went well, but some problems occurred. Based on data provided by Lygos, the anticipated rise in the minimum DO during the run should occur at near 100 hours after inoculation. For these experiments, despite the initial lag, this rise occurred around 100 hours except for F3, where the rise occurred at about 80 hours.

Dry cell weight data and corresponding OD measurement from the three fermentors are shown below in Figure 7 below.

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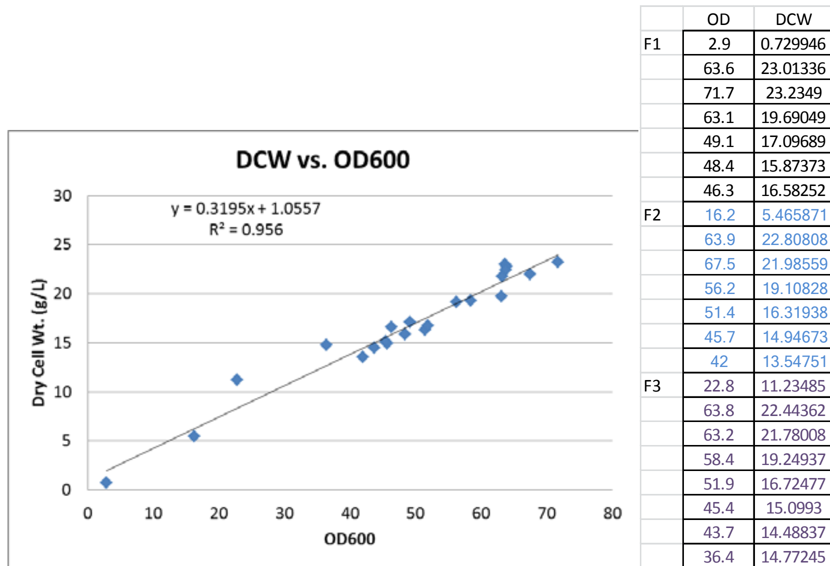

	OD	DCW
F1	2.9	0.729946
	63.6	23.01336
	71.7	23.2349
	63.1	19.69049
	49.1	17.09689
	48.4	15.87373
	46.3	16.58252
F2	16.2	5.465871
	63.9	22.80808
	67.5	21.98559
	56.2	19.10828
	51.4	16.31938
	45.7	14.94673
	42	13.54751
F3	22.8	11.23485
	63.8	22.44362
	63.2	21.78008
	58.4	19.24937
	51.9	16.72477
	45.4	15.0993
	43.7	14.48837
	36.4	14.77245

Figure 7. OV versus dry cell weight data.

A few rod-shaped cells (not yeast) were noted in F2 at 24 hours (see Figure 8) into the run and in F1 at 118 hours. Plating of broth from F2 confirmed two colonies (out of several hundred) that appeared different. Despite these observations, subsequent microscope observations showed no sign of these potentially contaminating microorganisms, and it appears that they were unable to compete with the Lygos' microorganism. At no time did it appear the contaminating microorganism rose to such numbers that they interfered with the run.

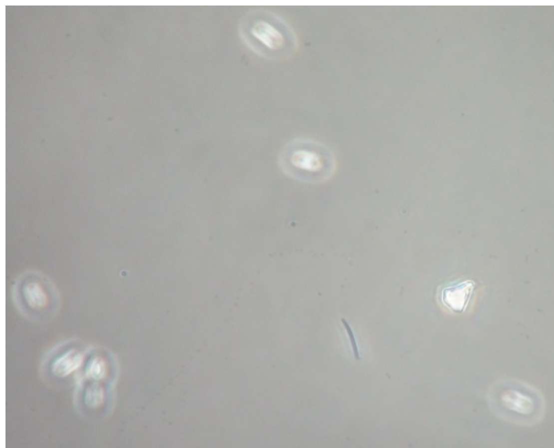

Figure 8. Photo taken of F2 broth at 24 hours.

Summary of mass balance data from the fermentation is shown in Table 1. Also included are mass of samples used for the HCl-diluted samples.

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Table 1. Summary of mass balance data

	F1	F2	F3
Total Glucose Solution Fed (g)	330	335	374
Total Base Used (g)	289	289	338
Initial Mass of Broth (g)[1]	483	482	481
Final Mass of Broth (g)	509	562	501
Total Mass Broth Removed (g)	419	489	658

[1]After inoculum addition and removal of initial sample

Finally, we also attempted to centrifuge the broth from this run through the small perforated basket (filter cloth pore size of approximately 30 μm) centrifuge, but were unsuccessful. We speculate that larger volume of material is needed to adequately test the unit.

Lygos indicated based on their analytical measurements of the intermediate product concentration that was achieved and so work on scaling up the fermentation to the larger fermentors could begin.

Task 2

The purpose of Task 2 was to generate a lignocellulosic sugar solution (containing about 7.3 kg of glucose) from pretreatment and enzymatic hydrolysis of corn stover. The solution was shipped to the LBNL/ABPDU for testing of Lygo's technology with this material in a separate CRADA. This material was produced by enzymatic hydrolysis of dewatered and washed deacetylated and dilute-sulfuric-acid pretreated stover. The dewatering and washing step produces clean cellulosic solids. This process configuration is well suited to production of multiple products from cellulosic and hemicellulosic sugars.

Deacetylation and sulfuric acid impregnation were performed in the 1900-L paddle reactor. Dry corn stover was added to the reactor along with a dilute sodium hydroxide solution (0.4% wt/wt). The slurry was heated to 80°C and held for 2 hours, and then the liquor was discharged. Rinse water was added to the tank and circulated for 30 minutes before being discharged. A dilute sulfuric acid solution was then added to achieve a 0.8% acid concentration in the reactor. After thoroughly mixing the acid and solids at room temperature for 2 hour, the slurry was pumped to a screw press and dewatered to approximately 40% solids.

The continuous horizontal pretreatment reactor (Metso Inc., Norcross, GA, USA) was preheated for one hour using the steam jackets and allowed to reach steady state at the desired temperature before the run begins. Feedstock was fed through the reactor at a rate of 25 dry kg/hr and squeezate was reinjected after the plug screw feeder. The reactor was operated at 160˚C with a residence time of 10 min to mimic the standard 2012 SOT conditions. After leaving the reactor, the material was discharged into an atmospheric pressure flash tank where it separates into a high-solids pretreated slurry stream (29.1% total solids) and volatile flash vent stream. The pretreated slurry was then discharged into 55-gal drums and stored in a cold room.

Sixty five (65) kg of pretreated material was re-suspended in water and loaded batchwise onto a perforated basket centrifuge (Western States Q-120 model) where entrained liquor was removed. Water was flowed through the solids cake until the discharging liquid contained <1 g/Lof xylose. The solids were then spun at

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2800 rpm to remove as much water as possible and the final cake was discharged at approximately 35 – 40% total solids. Additional material was prepared by re-suspending pretreated material in water and loaded it batchwise into the largest perforated basket centrifuge (Western States Q-320 model).

Enzymatic hydrolysis was performed in a 170-L paddle reactor that was cleaned and steam sterilized before use. Twenty three (23) dry kg of the washed pretreated solids were loaded into the reactor and the solids content was adjusted to 22% by addition of water. The slurry was heated to approximately 50˚C using the reactor's jackets. Finally, Novozymes CTec 2 was injected to the reactor using a pressure canister. The enzymatic hydrolysis ran for 5 days under constant agitation and a sample was taken daily for measurement of sugar concentration.

The residual solids in the enzymatic hydrolysate were removed using the Q-320 basket centrifuge. The sugar stream was collected in drums and then pumped back into the 170-L paddle reactor. The reactor was heated under vacuum to concentrate the sugar solution. Fifteen liters of a concentrated sugar solution was collected and shipped to LBNL. The component concentration in the final production are in the table below. The results of the fermentation work were not shared with NREL.

Cellobiose (g/L)	Glucose (g/L)	Xylose (g/L)	Galactose (g/L)	Arabinose (g/L)	Fructose (g/L)	Glycerol (g/L)	Acetic Acid (g/L)
32	485.8	82.14	5.08	7.66	64	2.6	4.2

Task 3

Run 1: 1,500 Fermentor

The purpose of this work was to perform a 1,000 L scale fermentation (maximum working volume) to produce the malonic acid intermediate according to the latest Lygo's fermentation protocol. The run began the week of September 5, 2016 and was completed the following week after a total of 144 hours of run time. The report provides all run records acquired during the run including online measurements of weight, temperature, pH, dissolved oxygen, off-gas analysis (O_2 and CO_2 monitoring), feed profile, and offline measurements of optical density and biomass dry cell weight. All samples (whole broth and HCl treated) from this run were shipped to Lygos on September 9, 2016.

Pilot-scale fermentation was performed in a 1,500-L fermentor following Lygos' protocols with the following exceptions.

1) Municipal water was used in place of deionized water for media preparation.

2) Salts including potassium phosphate and magnesium sulfate were added to media water directly in solid form instead of using concentrated solutions.

3) Prepared fermenter media containing water, dextrose, potassium phosphate, and magnesium phosphate was heat sterilized at 121°C for 30 min instead of sterile filtration. A concentrated urea solution at approximately 400 g/L was sterile filtered into the vessel after cooldown, to eliminate any potential Maillard reaction at temperature during sterilization. Antifoam was not prepared or added before sterilization, and was instead autoclaved and pumped into the fermenter aseptically (without filtration) just prior to inoculation.

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4) Prepared concentrated feed media containing water, glucose, and potassium phosphate was loaded into a previously sterilized tank and was subsequently heated to 80°C for one hour to pasteurize the media (avoid sugar degradation if sterilized at this concentration). A concentrated urea solution at approximately 400 g/L was sterile filtered into the vessel after cooldown, to eliminate any potential Maillard reaction at temperature during pasteurization. The concentrated feed was sterile filtered as it entered the fermenter vessel, as consumed.

Concentrated stock media solutions for vitamins and minerals were made with dionized water and sterile filtered into the fermentor and normal protocols were followed for feed media. The starting volume was 500 L in the fermenter and was limited to approximately 1,000 L by removing 250 kg of broth at 90 h run time. The k_La of the system was measured just prior inoculation at 65 h^{-1} using operating conditions of 250 slpm air, agitation of 250 rpm, and at 1.0 atm pressure. The resulting OTR_{max} at those conditions was estimated at 16 mmol/L-h.

Seed was inoculated with one vial of glycerol stock in 50 mL of HM Seed Media 04 in a 250 mL baffled shake flask and grown at 30^0C, 250 rpm to an OD of 12.5. The entire contents were used to inoculate the 5-L bench top fermenter for a second stage. This fermenter was maintained at 30^0C, agitated at 300 rpm, sparged with 5 L/min air and pH was controlled at 5.0. The seed was grown for 23 hours to an OD of 6.5 then the entire contents were used to inoculate the main fermentor (450B).

Plots of vessel operating conditions (temperature, pH, agitation rate, glucose addition rate, DO, OUR, CER, and fermenter and feed tank mass) are shown in Figure 9. The 5-L seed fermentor lagged approximately 12 hours behind what was expected and delayed the inoculation of the 500 L fermentor. The main fermentation generally ran as expected, but showed a longer than expected lag before the dissolved oxygen (DO) dropped enough to trigger the glucose feed. Glucose feed started at 24 hours when DO dropped and then rose to trigger injection for the first time. The cause of this behavior is unclear. Based on information provided by Lygos, it was expected that glucose feeding should begin near 12 – 15 hours after inoculation.

The caustic addition system performed well and did not have any problems throughout the run. It should be noted that the pH was initially adjusted from 4.1, but overshot the target of approximately 5. With no buffering capacity yet, a short injection spiked the pH to 6.6, which is above the acceptable range prior to inoculation. About 60 mL of 93% sulfuric acid was injected into the fermentor through a septum to lower the pH to 6.4, within the range.

After the fermentation was underway, pH control was set in automatic mode, and the resulting injections of caustic approximately every 3 – 5 minutes maintained pH at the setpoint. Offline pH measurement at 18 hours revealed that the fermentor online pH probe calibration had drifted about 0.3 units high. Offline pH was measured at 5.1, still coming down from the inoculation value of 6.4 on its way to the target 5.0, and the setpoint of the fermentor was adjusted to 5.3 to target a real value of 5.0. Subsequent offline measurements confirmed this was successful and offline pH held steady around 5.05 during the entire fermentation run. The caustic addition frequency was inferred from the fine oscillations in pH values, and the corresponding mass addition rate was calculated based on fermentor and feed tank mass differences.

Glucose feed media consumption rate was calculated by incrementing a counter each time the glucose pump was triggered by a DO spike and using the settings (10 min @ 0.130 L/min) for the calculation. Consumption rate was steady during the entire run, around 3 – 4 L/h, with a broad peak approaching 5 L/h around 85 hours. Fermentation broth samples were taken about every 24 hours as indicated by the OD dot-plot and it should be noted how no perturbation of parameters resulted. The removal of significant sample volumes during bench-scale runs resulted in the apparent decrease of metabolism, and that is absent here during the pilot-scale run because of the much larger working volume.

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The fermentor was held at a backpressure of 0.2 atm (g), and adding the atmospheric pressure at 5,400 ft at NREL, the total pressure was approximately 1.0 atm(a). The plot of pressure in Figure 9 shows slow ramp ups of pressure between 40 – 50 h and 60 – 68 hours. It was discovered that the sterile exhaust filter on the fermentor had begun to foul at these time points and because of deposits of fermentor broth aerosol. The exhaust filter was replaced at 50, 68, and 92 hours, during which time the exhaust flow to the mass spectrometer was interrupted as seen on the OUR/CER plots.

Inlet sparge air flow was reduced to about 200 slpm just prior to the first exhaust filter fouling event. The control valve was wide open and fermentor utility air pressure was nominal. The sterile filter was not inspected as this would have required completely stopping air flow. It was speculated that perhaps the sparger rod holes (3/32") became partially clogged with intermediate product. This could not be verified, and the system was left as-is. Following replacement of the exhaust filter and return of pressure to normal, the flow bumped up to 220 slpm, and it returned to 250 slpm after about 8 more hours with no explanation.

As the fermentor approached 89 hours, a drawdown of 250 kg from the tank occurred to keep sufficient headspace for continuing operation. This is also noted on the OUR/CER plot as exhaust flow momentarily stopped. The collected volume of broth was subsequently killed with bleach and caustic, and disposed of by municipal sewer after kill confirmation.

At 144 hours, the fermentation was halted by stopping pH control, glucose addition, and sparge air. Temperature was raised to 80°C and held for one hour to inactivate the yeast. This, however appeared to not entirely kill the organism when plated. A second 80°C heat cycle was run for two hours, which resulted in successful kill. The fermentor was then chilled as cold as possible with NREL's cooling water and held at 12°C for four days. The broth was then unloaded into 55-gal drums and placed in NREL's 4°C cold room prior to refrigerated truck shipment to Lygos.

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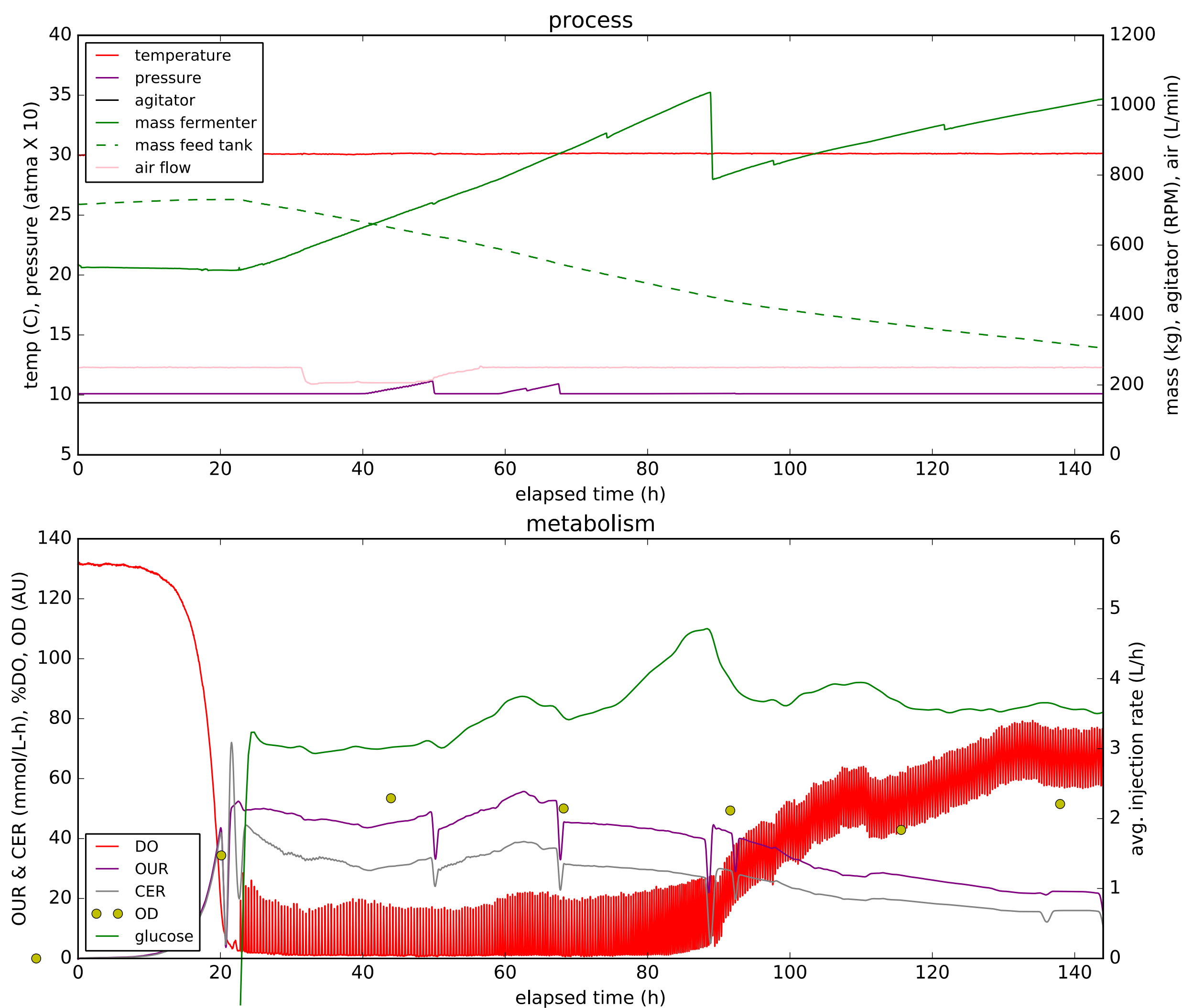

Figure 9. Fermenter operating conditions during the run. All measurements were online, except OD.

For information only, dry cell weight data and corresponding OD measurement were compared in Figure 10. Although a great linear fit was not seen, unlike the bench scale fermentations. But the value of the slope for a linear fit of the data, when the intercept is forced to zero, is very similar to be nch scale results.

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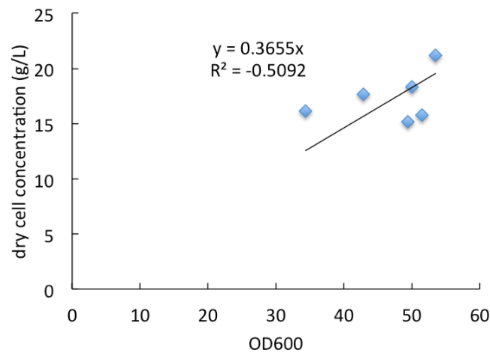

Figure 10. OD versus dry cell weight.

Broth samples were checked under the microscope and also plated. No samples indicated signs of contamination and all plates came back negative. The feed media tank with concentrated glucose was also plated before and after the run with negative results.

Run 2: 9,000-L Fermentor-Run 1

The purpose of this work was to perform a 7,000 L scale fermentation (maximum working volume) to produce the intermediate product. The run began the week of January 16, 2017 and was completed the following week after a total of 138 hours of run time. The procedure followed the same general protocols as used in the 1,000 L fermentation run, with necessary modifications for the larger fermentor.

The initial volume in the 9,000-L fermentor was 3,500 L. The k_La of the system was measured just prior inoculation at 66 h^{-1} using operating conditions of 1,000 slpm air, agitation of 80 rpm, at 1.0 atm pressure. The resulting OTR_{max} at those conditions was estimated at 16 mmol/L-h. Based on earlier testing of k_La on 3,500 L and 7,800 L of only water in the same vessel, the k_La was expected to significantly increase as the liquid level rose and also when the top agitator blade became submerged. Model estimates of k_La for initial and final volumes ranged between 70 – 125 h^{-1} and was agreed between NREL and Lygos that this range would meet requirements overall.

Seed was inoculated with one vial of glycerol stock in 50 mL of *HM Seed Media 04* in a 250 mL baffled shake flask and grown at 30°C, 250 rpm to an OD of 8.4 after 24 hours. Five mL was used to inoculate a larger 2,800 mL flask at 500 mL working volume with *HM Seed Media 04* and was cultured for 24 hours and reached an OD of 9.6. Microscopic evaluation at this point revealed only Lygos yeast cells were present. The entire contents were used to inoculate 50 L of *HM Seed Media 04* in the 160-L fermentor. The k_La on this fermenter was first measured at 55 h^{-1} at 220 rpm and 25 L/min, but adjusted to increase the k_La within 75 – 100 h^{-1}. This fermenter was maintained at 30°C, agitated at 230 rpm, sparged with 30 L/min air and pH was controlled at 5.0 using NaOH. Dissolved oxygen dropped from 100% to 0% by the end of the fermentation. After 18 hours, 37 kg of this broth was used to inoculate the 9,000-L fermentor. The two seed flasks and 50-L seed fermentor generally ran as expected and were transferred after 18 h in each stage. The 9,000-L fermentor was operated for 138 hour, then the brooth was heat attenuated and centrifuged to recover presipated product.

Plots of the 9,000-L fermentor operating conditions (temperature, pH, agitator speed, sparge air flow, exhaust air flow, glucose addition rate, DO, OUR, CER, and fermenter and feed tank mass) are shown in Figure 11. The fermentation initially ran as expected with DO dropping after about 10 hours. Glucose feed started at 19 hours when DO spiked to trigger injection for the first time. This nine hour lag between DO drop and first feed was expected to only last about four hours based on the first pilot plant run at 500 L scale. It is unclear why the lag was different for this run. Also, it should be noted that OUR/CER data may not characterize the true

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fermentation metabolism, as the main exhaust filter was clogged with product for a majority of the run, and the gas composition at the gas analyzer tap downstream of this filter may have not been representative.

Bacterial contamination was observed in the main fermenter starting with the initial time zero sample acquired just after inoculation. No sample was taken prior to inoculation, but the seed train showed no evidence of contamination, and it must be assumed the fermenter media had been compromised prior to inoculation. The contamination rose in population during the course of fermentation to approximately one cell for each yeast cell, but did not overtake the yeast growth.

Glucose feed media consumption rate was initially calculated by recording a counter each time the glucose pump was triggered by DO spike and using the settings (10 min @ 0.900 L/min). Feed rate was quite erratic, and a pressure gauge installed between the pump and sterile filter leading to the fermenter indicated clogging was occurring. The sterile filter was swapped out for a clean one every 2 – 6 hours during operation, and after also learning that there was a bacterial contamination inside the fermentor. The sterile filter was removed from the glucose addition system at 76 hours. The excessive backpressure resulted in considerable pump slippage (peristaltic pump), and the pump cycle counter significantly overestimated how much glucose was injected. Figure 11 instead plots the average glucose addition rate based on the loss in weight in the feed vessel. This signal has more noise, but gives a more accurate assessment.

The caustic addition system performed well and did not have any problems throughout the run. The large-scale setup used one valve cluster for both inoculation and for the caustic system, and thus pH was not adjusted to 5.0 until after inoculation as pH was within acceptable range at the beginning of the run. After inoculation, the caustic system was hooked up and set in automatic mode. The last run in the pilot plant gave us a starting point for the pH control loop tuning parameters, and the initial adjustment and subsequent automatic control achieved and maintained pH 5.0 without overshooting.

Caustic addition rate was calculated by observing both a trigger counter and caustic injection valve open time totalizer. The amount of caustic injected given a valve open time was inferred by the difference in physically measured tank level and the total valve open time over the entire fermentation run.

The fermentor was held at a backpressure of 0.2 atm, and adding the atmospheric pressure at 5,400 ft at NREL, the total pressure was approximately 1.0 atm. The plot of pressure in Figure 11 shows a pressure spike at 55 – 57 hour when the sterile exhaust filter plugged. This was expected from the experience gained during the previous run and disposable wipes were installed on the filter cartridges to enable reuse of the expensive sterile filters. However, the collection of solid powder on the wipes was not entirely effective, and a line connecting to the next fermentor vessel (used as drawdown tank) was opened to use its vent system in parallel. The backpressure on the blowdown vessel was set to maintain 0.25 bar in the fermentor in case the desired 0.20 bar could not be maintained, thus only venting through the secondary vessel when needed. This can be seen by the step shifts in pressure in Figure 11 between 0.20 and 0.25 bar.

Due to major problems feeding the fermenter glucose, and possibly also contamination slowing production, the vessel volume increase rate was much lower than expected, and no mid-run drawdown was necessary.

At 138 hours into the run, the fermentation was halted by stopping pH control, glucose addition, and sparge air. Temperature was raised to 80°C and held for two hours to inactivate the yeast. The broth was chilled to 11°C afterwards. Plating verified successful kill.

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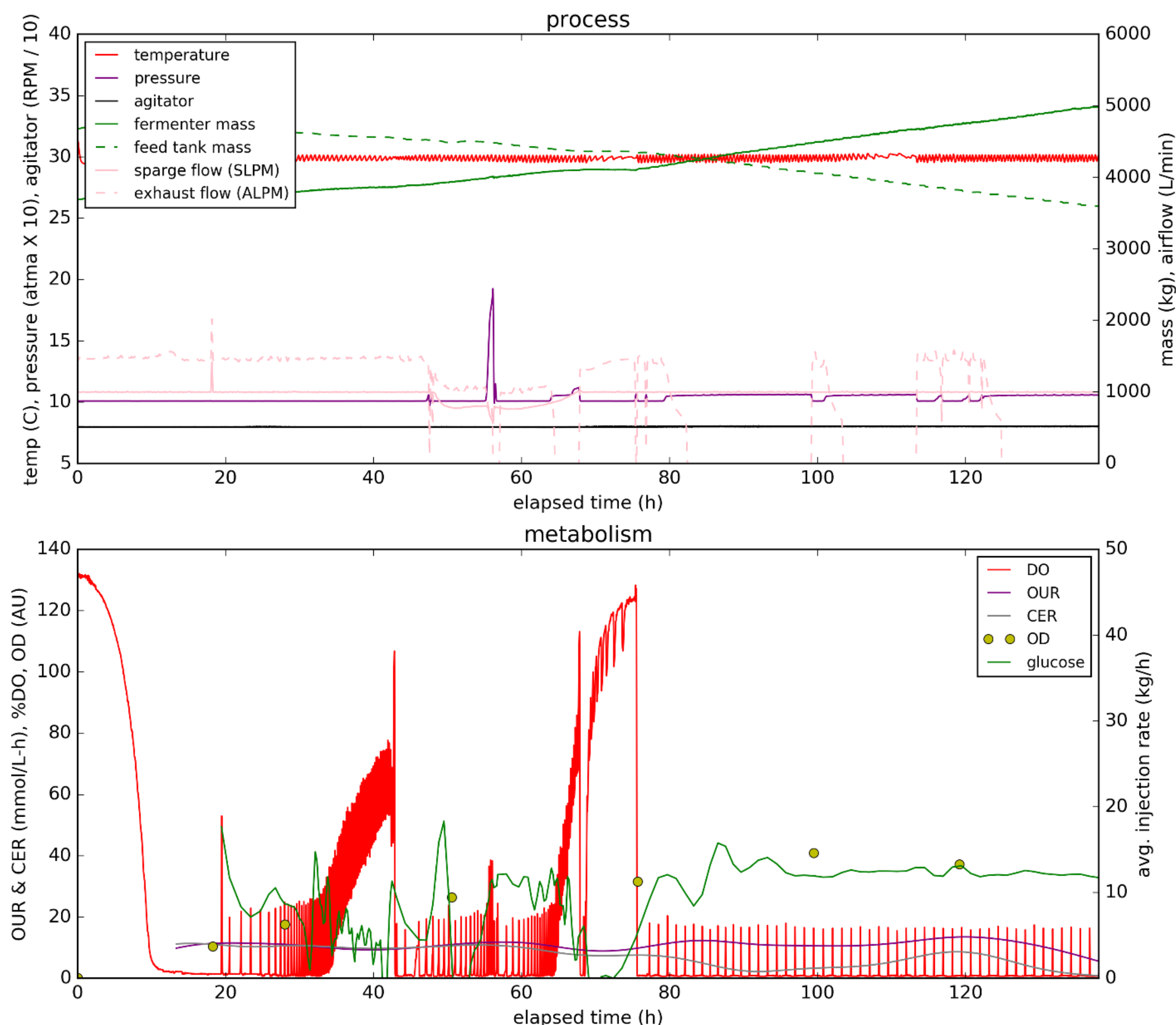

Figure 11. Fermenter operating conditions during the run. All measurements were online, except OD.

A low-gravity decanting centrifuge was rented from TEMA to process the fermentation broth and concentrate the intermediate product. An engineering representative from TEMA was on site during all processing activities, which occurred between 2 – 3 days after the heat attenuation of the broth.

TEMA adjusted centrifuge basket speed, scroll speed, and inlet flowrate until an optimum was found to produce the desired solids concentration, minimize the amount of cells in the solids, and minimize intermediate product loss in the centrate. The ideal supply flow rate for this unit was determined as 10 gal/min (measured using a magmeter). Specific separation data and samples were collected by TEMA and Chi and are not available for this report from NREL.

During processing, Lygos noted that the pH had not been adjusted to 9 yet. NREL was unaware that pH adjustment was necessary prior to processing—only for final product preservation. About one quarter through processing the broth, caustic was added to the fermentor and pH was adjusted to 10.3.

The resulting concentrate was reslurried with water, pumped into a 1,000-L agitated holding tank, and reprocessed in the centrifuge to further wash the cells and broth from the intermediate product. After the second pass through the centrifuge, the produced slurry was diluted below 40% solids, adjusted above pH 9, and stored in 55-gal drums. The entire operation processed the fermentor contents in two batches, and produced two

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drums, both approximately 80% full. The second drum produced was processed above pH 9 the entire time, where as, the first drum is a combination of pre- and post-pH adjusted broth.

There were two problems during this run that did not allow us to call it successful. The 9,000-L fermentor and feed tank both showed microbial contamination, and the glucose filter frequently clogged leading to too little glucose fed to the fermentor. Investigations have uncovered the most likely causes of troubles, and solutions have been identified were implemented in the follow on run.

The root cause of the contamination problems was determined to be faulty steam traps on the large-scale fermentor systems. Age and solids residue caused 3 – 4 steam traps out of 20 total per system to fail open or become clogged—both of which likely resulted in cold spots during sterilization cycles. The steam traps have been replaced or cleaned. All fermentor connections have been inspected, cleaned, and reinstalled to ensure all solids residue has been removed from the system to avoid future steam trap fouling. In addition, temperature-indicating crayons will be used in the future to verify all steam traps reach required sterilization temperature.

The root cause of frequent sterile glucose feed filter clogging was fine ash particulate from the granulated glucose itself. Although a very small amount of brown biomass-like matter was caught by an 80-mesh strainer upstream of the sterile filter, the residue caught on the filter under microscopic examination was a dark, grey, fine grit-like substance with a particle size of approximately 5 – 10 µm. Not enough residue was recoverable for particle size analysis. A separate, independent experiment also encountered problems using the same glucose source (Cerelose), where 0.45 µm sterile filters clogged with the same residue even after first passing through a 20 µm prefilter. It is recommended that future Lygos runs install a much larger capacity sterile filter or simply omit the filter and rely on pasteurization of the feed tank. The first pilot plant run did not show any sign of microbial contamination in the feed tank after pasteurization, and the latter is recommended for future runs.

Exhaust filter clogging remains a minor problem. When the exhaust system was reconfigured to use two fermentor systems in parallel and with cascading pressure control loops, filters only required changing or cleaning every 24 hours. It is recommended to set up the fermentation system with parallel filters at the beginning, and/or bubble the exhaust through another tank with water prior to sterile filtration. A scrubber system inside the biological containment zone will be required to handle particulate at larger scales.

Run 3: 9,000-L Fermentor-Run 2

This section documents the results of a second run in a 9,000-L fermentor employing the lessons learned in the first run. This run began the week of Feburary 27, 2017.

Plots of vessel operating conditions (temperature, pH, agitator speed, sparge air flow, glucose addition rate, DO, OUR, CER, and fermenter mass) are shown in Figure 12. The main fermentation initially ran as expected, and DO dropped after about 10 hours. Glucose feed started at 18 hours when fermentor backpressure was elevated from the initial 1.0 bar to 1.2 to increase the OUR. Pressure was again increased to 1.4 bar, agitation speed increased to 100 rpm, and airflow varied between 1,700 – 2,400 slpm to ensure a high enough OUR was achieved.

At 28 hours, a major loss of sparge air flow occurred and was due clogging of the sparger tube orifices. Since the sparger tube could not be replaced with the fermentor full, sterile air was rerouted to the bottom harvest valve with a hose. This change did not appear to adversely effect the OUR.

No contamination was reported in the sugar feed tank during the entire run, nor in the fermentor initial media. Bacterial contamination was seen under the microscope in samples taken after the rerouting of sparge air flow. Despite aseptically installing this bypass hose using alcohol spray, some unknown microorganisms were likely blown into the fermentor. This was a rather small contamination and never overtook the Lygos yeast.

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Glucose feed media was injected using a peristaltic pump set to 0.90 L/min for 10 min every trigger spike in DO. Consumption rate was measured and totalized during this run using a Coriolis meter, and the average hourly feed rate is plotted in Figure 12 with an average of 28 kg/h (density of 1.23 g/cm^3). Feed rate was fairly smooth this run, with larger variations caused by the sparger problem and also seen during the drawdowns. The 6” long 0.2 μm sterile filter from previous runs was swapped out with a new 30” cartridge, which proved to greatly help and never clogged during the run. Feed tank weight change was accidentally not recorded, since a similar but different tank was used to batch and feed from this run.

Glucose feed media concentration appeared to be lower than desired, as only about 585 g/L glucose was measured on the HPLC. The target was 650 g/L. This dilution could be caused by an error during batching, or additional water entering the tank after batching. The glucose concentration did not drift downwards during this run, indicating water was likely not entering the vessel through the agitator mechanical seal as suspected during previous runs. However, steam may have been condensed in the fermentor during the pasteurization step when valve clusters may have injected steam during the cycle. The initial tank sample was collected after the pasteurization step to also plate for contamination, and a sample prior to that step was not available to verify initial batch concentration.

The caustic system performed well, but parameters required adjustment a couple times during the run when the pH started drifting down. The system struggled at times to pulse in enough caustic at the initial settings when the fermenter backpressure was purposely increased to aid in OUR. Maximum pulse time was increased to help inject more caustic per pulse.

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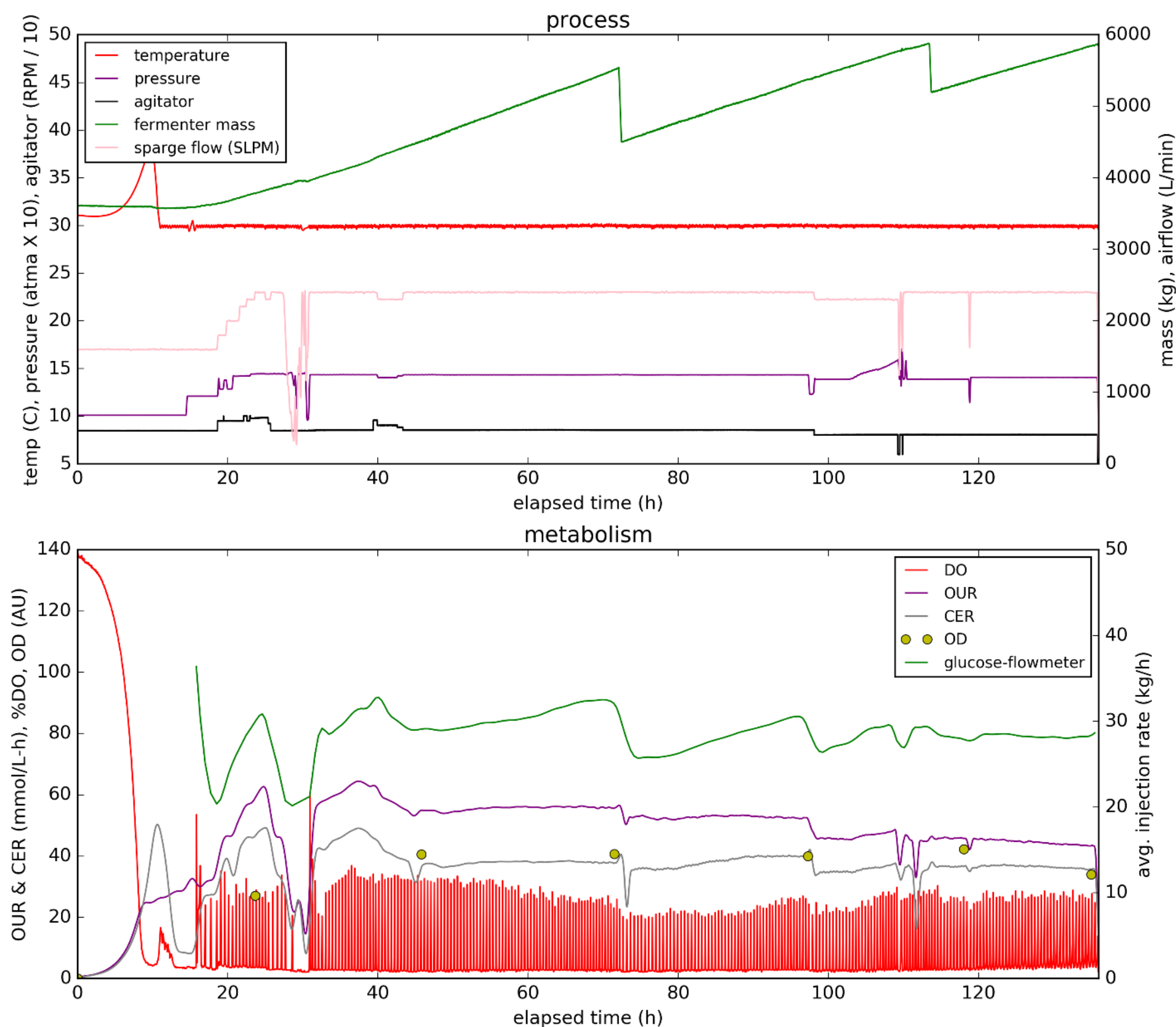

Figure 12. Fermenter operating conditions during the run. All measurements were online, except OD.

Filtration of the fermentor exhaust had caused clogging problems and pressure spikes during the first two pilot runs. To combat this problem, the fermentor exhaust was first bubbled through and adjacent vessel with a small head of water. This setup worked very well and never once clogged a filter. There was one pressure spike resembling the previous problems between 105 – 110 h, but this event was actually caused by a rare failed pneumatic air line to the exhaust control valve.

Mid-run drawdowns were necessary twice, first at 72 hours and then at 113 hours. Per the fermentor weight measurements, 1,054 kg was removed but 1,494 kg of this broth was added to an empty fermentor of identical design. The second drawdown removed 679 kg from the main fermenter but 1,012 kg was reported by the load cells of the receiving vessel.

At 136 hours, the fermentation was halted by stopping pH control, glucose addition, and sparge air. Temperature was raised to 80°C and held for two hours to inactivate the yeast. The broth was held between 20 – 40°C afterwards. Plating verified successful kill.

The low-gravity decanting centrifuge was again rented from TEMA and used to process the fermentation broth and concentrate the intermediate product. Fermentor broth was pH-adjusted from 5 to 9.0 – 9.3 using a slow pulsed injection of caustic prior to running through the centrifuge. The fermentor agitator was set to 50 rpm. Then the broth slurry was pumped out of the fermentor using a rotary lobe pump and magnetic flow meter into

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the TEMA unit operating at low G force to primarily discard cell material (detailed settings recorded by Lygos). Feed rate varied between 4 – 6 gal/min and was limited by the centrifuge motor capacity. A plastic hopper collected solids and centrate was sent directly to the disposal. Water was added to the hopper and the solids were reslurried by a shovel before pumping into an adjacent fermentor. Table 2 lists the amounts of water used, vessel consumption, and totalized flow from the vessel outlets during the operations. Note that a garden hose sprayer was necessary to reslurry solids once the vessel levels were below minimums for agitation, and that water was not measured but was on the order of 100 – 200 L. Also, the final amount of slurry in the vessel during each pass could not be drained into the centrifuge, as it was impossible to keep solids settling out and clogging. Approximately 100 kg of material was lost each pass due to settling.

Table 2: Measured Weights and Totalizers During Operation

	1st Pass	2nd Pass	3rd Pass
Water added to feed for reslurry (L)	0	2354	932
Vessel weight consumed (kg)	5784	3766	3421
Totalized flow from vessel (L)	7938	4758	4549

does not include water added directly to tanks via garden hose sprayer

Once this first pass was complete, the reslurried product was again pumped into the TEMA unit, this time operating at higher G force and similar feed rate (centrifuge settings recorded by Lygos). The solids were reslurried and pumped into the original fermentor that had been rinsed clean after emptied. A third pass through the TEMA unit was done with the again reslurried solids at the same settings as the second pass. This time, the solids were shoveled into drums for collection. The feed pH for the third pass was measured at 8.47 and no adjustments were made.

During the second pass through the centrifuge, there were some problems feeding the slurry when the pump and hoses became clogged with thick solids and the centrifuge also tripped out on high motor load. The target volume solids concentration (when a sample vial of material is spun down in the laboratory centrifuge and fraction of solids volume vs total volume is measured) was 25%, but the measured solids concentration was only 15%. The tank agitator was increased from 50 to 80 rpm and allowed to mix for 15 min before taking a new sample. This time the solids loading was actually measured at 40%, indicating we had poor tank mixing at 50 rpm. Water was added to bring the solids concentration down to 25%, and no problems were observed again with the feed equipment or centrifuge. Sufficient water was added to reslurry the solids in the hopper during the second pass to reach 25% solids without further water addition prior to the third pass.

Approximately 2,500 L is required to reach the lower fermentor agitator, and the starting volume for the first pass was approximately 5,400 L. When the level fell below 3,000 L, air was added through the sparger tube (400 slpm) to keep the slurry somewhat suspended until until the volume reached approximately 400 L. Then when aeration could no longer provide sufficient agitation a garden hose sprayer was used to mix up the solids on the bottom of the tank. Table 3 below lists the final product weights. Samples of materials have been shipped to Lygos for chemical and gravimetric analysis.

Table 3: Final Quantities of Recovered Product

Drum 1 net mass (kg)	283.5
Drum 2 net mass (kg)	289.0
Drum 3 net mass (kg)	278.5
Total net mass (kg)	851.0

Lygos' personnel conveyed to NREL that this run successfully met their performance targets.

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Summary

Lygos uses biology to convert low-cost starch or cellulosic sugars into high-value chemicals targeting chemicals that are difficult and expensive to make petrochemically, and that can be produced microbially for less than petrochemical competitors' raw material cost. The subject of this work and Lygos' first product is malonic acid, a building-block chemical with applications in electronics manufacturing, flavors/fragrances, and agrochemicals, among other industries. Lygos uses an acid-tolerant yeast, *Pichia kudriavzevii*, engineered to produce malonic acid at high yields and rates from sugar; in contrast, all other malonic acid producers use a non-renewable, energy intensive, and environmentally hazardous sodium cyanide-based process.

Te current challenge is to demonstrate process scaling and robustness. Lygos has developed an integrated fermentation and downstream purification process at bench-top scale and fermentations have been run up to 15-liters and downstream processing is capable of producing 10–25 g of purified malonic acid per run. However, scaling is necessary for demonstration of process robustness (e.g., process reproducibility, fermentation yield and productivity) and for acquisition of engineering data necessary for commercial production (e.g., heat removal from fermentation). Demonstration of fermentation process scaling is also an essential component of technology de-risking necessary for advancing the technology readiness level in support of further commercialization efforts; for example, a potential strategic partner has indicated that data from a 1,000-liter fermentation would enable a more accurate assessment of the ability to transfer Lygos' technology/process to their facility.

There were two objectives for the work performed at NREL. The first was to produce lignocellulosic-derived sugars from corn stover for testing of low-cost sugar as an alternative to high-cost conventional sugar sources (e.g., starch- or cane-derived sugars). The corn stover was deacetylated and then dilute-acid pretreated in the Integrated Biorefinery Research Facility's large horizontal reactor. The resulting slurry was enzymatically hydrolyzed and then concentrated to over 300 g/L total monomeric sugars. The sugar solution was packaged and shipped to the LBNL ABPDU where the bench-scale fermentation work on this material was performed.

The second and major objective of this project was to demonstrate scale up of Lygo's biological malonic acid production technology in 9,000-L bioreactors and to produce sufficient product for further downstream technology and market development efforts. After first demonstrating that we (NREL) could achieve yield and titer targets in small bioreactors (~ 0.5 L scale) previously demonstrated by Lygos, the fermentation was performed in a 1,500-L bioreactor, and again, yield and titer targets were met at this scale. Based on this successful demonstration, two separate fermentation runs were performed in 9,000-L aerobic bioreactors. Unique solid-liquid separation equipment was also brought in and used to recover product from these large-scale runs. While some problems were encountered during the first run, the experiences gained from this attempt at scaling up the technology were used to perform a second highly-successful run in the 9,000-L bioreactor that achieved yield and titer targets and produced a large amount of intermediate product.

Lygos's biological process for producing malonic acid was successfully scale-up from bench to large-scale bioreactors furthering Lygos' goals to commercialize this technology. In addition to achieving performance targets in the large 9,000-L bioreactor, a large quantity of intermediate product was produced for further downstream recovery and purification work. From which, the final product will be used product testing and market development; however, this work is being performed by Lygos and is outside the scope of this project.

Subject Inventions Listing:

None

ROI #:

None

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Responsible Technical Contact at Alliance/NREL:

Dan Schell

Name and Email Address of POC at Company:

Jeffrey Dietrich (jadietrich@lygos.com)

DOE Program Office:

Bioenergy Technologies

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