DOE Bioenergy Technologies Office (BETO)
2021 Project Peer Review

A Two-Chamber Growth and Production System for Robust Continuous Bioprocessing

March 10th ,2021

Technology Area: Biochemical Conversion and Lignin Utilization

Principal Investigator: Ouwei Wang Ph.D.
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Eric Sundstrom Ph.D.
Organization: Pow Bio, Visolis, LBNL

Project Team

Talented team with board expertise to commercialize continuous bio-manufacturing

Dr. Ouwei Wang, Lead PI, CTO Pow Bio

Dr. Thomas Rüegg, Scientist, LBNL,
Currently at Jungle Bio lab, LLC

Dr. Eric Sundstrom, Scientist, LBNL/ABPDU

Dr. Deepak Dugar, Founder and CEO, Visolis

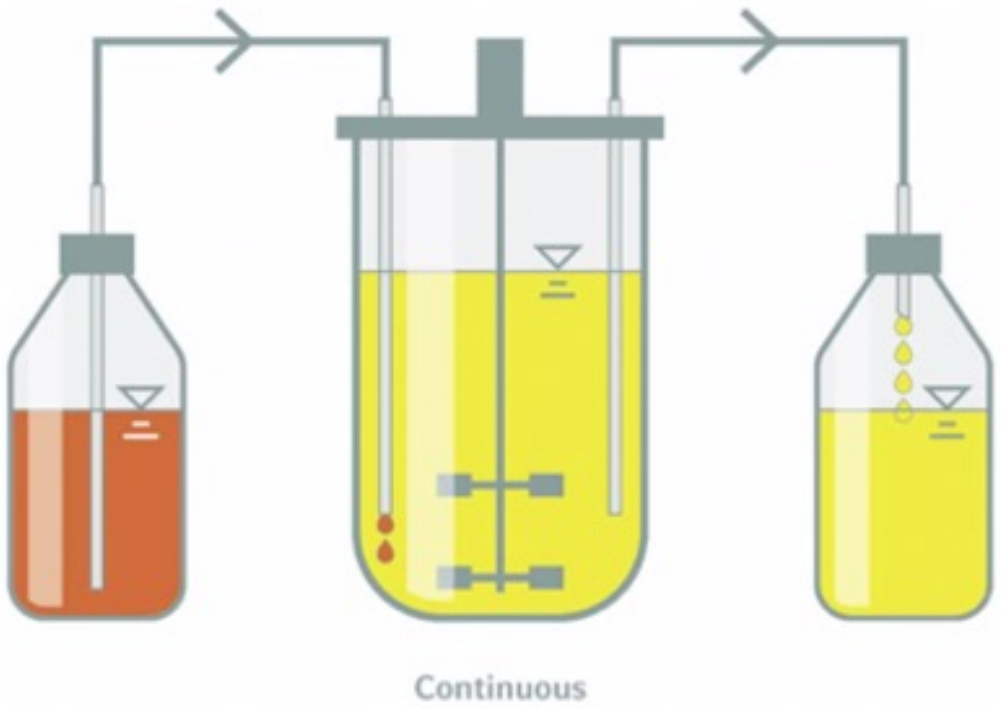
Pow Bio is the prime award recipient
Visolis and LBNL are sub-contractors of the award

Petrochemical industry forms the backbone of modern society

- Enables the manufacturing of ubiquitous products
- Economically cost-effective
- Continuous Production Process
- Unsustainable

Bio-Based alternatives have largely not been able to compete on price

Continuous fermentation can reduce production cost



Traditional (fed) batch is slow and serial in nature

Continuous fermentation

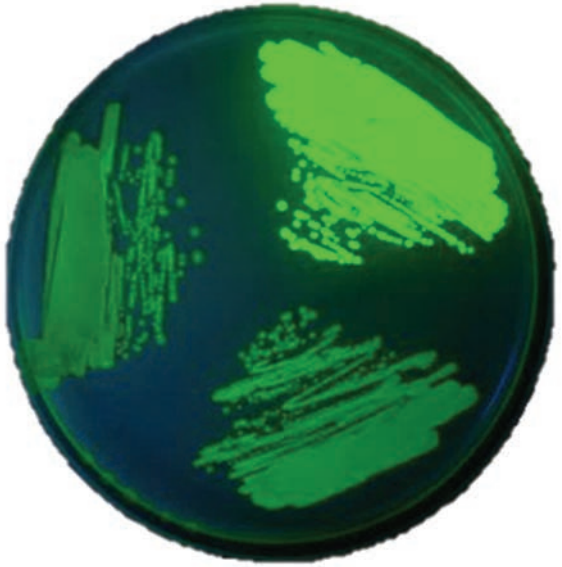
- Increases productivity, minimizes downtime
- Reduces CapEx and OpEx
- Mimics petrochemical refinery processes

Continuous fermentation with distinct growth and production phases

- Dramatically improve yield and productivity
  - No longer need to waste expensive carbon source on biomass
  - shunting all carbon to product during the production phase.
- Extend fermentation times
  - Minimal metabolic burden (if any) during growth phase
  - Low chance of “cheater” cell propagation during production phase

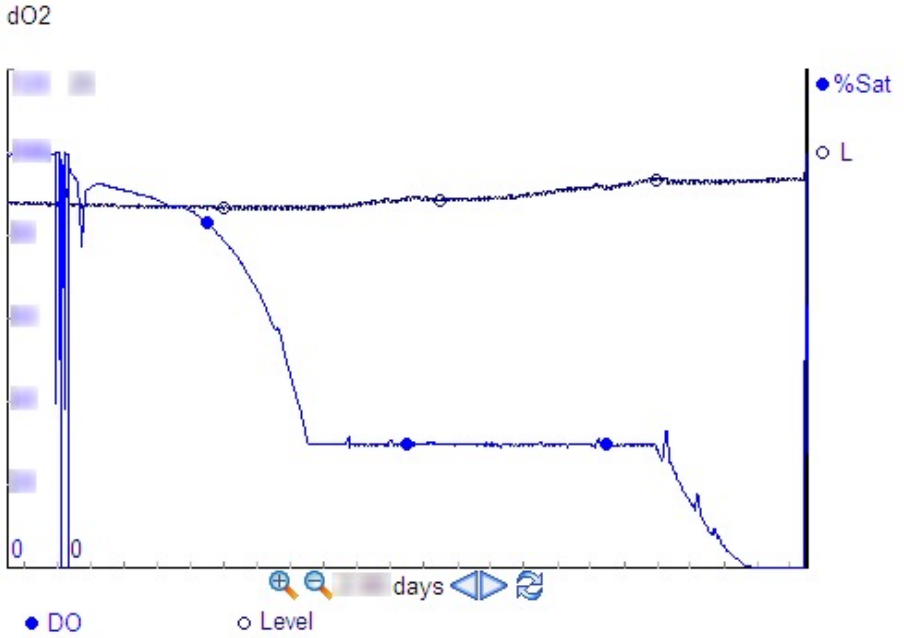
However, continuous fermentation is often considered unreliable

Genetic instability of the production host lead the production strain to “break” overtime



Stability : Loss of function

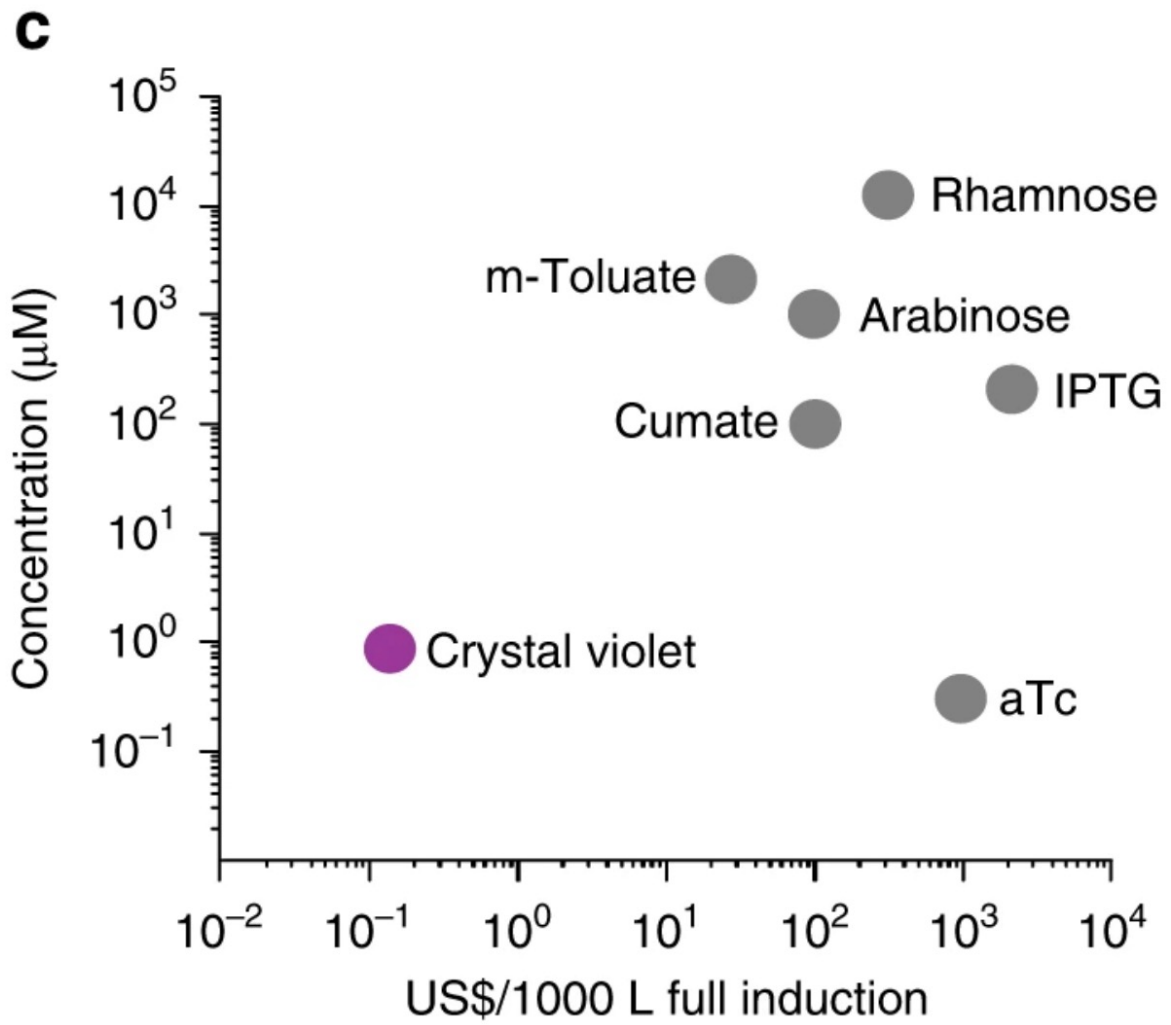
Accidental bioreactor contamination, even with a single spore from dust, could cause continuous reactor to collapse



We aim to solve these problems by combining three emerging technologies that will enable an efficient novel bioprocess:

1. An economical contamination control system - Biocide/Biocide resistance
   - Ensure contamination free continuous fermentation (Task 2)
   - Responsible Party: POW.BIO INTELLIGENT FERMENTATION
2. A highly controllable and economical genetic switch - Jungle Express
   - Ensure stringent separation of growth and production phases (Tasks 3 - 5)
   - Responsible Party: BERKELEY LAB, VISOLIS CARBON NEGATIVE MATERIALS
3. A novel fermentation process – Two-chamber fermentation
   - Ensure mutation free continuous fermentation (Tasks 6 - 8)
   - Responsible Party: POW.BIO INTELLIGENT FERMENTATION, BERKELEY LAB
4. All implemented in Visolis' platform molecule (PM) production system (Tasks 9-13)
   - Responsible Party: POW.BIO INTELLIGENT FERMENTATION, BERKELEY LAB, VISOLIS CARBON NEGATIVE MATERIALS

Jungle Express – Economic and efficient at any scale

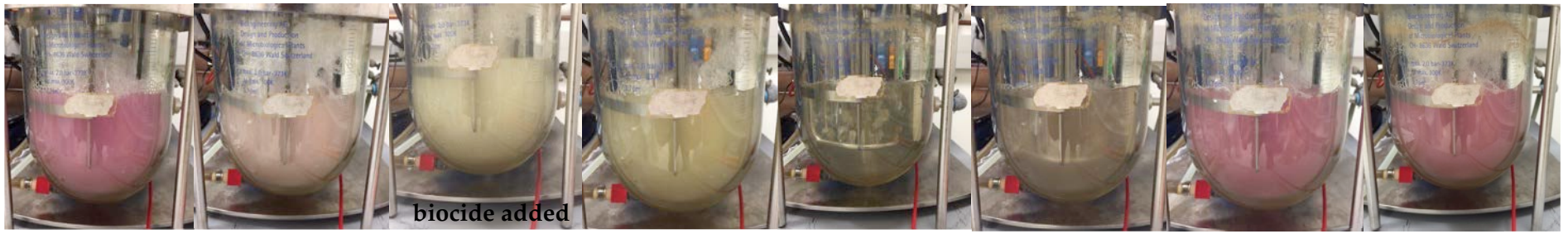


	media independent	economically feasible	control
Constitutive expression	✓	✓	✗
Conventional chemical induction	✓	✗	✓
Media-dependent induction (e.g. phosphate)	✗	✓	✓
Jungle Express	✓	✓	✓

Ruegg et al, Nature Communications 2018

Contamination-Free Continuous Processing

- A RFP-expressing *E. coli* was deliberately contaminated at day 1.
- Contaminants take over the tank (Note color change).
- After application of Pow's contamination treatment (chlorite), tank reverted back to its initial axenic state.



Fermentation development at ABPDU

Bench-scale validation and pilot-scale demonstration

End-to-end process development capabilities:

- Fermentation from 250mL single-use up to 300L pilot
- Online and offline analytics
- Flexible configuration for continuous operation
- Experienced team of fermentation scientists and engineers
- Planned activities: tech transfer and process optimization at 250mL and 2L scale, demonstration at >10L scale

VISOLIS
CARBON NEGATIVE MATERIALS

Synthetic Biology Enabled Manufacturing

Deepak Dugar, President & Founder
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www.visolisbio.com

Project Objective

Demonstration of continuous production of a valuable bio-intermediate, Visolis' platform molecule (PM), at **>2 g/L/hr for 72 hrs at > 500 mL** scale with cellulosic hydrolysate.

TECHNICAL SCOPE SUMMARY:

The proposed project aims to develop and demonstrate a two-stage bioprocess for continuous biochemical production of PM by combining three emerging technologies: (1) a highly controllable and economical genetic switch technology that minimizes genetic drift and enables efficient decoupling of growth from production, and (2) use of a biocide/biocide-resistant system that can treats and prevents biological contamination during prolonged continuous fermentation, and (3) a two-chamber fermentation process to physically separate growth from production. Taken together, these technologies aim at reducing CapEx and OpEx to enable bio-production to be competitive with petrochemical production.

Significant improvement of process economics relative to a comparable bioprocess

Scale out vs. Scale up – much lower capex
Continuous process – reduced downtime and opex

Parameters	Traditional Batch	SMART COMBO	% Reduction
Productivity (g/l/hr)	1.0	3.0	
Fermenter Size	5 x 1,000,000 L	900,000 L	82%
Fermenter CapEx (k$)	7483	1432	81%
CapEx ($/ton)	211	130	38%
Variable costs ($/ton)	1735	1176	32%
Fixed costs ($/ton)	302	225	25%
Production costs ($/ton)	2248	1531	**32%**

Project Management

- The final project goal is the demonstration of continuous production of a valuable bio-intermediate, at >2 g/L/hr for 72 hrs at > 500 mL scale with cellulosic hydrolysate.

- Three budget periods each unlock a critical technical milestone
  - Budget Period 1: Baseline Verification (task 1)
  - Budget Period 2:
    - Strain engineering: Construct a mutation, contamination resistant production strain (tasks 2-5, 8)
    - Process engineering: Construct a proof-of-concept mutation resistant continuous system (tasks 6-8)
  - Budget Period 3:
    - Optimizing the two-stage continuous bioreactor process for PM production (tasks 9, 11, 12)
    - Pilot Scale Continuous PM Production (tasks 10, 13, 14)

Task and Responsibility
(proprietary information removed)

Task Number	Task or Subtask Title	Milestone Type	Milestone Number	Milestone Description	Milestone Verification Process	Responsible team	Anticipated Quarter
1	Initial Verification	Go/No-Go, Milestone	G/NG #1, M1	*Jungle express control of PM production at x g/L/hr. and contamination free continuous bioreactor for > 1 wk.*	DOE verification initial performance data	All	Q1
2	Biocide Resistant PM Strain	Milestone	M2	Construct a biocide resistant strain, achieves PM titers of > x g/L in shake flasks.	Progress Report to DOE	Pow	Q2
3	Expression Tool Kit	Milestone	M3	Produce a promoter Library with expression strength span x magnitude	Progress Report to DOE	Visolis, LBNL	Q3
4	Optimizing Production #1	Milestone	M4	Improved production to x g/L in x L bioreactors	Progress Report to DOE	Visolis, LBNL	Q4
5	Optimizing Production #2	Milestone	M5	Improved production to x g/L in x L bioreactors	Progress Report to DOE	Visolis, LBNL	Q5
6	Continuous Bioreactor Development	Milestone	M6	Demonstrate operation of 2-stage bioreactor for > 1 wk.	Progress Report to DOE	Pow	Q6
7	Bioreactor Ready Strain	Milestone	M7	PM production at >x g/L/hr. in fed-batch bioreactor for 24 hr	Progress Report to DOE	Visolis, LBNL	Q7
8	Intermediate Validation	Go/No-Go, Milestone	G/NG #2, M8	*PM production at > x g/L/hr. in fed-batch; Operation of a continuous two-stage bioreactor (50-100 mL growth tank, 100-500 mL production tank) for >1 wk.*	DOE validates productivity in on-site visit	All	Q7

Task and Responsibility (cont.)

9	PM Production #1	Milestone	M9	PM production in a continuous bioreactor at >x g/L/hr for x hours.	Progress Report to DOE	Pow, Visolis	Q8
10	PM Production #2	Milestone	M10	PM production in a continuous bioreactor at >x g/L/hr for x hours.	Progress Report to DOE	Pow, Visolis	Q9
11	Robustness Analysis #1	Milestone	M11	Demonstration of recovery (at >x% of initial titer) of PM productivity with treatment upon deliberate contamination	Progress Report to DOE	Pow, Visolis	Q10
12	Robustness Analysis #2	Milestone	M12	Demonstration of bioprocess lifetime of > x hrs.	Progress Report to DOE	Pow, Visolis	Q11
13	Pilot Scale Operation, Final Validation	Milestone	Final Project Goal	*Cellulosic PM production at pilot scale (to>10L) in a continuous bioprocess at >2 g/L/hr. for >72 hrs. producing > 5 kg of PM*	DOE validates productivity in on-site visit	All	Q12

Risk Management Examples

Risk			Risk Level explanation	Mitigation strategy
1	General Technical Risk		Unforeseen technical risk is always a risk for all projects. i.e. Incompatibility of proposed technologies	Open and clear communication among team members. Risk diversification
	Probability Likely	Impact Major	Risk Level Summary = High	Final risk = Moderate
2	Personnel Risk		PI employment shift	Briefly shift task and responsibility to a different team member to ensure continuity of the project
	Probability Unlikely	Impact Major	Risk Level Summary = Moderate	Final risk = Low
3	COVID related company shutdown		Lab shutdown due to COVID	Implementation of COVID policy, reduced headcount in lab, team shift
	Probability Likely	Impact Moderate	Risk Level Summary = High	Final risk = Low

Risk Level Summary represents the final risk level based on probability and impact

Approach

The final project goal is the demonstration of continuous production of a valuable bio-intermediate, Visolis' Platform Molecule (PM), at >2 g/L/hr for 72 hrs at > 500 mL scale with cellulosic hydrolysate. We will harmonize four technologies in a proof-of-concept process and take it to pilot scale for the biological production of PM.

- A highly controllable and economical genetic switch - Jungle Express
  - Ensure stringent separation of growth and production phases
- An economical contamination control system - Biocide/Biocide resistance
  - Ensure contamination free continuous fermentation
- A novel fermentation process – Two-chamber fermentation
  - Ensure mutation free continuous fermentation
- All implemented in Visolis' production system

Potential Challenges of the Technical Approach

- Technology incompatibility
  - For example, biocide resistant system and PM production may not be initially compatible and lethal for the cells. Genetic fine tuning could be needed.
- Process optimization
  - Fermentation process development is notoriously resource and time consuming. Continuous fermenter is expected to difficult to optimize due to is length process time
- Scale up
  - Scaling up is could be unpredictable in all bioprocess. It is important to keep end in mind. “Start from the end.”

Go/No-Go Decision Points

- Three G/NG points at end of each budget period.
  - First G/NG is to validate and verify all project teams background technology can work reliably as descripted in the proposal.
  - Second G/NG is to confirm significant progress in strain construction and process development.
    - **Go/No-Go #2, Milestone 8:** *Demonstration of PM production from Jungle Express promoter in fed-batch at x g/L/hr. and operation of a continuous two-stage bioreactor (50-100 mL growth tank, 100-500 mL production tank) for >1 wk. with cellulosic hydrolysate feed without contamination.*
  - Third and final G/NG is to confirm the project final aim is satisfied
    - **Final G/NG and Project Goal:** *Demonstration of PM production from cellulosic hydrolysate at pilot scale in a continuous bioprocess at >2 g/L/hr. for >72 hrs. at >10 L pilot scale*

Impact

Biomanufacturing offers great promise, yet barriers exist that limit product to market

- Addressing challenges related to biomanufacturing scale up could be the key to unlock biomanufacturing as a reliable national resource
- As both Pow and Visolis are from industry, we have first-hand experience with the urgent need to scale-up, thus this project are inherently addressing an industry-relevant challenge

Reliable continuous biomanufacturing break these barriers and super charge the process from laboratory to market

Continuous process:

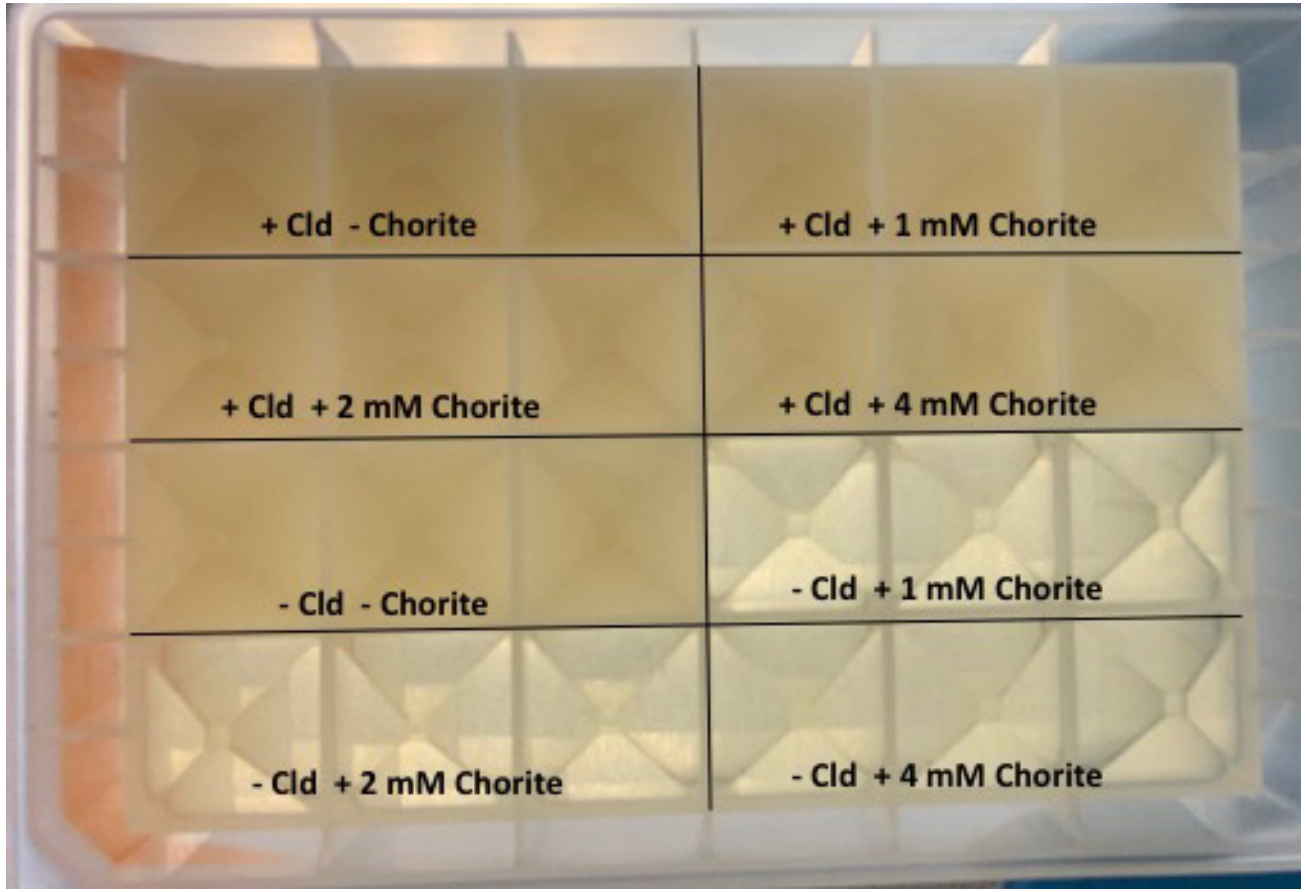
1. Allows automation, unlike slow, serial in nature traditional batch fermentation
2. Amplify productivity per tank. Scale-out vs. Scale-up
3. Minimize downtime. Enhance production economics by increasing productivity

Progress and Outcomes

- Project BP 1 started Oct 2019
  - Task 1, G/NG 1 were successfully completed on time
- Project BP 2 started May 2020
  - Task 2, milestone 2 completed
  - Task 6, 50% completed
- Progress delay in Task 3-5 due to:
  - Pandemic company shutdown
  - National lab headcounts restriction
  - Personnel shift
  - Milestones no-cost extension applied, expecting to catch up

Outcomes Summary

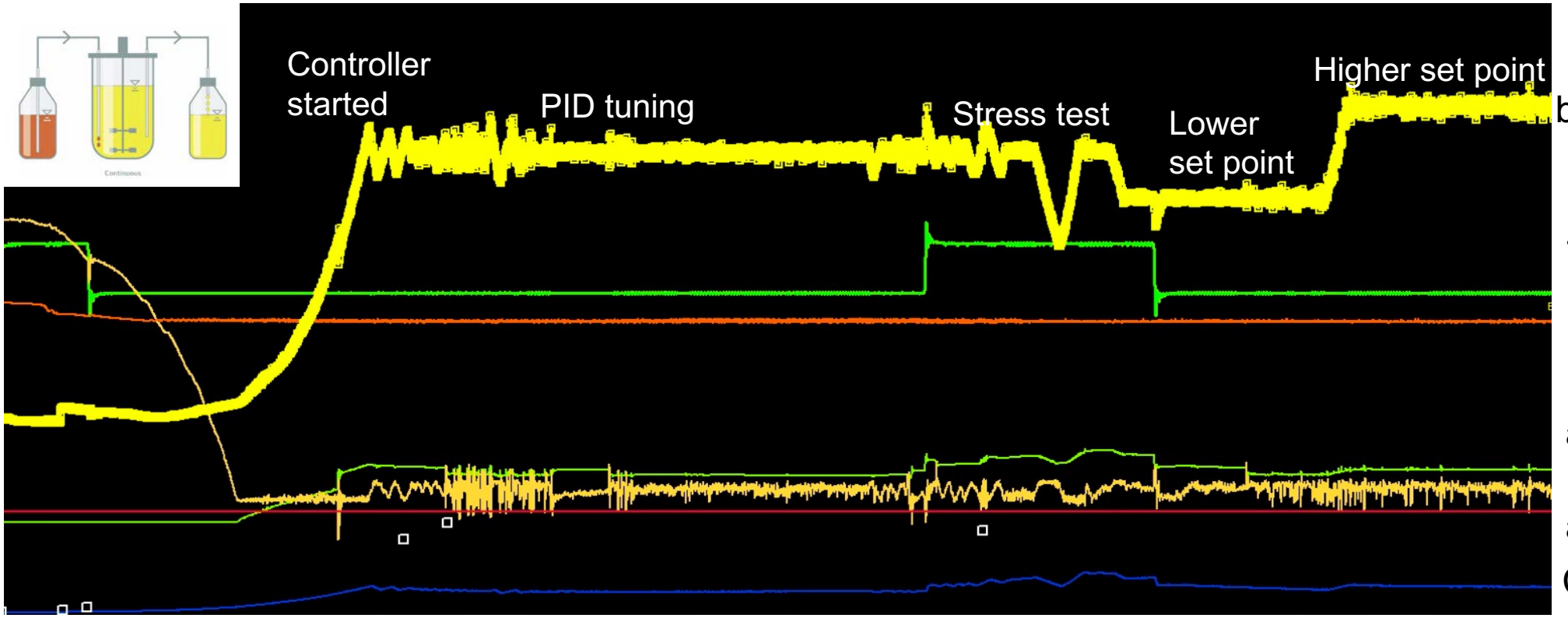
- Task 1 - successfully demonstrated baseline metrics to the DOE verification team
- Task 2 – successfully demonstrated a biocide resistant strain with a high PM titer
- Task 3 – in progress, 80% completed.



A growth experiment showing improved chlorite resistance in Visolis' production strain
Top 2 rows contains a biocide (chlorite) resistant PM producing strain.
Bottom 2 rows contains the parental PM producing strain.

A PID controlled Turbidostat is established

A turbidostat is a type of continuous fermenter with constant level of biomass concentration (see the yellow line below). This is a foundational step to build our continuous, two-chamber fermenter. Now controlled cell density can be achieved



Summary

- Bio-Based fermentation products have largely not been able to compete on price
- Addressing challenges related to biomanufacturing scale up could be the key to unlock biomanufacturing as a reliable national resource
- Continuous fermentation increases process productivity and economics, but the strain mutation and contamination issues limit its implementation
- The project team aims to solve these problems and realize a robust continuous fermentation process in Visolis' PM production system

Summary (cont.)

- Completed tasks 1 to 3, and Task 6.1
  - Initial project verification
  - Constructed a chlorite resistant strain with PM titers of > 1 g/L in shake flasks
  - Generated of a promoter library for expression over 2 orders of magnitude with integrated Jungle Express repressor
  - Established a PID controlled Turbidostat

- Project delayed due to the pandemic, but strong project management and risk mitigation strategy are in place to reduce pandemic impact and ensure the project is on track to deliver intermediate validation and final project goal (>2 g/L/hr for 72 hrs at > 500 mL scale with cellulosic hydrolysate in a continuous fermenter)

Quad Chart Overview (AOP Project)

Timeline

- 10/01/2019 - 9/30/2021?

	FY20	Active Project
DOE Funding	(10/01/2019 – 9/30/2020)	$1,718,821.00

Project Partners*

- Visolis, Inc
- Lawrence Berkeley National Laboratory

Barriers addressed

Advanced Bioprocess Development

Project Goal

The proposed project aims to develop and demonstrate a two-stage bioprocess for continuous biochemical production of PM by combining three emerging technologies: (1) a highly controllable and economical genetic switch technology that minimizes genetic drift and enables efficient decoupling of growth from production, and (2) use of a biocide/biocide-resistant system that can treats and prevents biological contamination during prolonged continuous fermentation, and (3) a two-chamber fermentation process to physically separate growth from production. Taken together, these technologies aim at reducing CapEx and OpEx to enable bio-production to be competitive with petrochemical production.

End of Project Milestone

Demonstration of continuous production of a valuable bio-intermediate, PM, at >2 g/L/hr for 72 hrs at > 500 mL scale with cellulosic hydrolysate.

Funding Mechanism

Funding Opportunity Announcement (FOA) Number: DE-FOA-0002029

Topic Area 7: Advanced Bioprocessing and Agile BioFoundry

*Only fill out if applicable.