DOE Bioenergy Technologies Office (BETO) 2023 Project Peer Review

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

April 4th, 2023
Technology Area Session: Agile BioFoundry

Principal Investigator: Ouwei Wang Ph.D.
Organization: PowBio, Visolis, LBNL

Project Team

Talented team with broad expertise to commercialize continuous bio-manufacturing

Dr. Ouwei Wang, Lead PI, CTO Pow Bio

POW.BIO REG

Dr. Eric Sundstrom, Scientist, LBNL/ABPDU

BERKELEY LAB ABPDU

Dr. Maggie Stoeva, Senior Scientist, Pow Bio

POW.BIO Pendulum

Dr. Thomas Rüegg, Founder, Jungle Biolabs

BERKELEY LAB JungleBio

Dr. Brian Lee, COO, Pow Bio

POW.BIO MIT UCSF University of California San Francisco VISOLIS

Dr. Deepak Dugar, Founder and CEO, Visolis

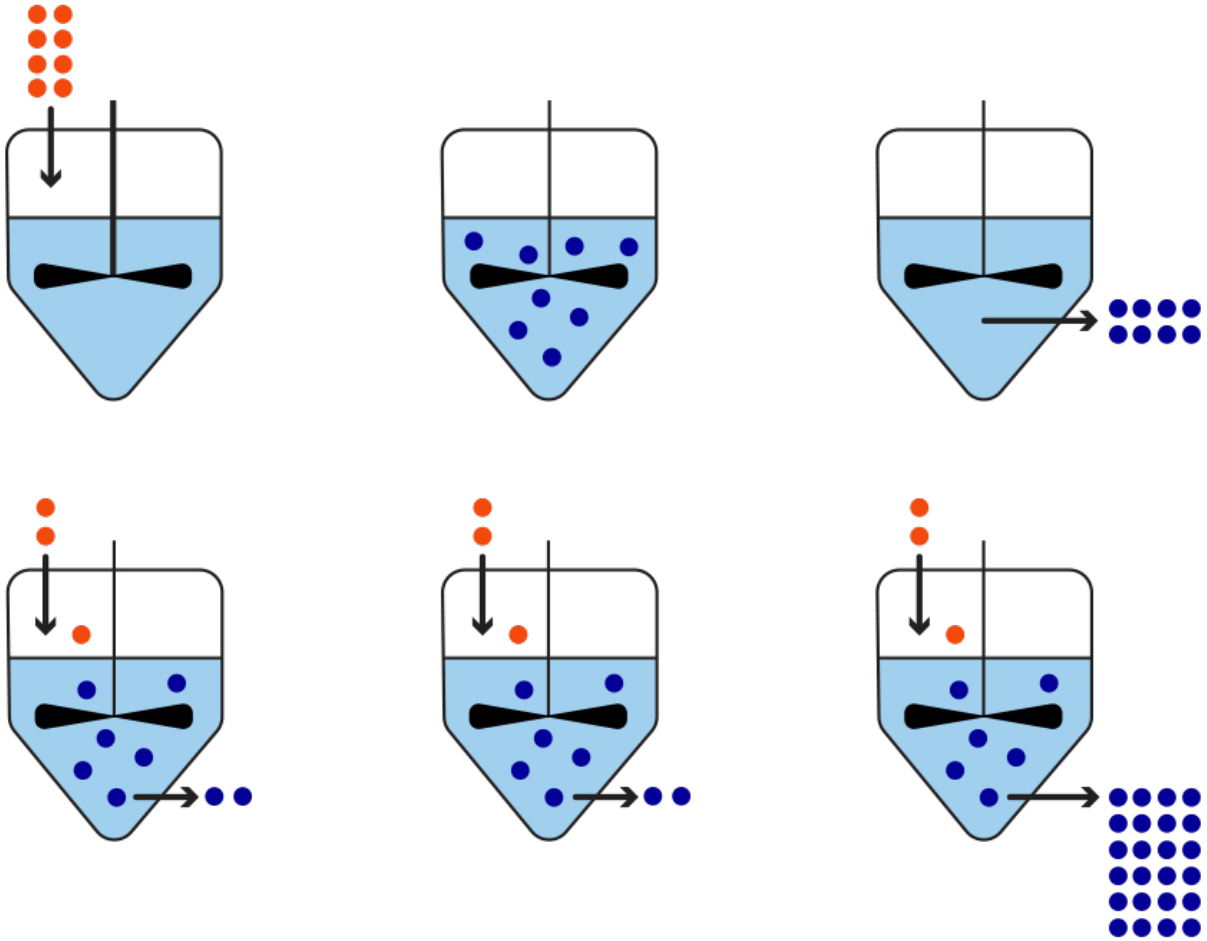
MIT iit delhi DSM pwc VISOLIS

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

Benefits of Continuous Fermentation

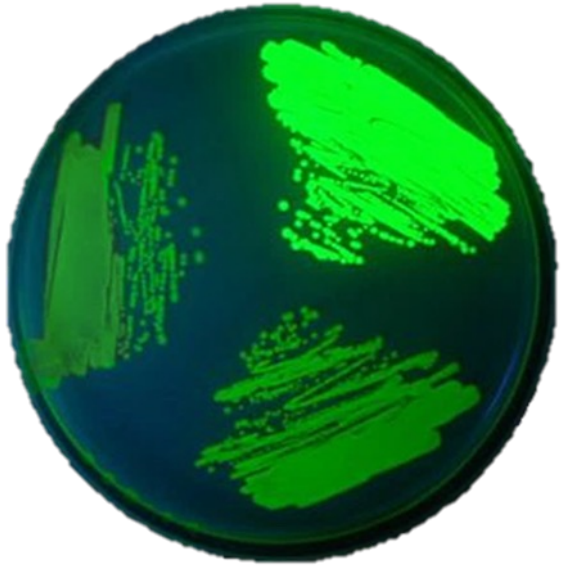


Fed-Batch process:
- Slow, repetitive and serial in nature
- Low productivity
- Productive biomass is wasted

Continuous process:
- Multi-fold higher productivity
- Minimal downtime
- Drastically lowered CapEx/OpEx
- Allows for automation

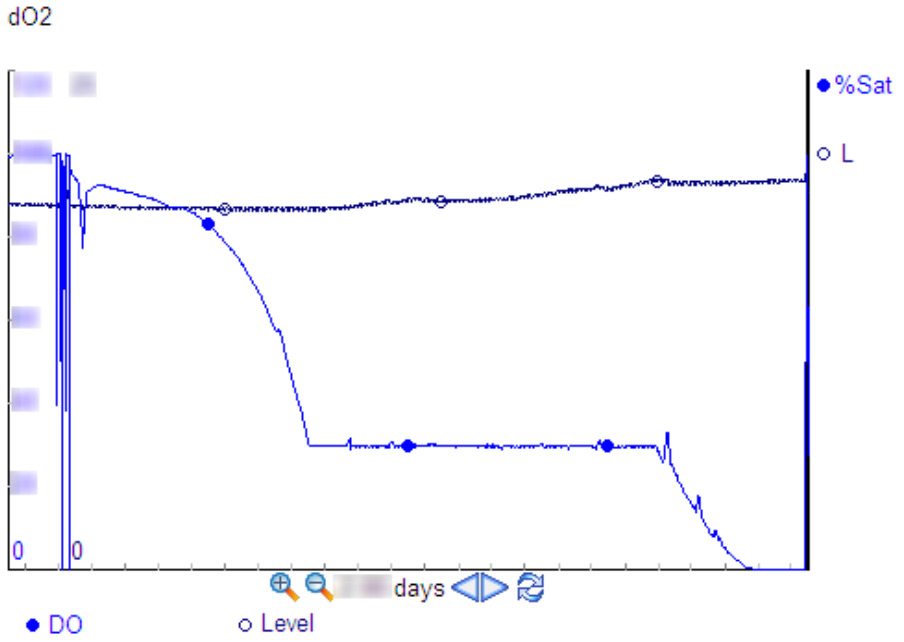
Obstacles to continuous biomanufacturing

Genetic instability of the production host leads to loss of function over time



Stability : Loss of function

Accidental bioreactor contamination can cause continuous reactor to collapse



We aim to build a robust, contamination resistant continuous production system

- A highly controllable and economical genetic switch - Jungle Express
- An economical contamination control system - chlorite/ClO2
- A novel fermentation process – two-chamber fermentation
- All implemented in Visolis' platform molecule, MVL, production system

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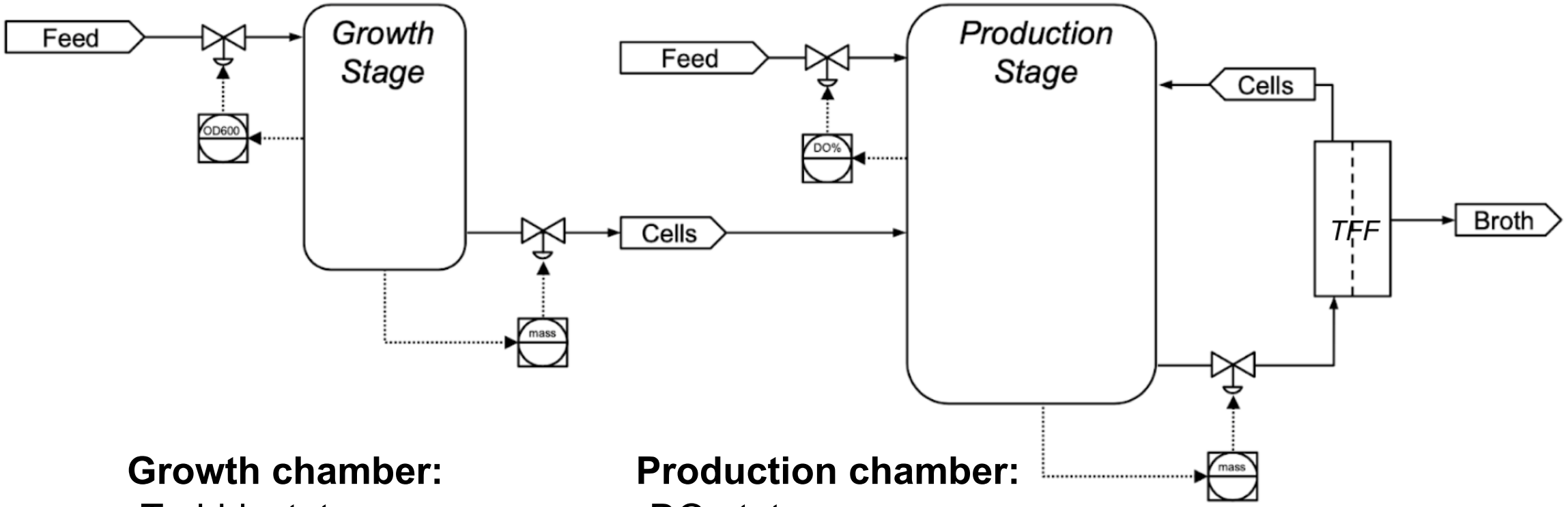
INTELLIGENT FERMENTATION

VISOLIS
CARBON NEGATIVE MATERIALS

JungleBio

1- Approach

Novel Two-Chamber Design



Growth chamber:
- Turbidostat
- Production *repressed*
- Plasmid maintenance system

Production chamber:
- DO-stat
- Growth repressed; production pathway coupled to survival
- Cell recycling

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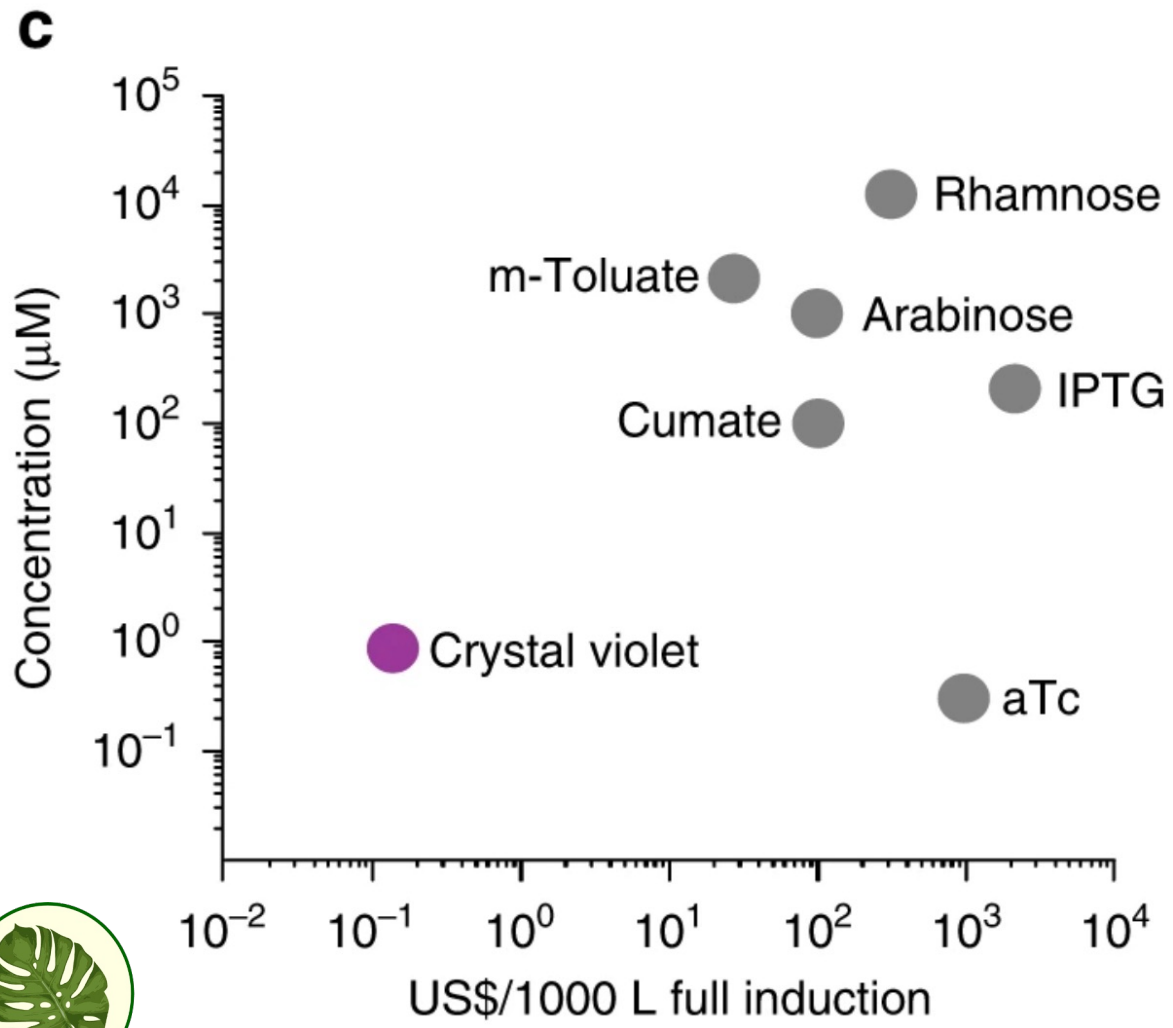
Tightly Regulated and Economical Genetic Switch



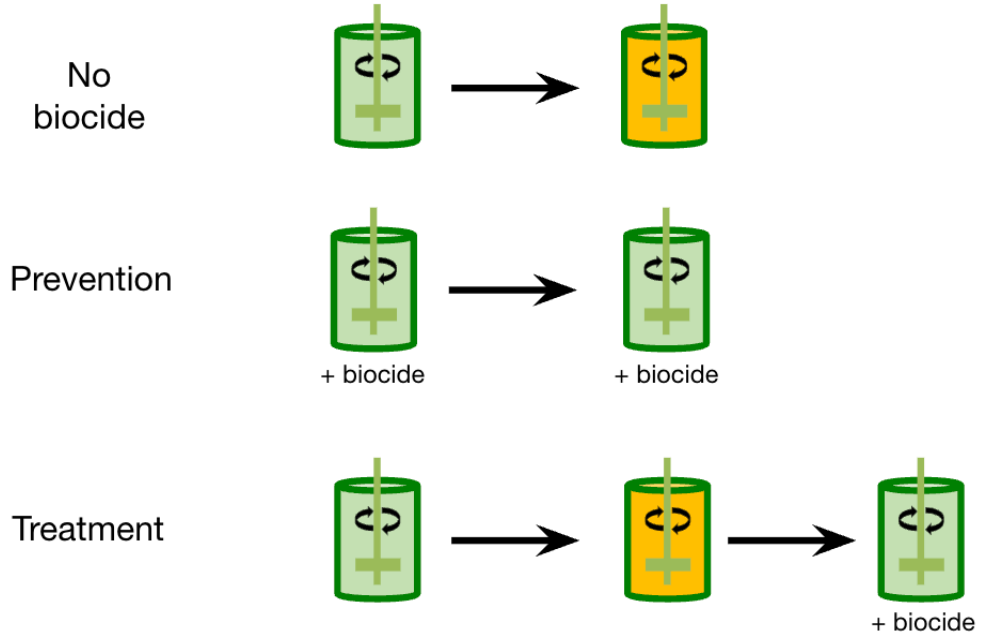
	media independent	economically feasible	control
Constitutive expression	✓	✓	✗
Conventional chemical induction	✓	✗	✓
Media-dependent induction (e.g. phosphate)	✗	✓	✓
Jungle Express	✓	✓	✓

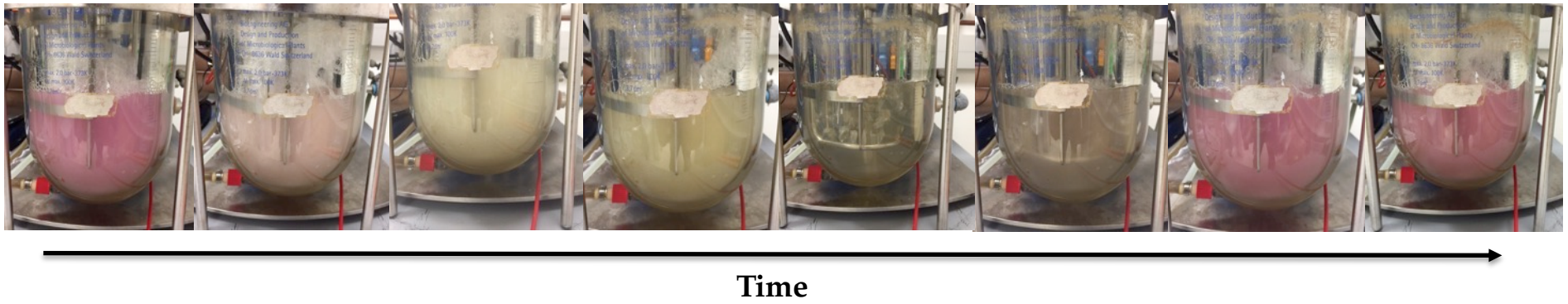
JungleBio

Ruegg et al, Nature Communications 2018

Economical Contamination Control System

- Chlorite dismutase (Cld) detoxifies chlorite
- cost ~$1 per kg
- directed evolution identified mutation resulting in high chlorite resistance





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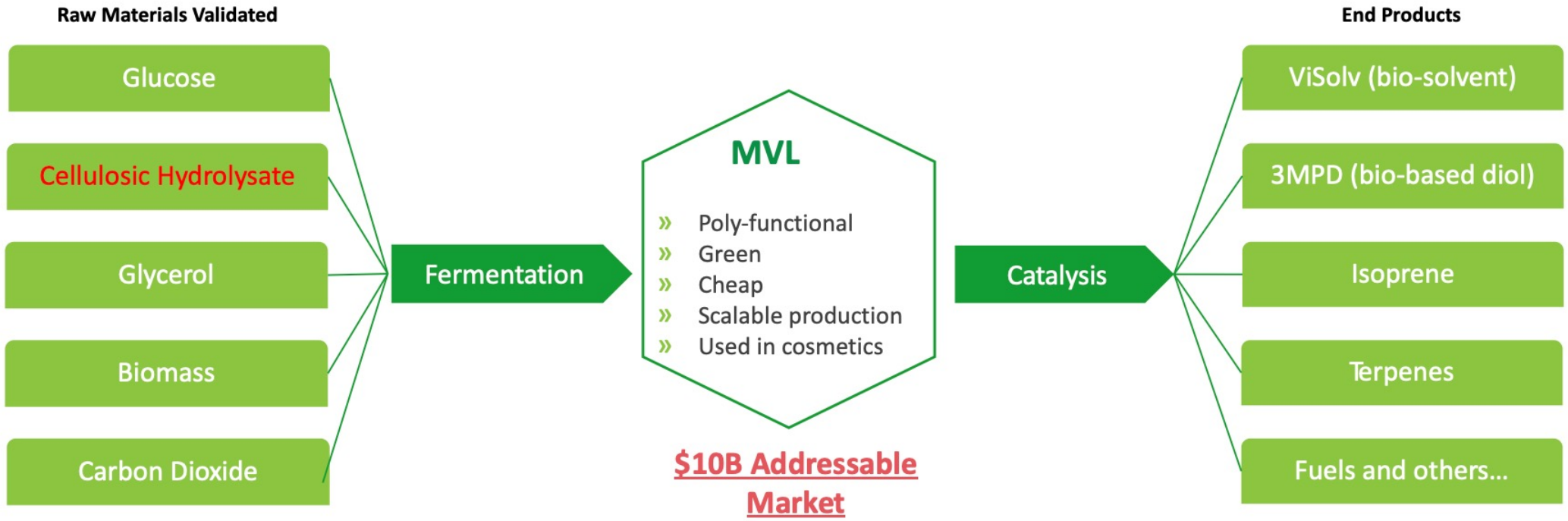
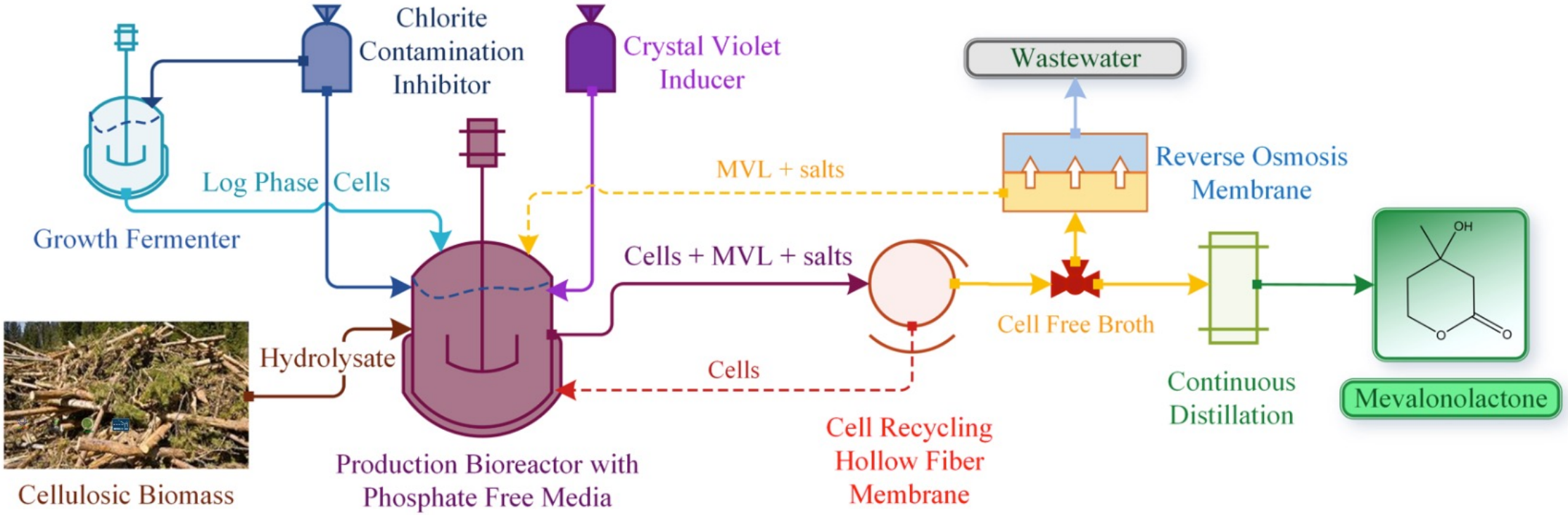
MVL Platform



VISOLIS
CARBON NEGATIVE MATERIALS

SMART COMBO

(**S**table **M**ultistage **A**xenic **R**egula**t**ed **Co**ntinuous **M**icrobial **Bio**processing)



Final goal: Demonstration of cellulosic MVL production at pilot scale in a continuous bioprocess at > *2 g/L/h for* > *72 h* to produce > *5 kg* of MVL

Summary of Progress and Achievements

- *1st Go/No-Go:* Baseline verification
  - Milestone: rate = 0.15 g/L/h, titer = 1 g/L, continuous bioreactor 1 wk
  - Achieved: rate = 0.44 g/L/h, titer = 32 g/L, continuous bioreactor > 1 wk

- *2nd Go/No-Go:* Confirm significant progress in strain construction and process development
  - Milestone: rate = 1.0 g/L/h for 24 h, contamination free two-stage bioreactor 7 days
  - Achieved: rate = 1.05 g/L/h 27 h, contamination free two-stage bioreactor 11 days

- *3rd Go/No-Go:* Final project aim, optimization and scale up
  - Milestone: rate = 2 g/L/h for >72 h with cellulosic hydrolysate, 5 kg MVL produced
  - Progress:
    - rate > 2 g/L/h for 101 h with glucose at 2 L scale (peak 2.18 g/L/h)
    - process has scaled to 15 L with productivity > 2 g/L/h for 114 h
    - Demonstrated production on cellulosic hydrolysate comparable to glucose

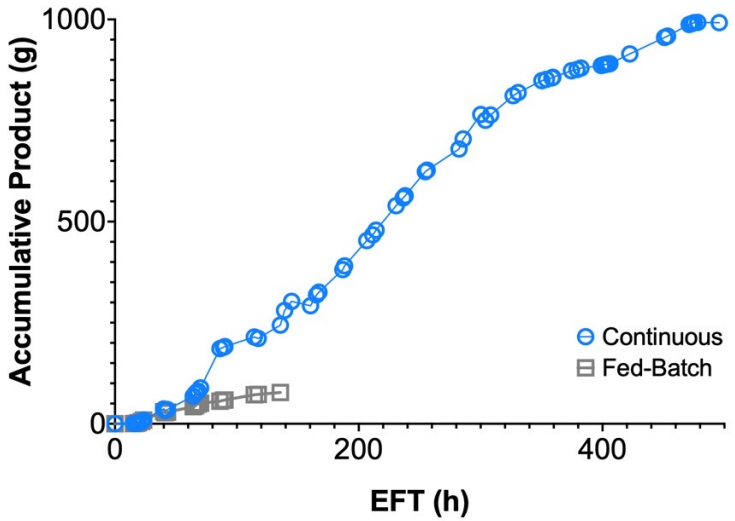
Risks and Mitigations

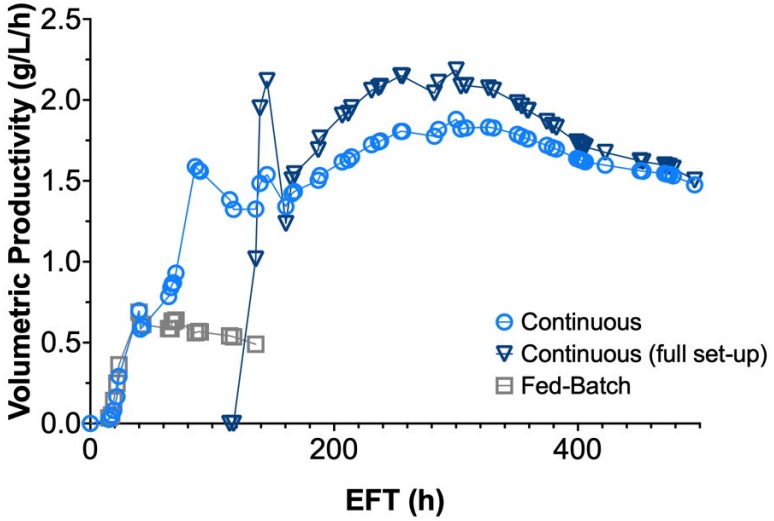
1. Tech transfer of two-chamber system to ABPDU for scale up fails
    - Tech transfer has already initiated and ABPDU was able to stably replicate the turbidostat growth chamber
    - Pow has pilot scale bioreactor in house to perform scale up if necessary
    - Process has been scaled up to 30 L by Pow, with a productivity of 2 g/L/ for >72 h
    - Pow and ABPDU are closely located, allowing Pow personnel to assist with tech transfer to ABPDU

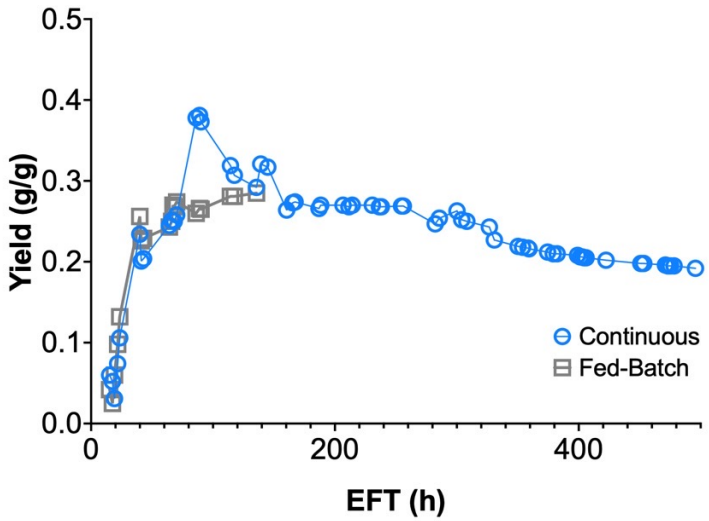
2. Productivity with cellulosic hydrolysate is significantly lower than on pure glucose.
    - Demonstrated by Visolis in fed-batch to have same titer and yield as glucose
    - Demonstrated production in two-chamber system as part of the intermediate validation at 2 L scale
    - Screened several different hydrolysate sources and identified one that is compatible for MVL production in fed-batch

2 – Progress and Outcomes

Productivity Milestones Reached

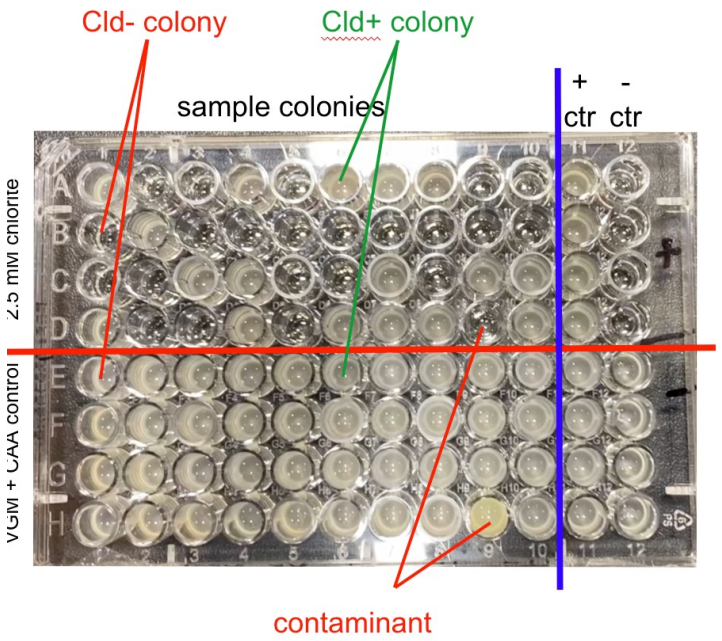


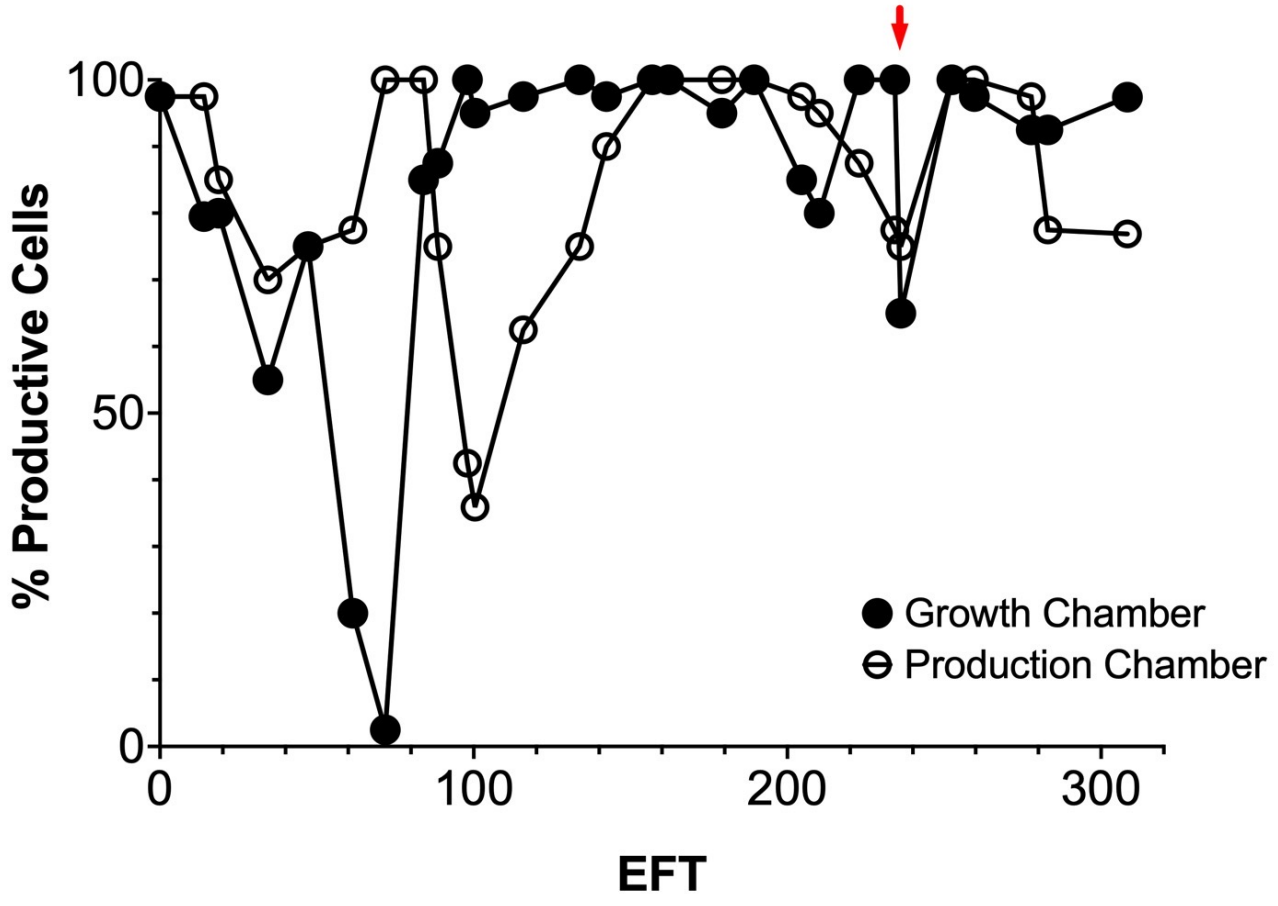




- *Milestone*: >150 h TOS; >2 g/L/h productivity for 72 h
- *Achieved*: 500 h TOS; > 2 g/L/h for 101 h in continuous mode
- 992 g of MVL produced in a 2 L system
- ~5x more product and ~3x higher productivity over Fed-Batch
  - Similar yield achieved

Successful Demonstration of Contamination Control





- Deliberate contamination with GFP *E. coli*
- *Milestone*: >95% Cld+ after 7 days
- *Achieved*: >95% Cld+ after >11 days

Co-consumption of C6 and C5 sugars

- Engineered strain is capable of efficiently co-utilizing glucose-rich or xylose-rich cellulosic sugar streams
- Production metrics similar to the commercial fermentation process utilizing glucose as the feedstock (MVL titer of 50-70 g/L and an overall yield of 0.3-0.4 g MVL/g sugars).

Table 1. Composition of the different cellulosic hydrolysate feedstock used in the fermentation run.

Sample #	Glucose (g/L)	Xylose (g/L)	Arabinose (g/L)	Acetate (g/L)	EtOH (g/L)	MVL (g/L)	Yield (g MVL/g Sugar)
1	66	328	0.5	9.5	0.7	50.3	0.31
2	500	98	3	10.8	2.3	74.6	0.40
3	58	251	2.6	31.8	0.5	57.2	0.37
4	245	178	2	25	0.8	58.7	0.39
5	430	285	1.4	2.5	0.4	75.3	0.38

Plasmid Maintenance System with High Stability

- The stability of the biosynthetic pathway is crucial for high MVL titers/productivities
- ***Goal:*** highly stable, antibiotic-free plasmid maintenance system for the Growth Chamber
- strains achieve similar titers and productivities in fed-batch (30-70 g/L, 1 g/L/h for 40 h)

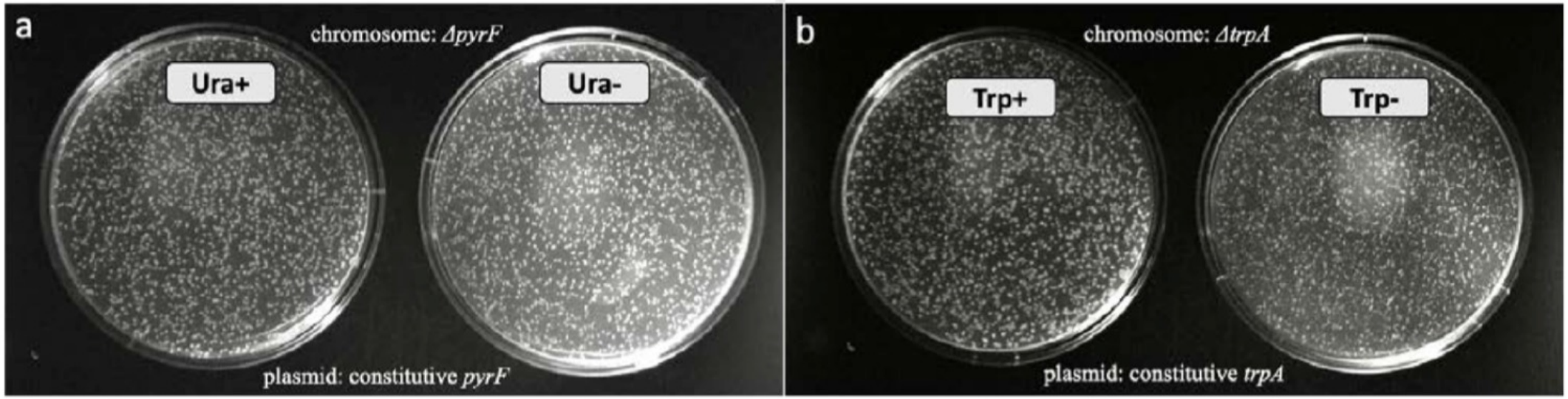
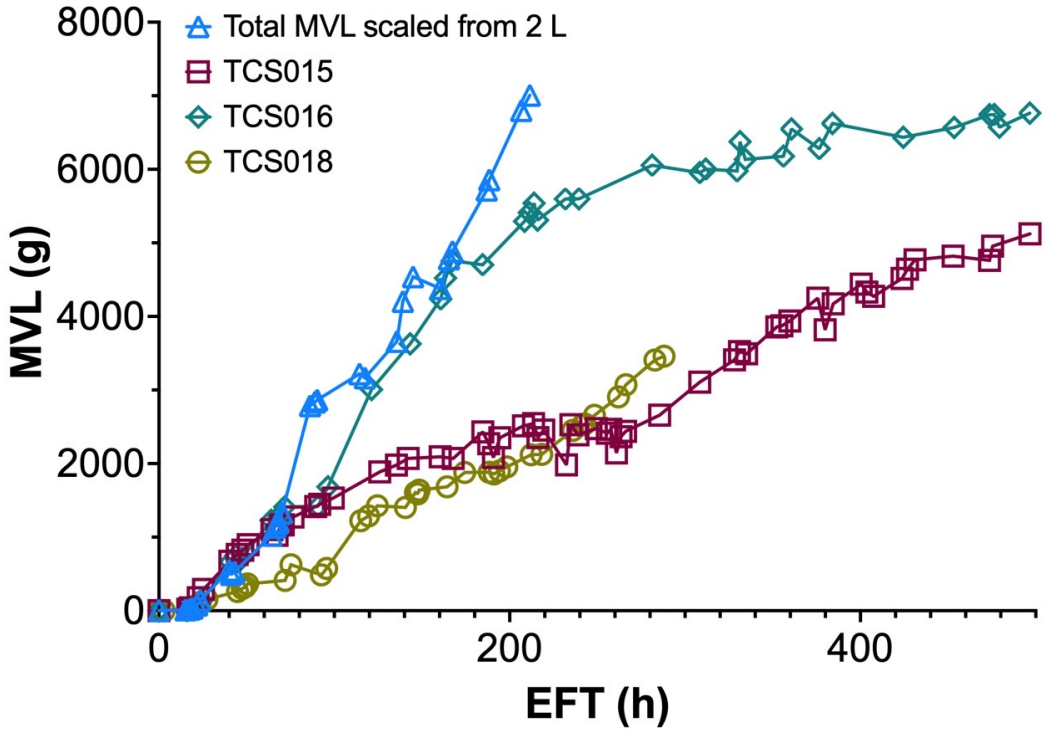
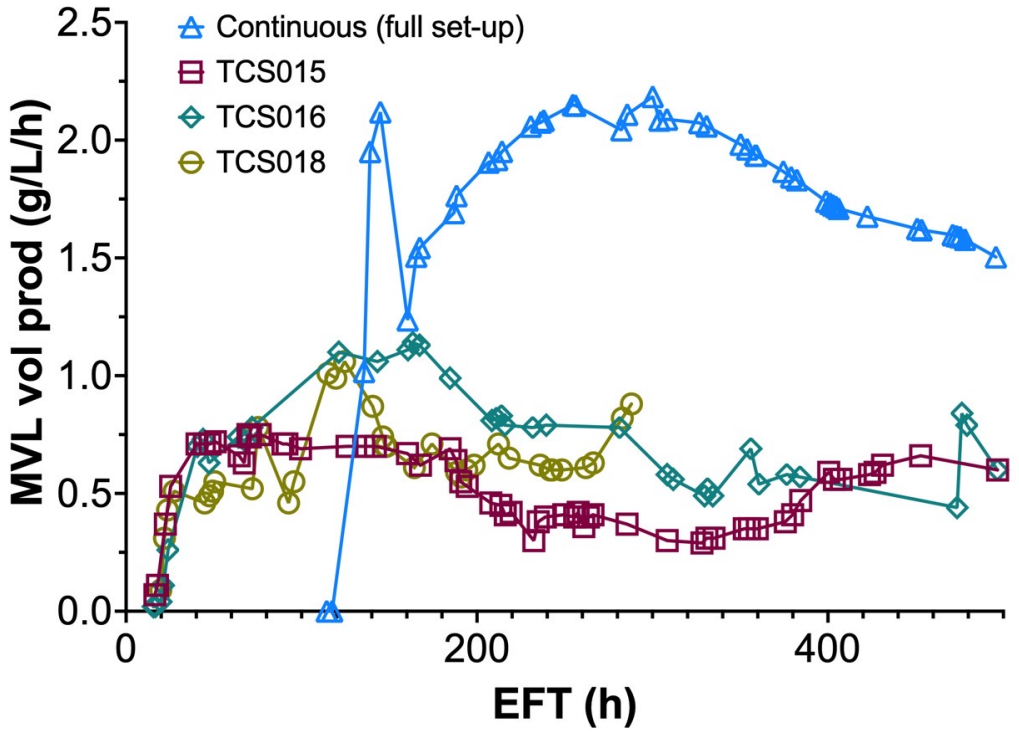


Figure 3 | Plasmid stability after 7 days fermentation. Stability of the newly constructed auxotrophic MVL plasmid maintenance system was assayed by plating cells on media with and without uracil (a) and tryptophan (b) after a 7-day MVL production run in ambr250 bioreactors. Similar colony numbers on non-selective and selective conditions show that the plasmids are highly stable.

Scaling to 30 L

- *Milestone*: Demonstration of cellulosic MVL production at pilot scale in a continuous bioprocess at > *2 g/L/h for >72 h*
- *Progress*: scaled to 15/30 L with run times of 500 h, produced >20 kg over 4 runs
- Further optimization of solid and liquid retention times





3 – Impact

Robust Continuous Fermentation Unlocks the Potential of Biomanufacturing

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

Lack of infrastructure

Limited fermentation capacity; lack of tank weeks; lack of production facility

Lack of expertise

Inaccessible knowledge; a dark art; Outdated mentality

Poor production economics

Impossible production economy, cannot scale up

- Addressing challenges related to biomanufacturing scale up and capacity are the key to unlocking biomanufacturing as a reliable national resource
- 1 provisional patent application and 1 commercial partner initiated

Economic Impact of Continuous Process

	Traditional	Pow.bio Continuous	Technology Impact
Production:	10 kta	10 kta	For identical production capability
Investment:	$187.4M	$40.3M	78% reduction in capital
COGS:	$3.36/kg	$1.63/kg	51% lower cost in production
Volume:	47 m3	54 m3	For identical investment
Production:	0.5 kta	4.1 kta	8x more products
Investment:	$25M	$25M	4.4x lower cost in production
COGS:	$9.27/kg	$2.08/kg	

Summary

- Bio-Based fermentation products have largely not been able to compete on price
- Addressing challenges related to scale up and capacity are the key to unlocking biomanufacturing as a reliable national resource
- Continuous fermentation increases process productivity and economics, but strain mutation and contamination issues limit its implementation
- The project team aims to solve these problems and realize a robust continuous fermentation process
  - Routine operation of continuous process > 500h
  - At 2 L scale, strain stability and contamination overcome; process productivity at milestone
  - Strain engineering for plasmid stability in the Growth Chamber, and production-coupled survival in the Production Chamber, and co-utilization of cellulosic sugars
  - Scale up to 30 L in progress, productivity milestone achieved

Quad Chart Overview

Timeline

- *10/01/2019*
- *10/01/2023*

	FY22 Costed	Total Award
DOE Funding	*(10/01/2021 – 9/30/2022) FY22 = $491,444 Total = $2,468,821*	*FY22 = $674,810 Total = $3,100,272*
Project Cost Share *	$631,451	

TRL at Project Start: 3
TRL at Project End: 7

Project Goal

The project aims to develop a two-stage bioprocess for continuous production of MVL by combining three emerging technologies: (1) a highly controllable and economical genetic switch, (2) the use of a biocide/biocide-resistant system, and (3) a two-chamber fermentation process to physically separate growth from production. Taken together, these technologies reduce CapEx and OpEx, rendering bio-production competitive with petrochemical production

End of Project Milestone

Demonstration of continuous MVL production at >2 g/L/h for 72 h 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

Project Partners*

- Visolis, Inc.
- Jungle Bio
- ABPDU, LBNL

*Only fill out if applicable.

Additional Slides

TEA – Assumptions

Black-Box, unsegregated model

- Cells have only two metabolic states (growth and production)
- Metabolic state is perfectly controllable by operators (inducible at any point in the process)
- Cells have constant specific consumption rates (qs) in each metabolic state (qs only changes with induction)
- Cells have constant metabolic yields in each metabolic state (Yx/s and Yp/s only changes with induction)
- Agitation and aeration is sufficient to achieve good mixing
- Maximum cell density is capped by OTR

TEA – Assumptions

Growth: Max Yield: 0.5 g DCW/gGlc

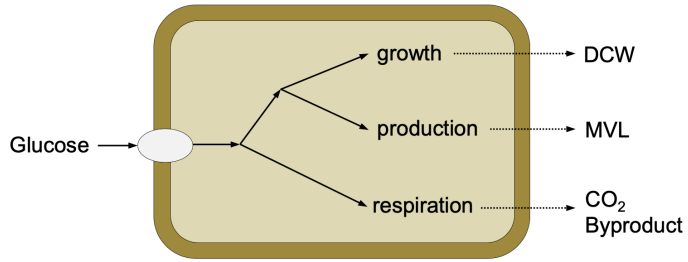
$$\mathbf{1}Glu + \mathbf{3.011}O_2 + \mathbf{0.846}NH_3 \xrightarrow{X} \mathbf{3.255}DCW + \mathbf{2.745}CO_2 + \mathbf{4.441}H_2O$$

Production: Max Yield: 0.48 g MVL/gGlc

$$\mathbf{1}Glu + \mathbf{1.349}O_2 \xrightarrow{X} \mathbf{0.664}\mathrm{MVL} + \mathbf{2.013}CO_2 + \mathbf{2.678}H_2O$$

Respiration (Maintenance/Stress/Byproduct):

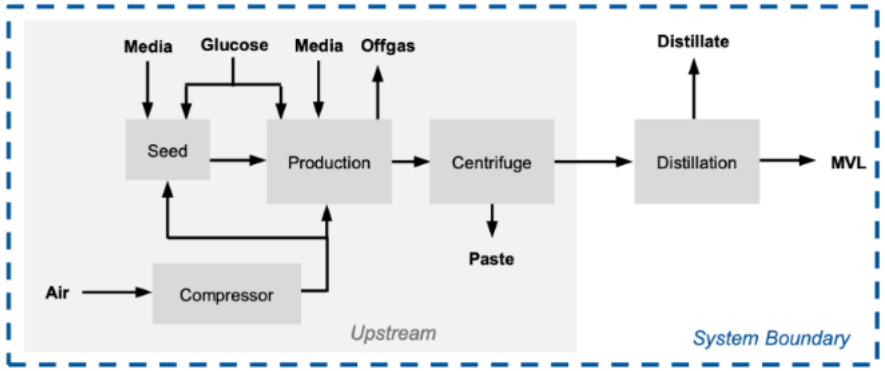
$$\mathbf{1}Glu + \mathbf{6}O_2 \xrightarrow{X} \mathbf{6}CO_2 + \mathbf{6}H_2O$$



TEA – Assumptions

Process Flow Diagram

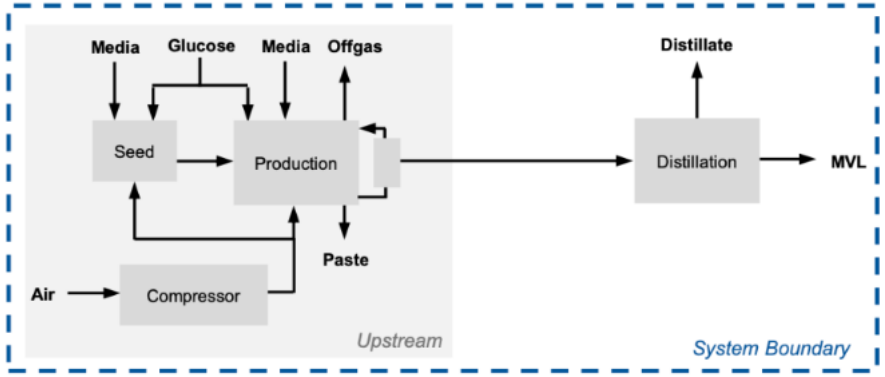
Fed-batch



Product Specification:

80 wt% MVL in the product stream

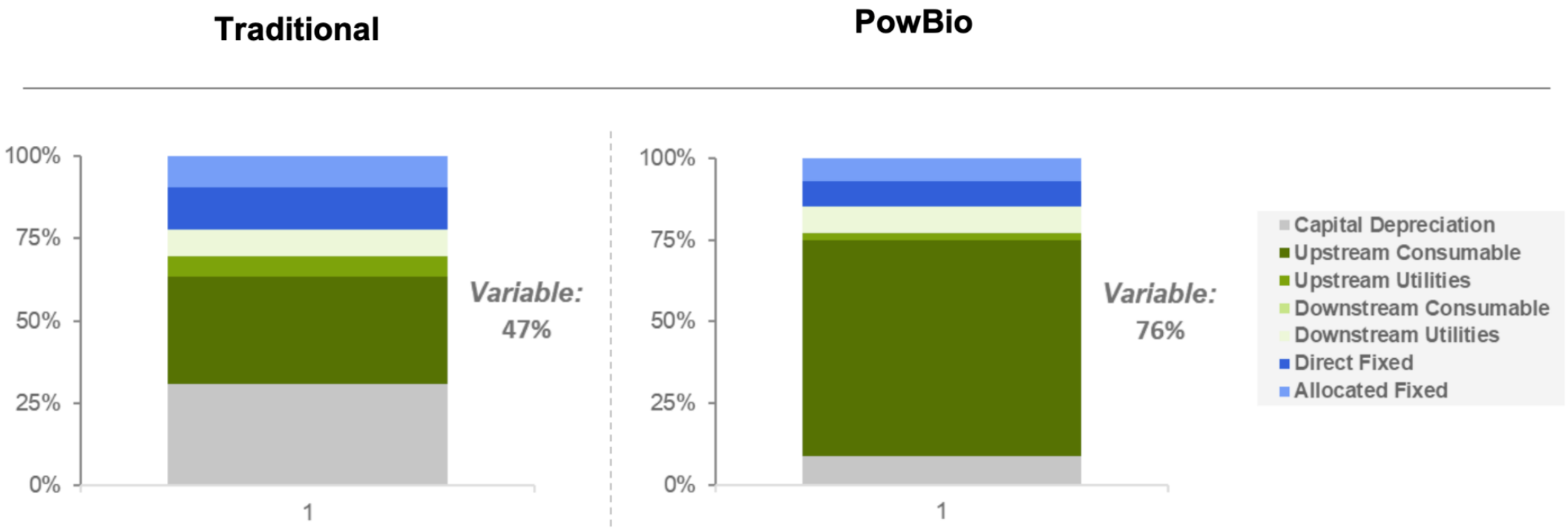
Continuous



Product Specification:

80 wt% MVL in the product stream

Comparative Cost stack



Compared to the equivalent fed batch system the PowBio process has…

- Reduced percentages of capital and fixed cost
- Increased dominance of variable costs to the overall COGS

Publications, Patents, Presentations, Awards, and Commercialization

- 1 provisional patent application: Multi-chamber Cell Culture System and Method, filed Dec 24, 2021

- Platform is being commercialized by PowBio. PowBio is already working with a client to develop a continuous manufacturing process based upon these results