Xylome
Renewing tomorrow today

Award DE-EE0008497

First public presentation

# Biodiesel and higher value products from stillage fiber

## Timeline (nominal)

- Project start date 06_01_2019
- Project end date 12_31_2022

| | FY20 Costed | Total Award |
|---|---|---|
| **DOE Funding** | (10/01/2019 – 9/30/2020) $434,360 | (negotiated total federal share) $1,040,426 |
| **Project Cost Share** | $305,784 | $323,000 |

## Project Partners*

- Partner 1 Confidentiality agreements
- Partner 2 Limit disclosure

## Project Goal

To make biodiesel precursors from dissolved organics and corn fiber present in grain ethanol stillage.

By engineering hyper-lipogenic strains of *L. starkeyi* to express and secrete cellulases and xylanases.

## End of Project Milestone

Demonstrate fermentation with our top strain on stillage in a commercial pilot-plant.

We are currently near end of Phase 2

## Funding Mechanism

DE-FOA-0001916: Bioenergy Engineering for Products Synthesis (BEEPS) 2018; Biofuels and Bioproducts from Wet Organic Waste Streams

# DOE Bioenergy Technologies Office (BETO) 2021 Project Peer Review

Topic 4: Biofuels and Bioproducts from Wet Organic Waste Streams (WWTE)
March 30, 2021

University of Wisconsin
Research Park
Madison, Wisconsin

Xylome
*Renewing tomorrow today*

**Tom Jeffries**

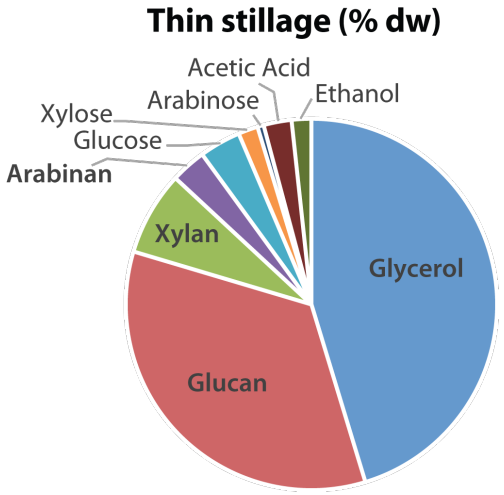
# Biodiesel and higher value products from stillage fiber

What is stillage fiber?

## Stillage fiber, solubles, protein and corn oil are left over after fermentation of corn grain

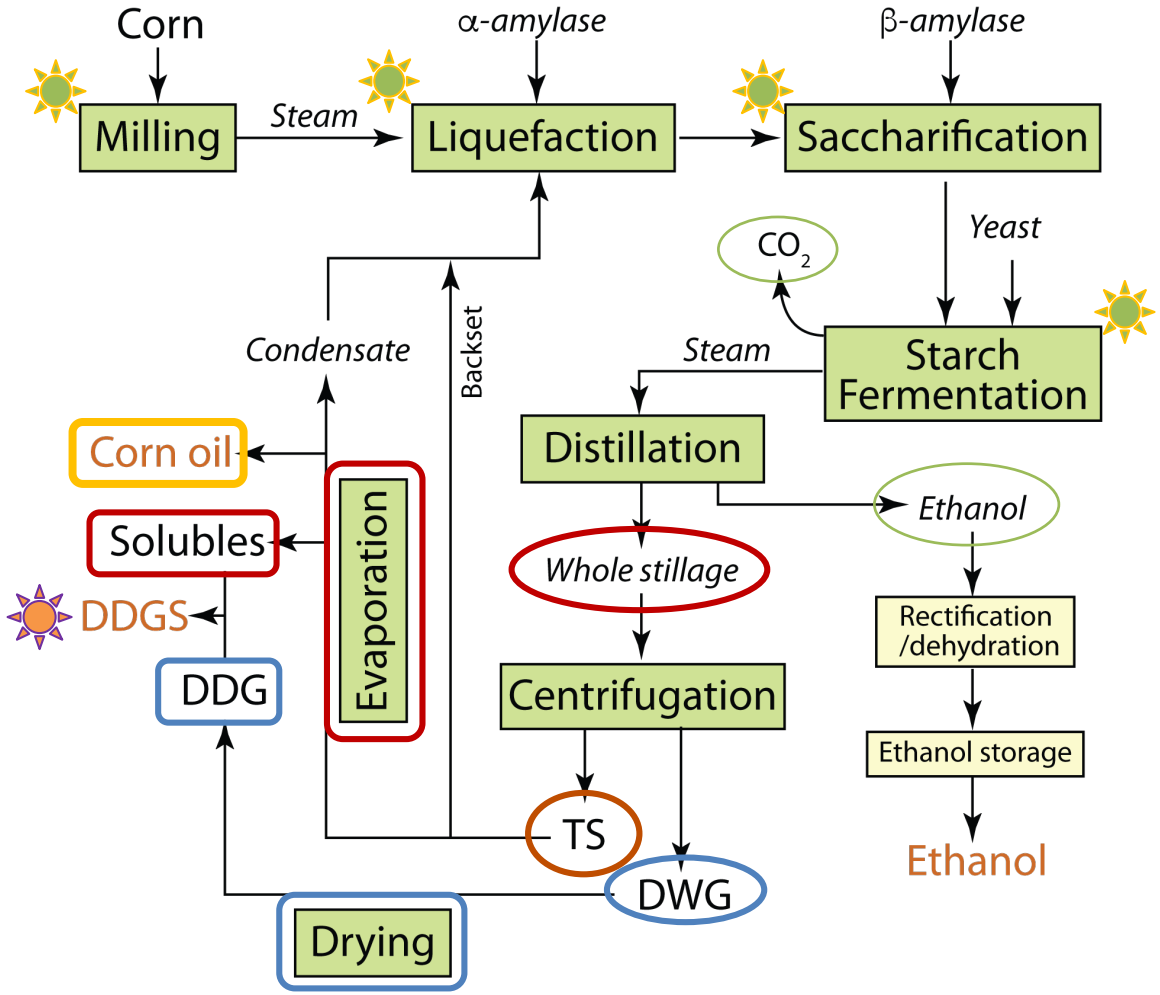
55% starch
20% fiber
18% crude protein
7% oil

Thin stillage is produced at ≈400 gallons/min



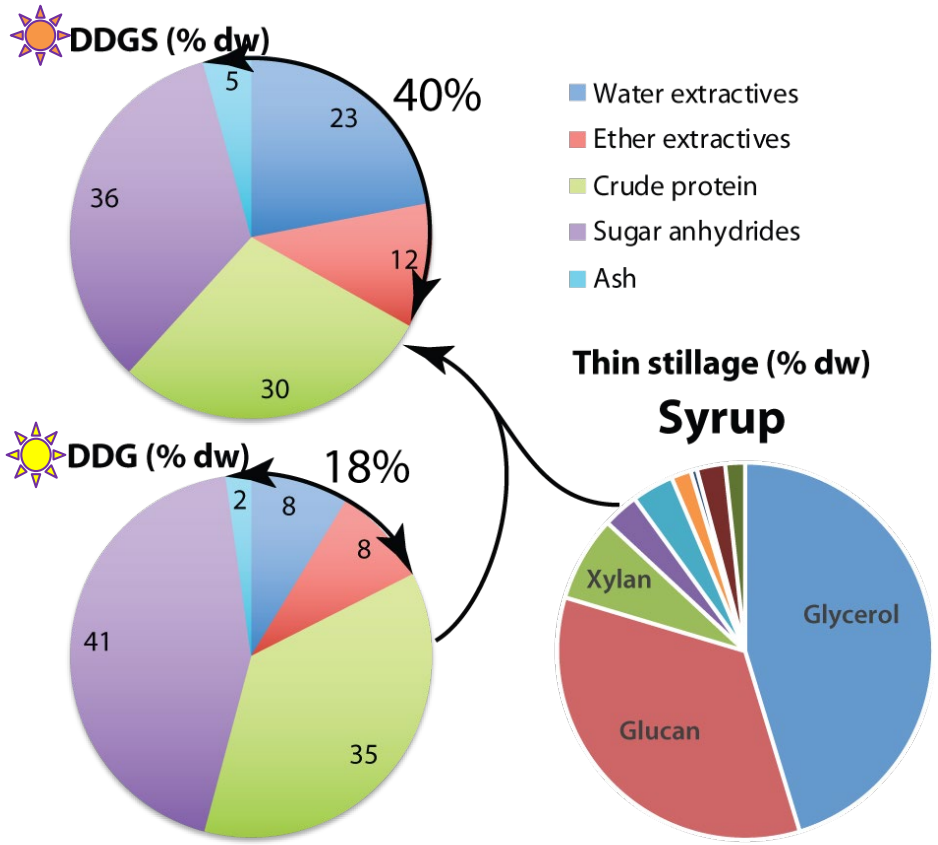

>125 million gal/yr at a typical plant

## Traditional dry-mill process



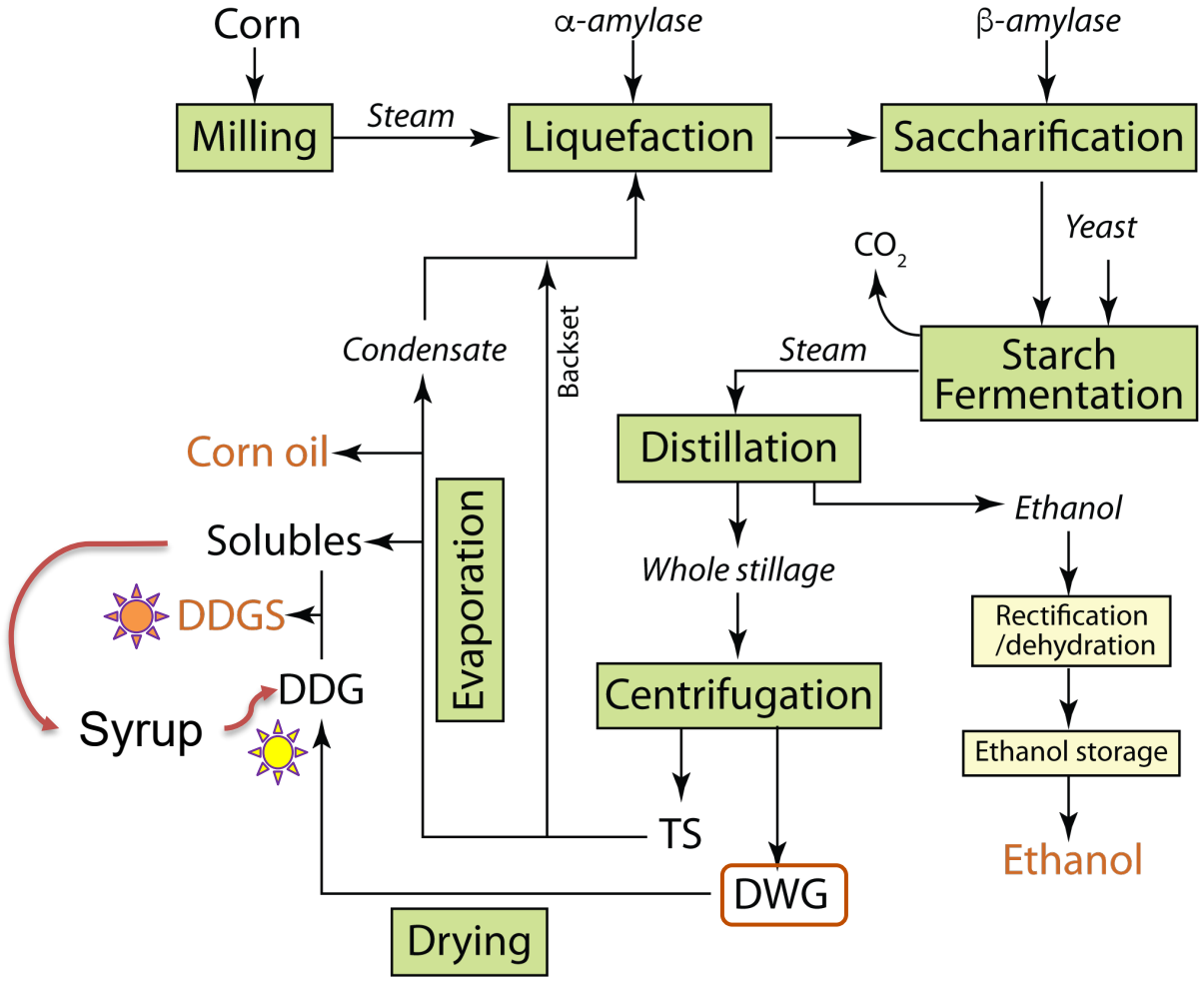
**The stillage syrup is combined with DDG to make DDGS, which is fed to cattle. The fiber and solubles contents restrict use of protein by poultry, swine and fish.**

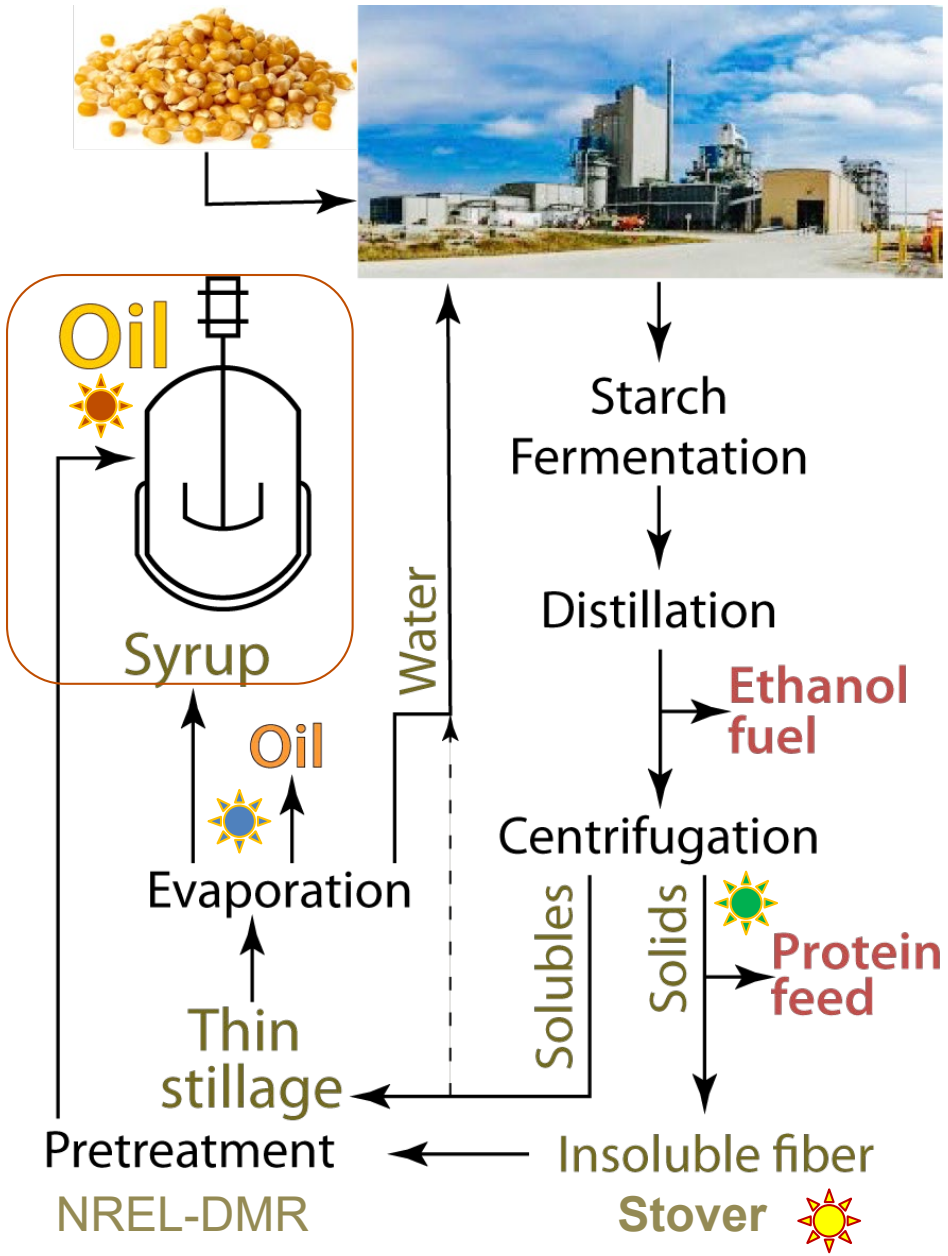
Syrup and fiber have little nutritional value




**Newer processes separate protein from fiber to increase by-product values**

Traditional dry-mill process






## **Xylome's technology** builds on newer practices to save costs while moving grain ethanol to **cellulosic biodiesel**

- Thin stillage and insoluble fiber are wet waste products from dry grain fermentations
- In newer processes **Protein** is separated from **insoluble fiber**, which increases the protein value as feed for poultry, swine and fish
- Evaporation recovers:
  - Corn oil which is used for biodiesel
  - Stillage syrup which is fermented by *Lipomyces*
- Xylome's technology will convert stillage syrup and Insoluble fiber into biodiesel
- Bioconversion of **stover** will further increase the amount of biodiesel from corn grain
- Fractionation and bioconversion will increase the overall product values and yield

## In our lab we run 2.0 to 5.0 L stirred bioreactors and 10 L bubble bottle fermentations

We developed our hyperlipogenic strain through NSF Phase I and Phase II SBIR grants

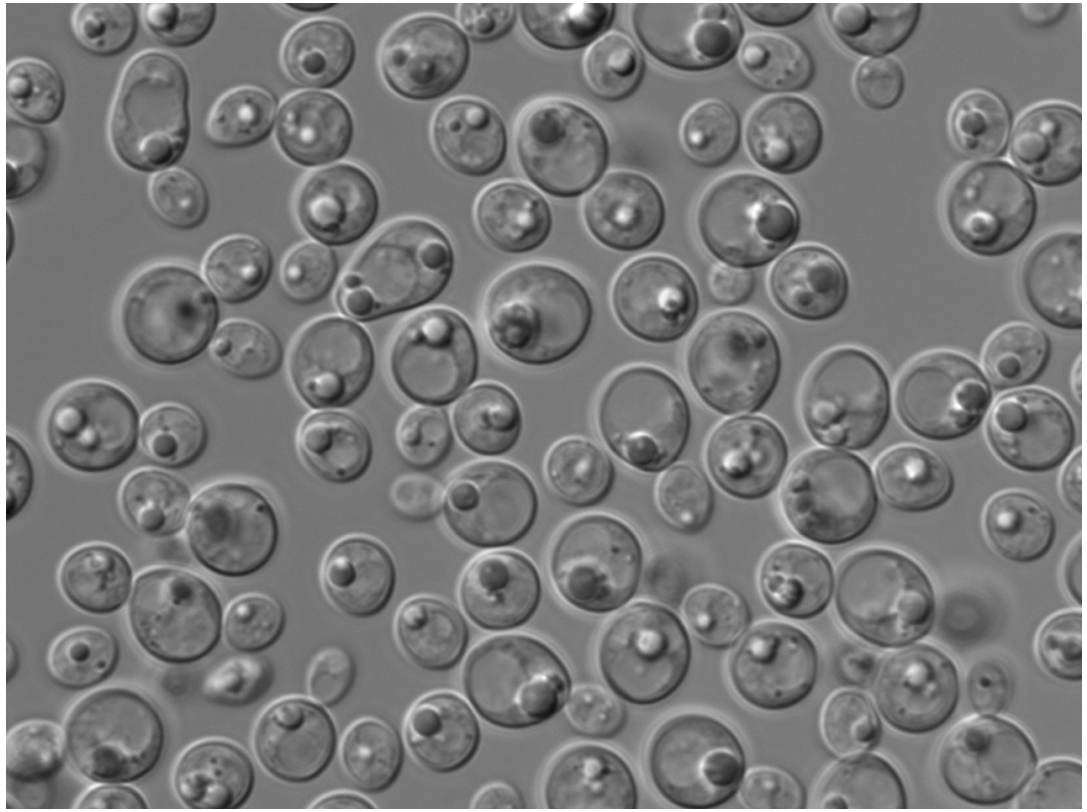
This depicts a fermentation run performed in May of 2019.

Our current yields are significantly higher

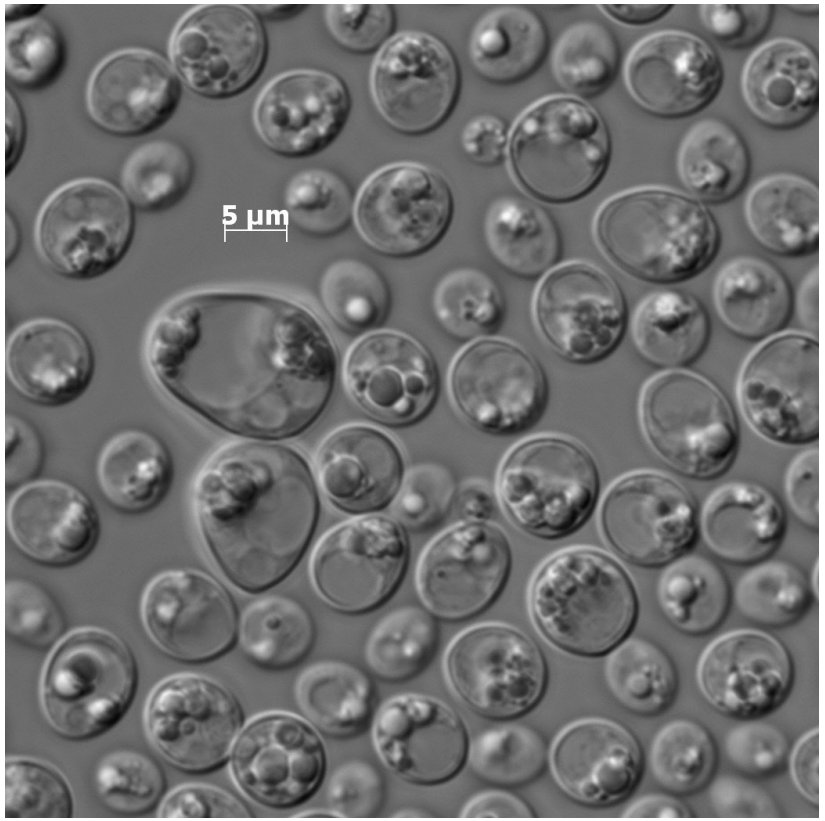
Larger volume fermentations are performed at a contract manufacturer

# Strain development resulted in significant increases in lipid production rates and yields

Wild-Type Y11557

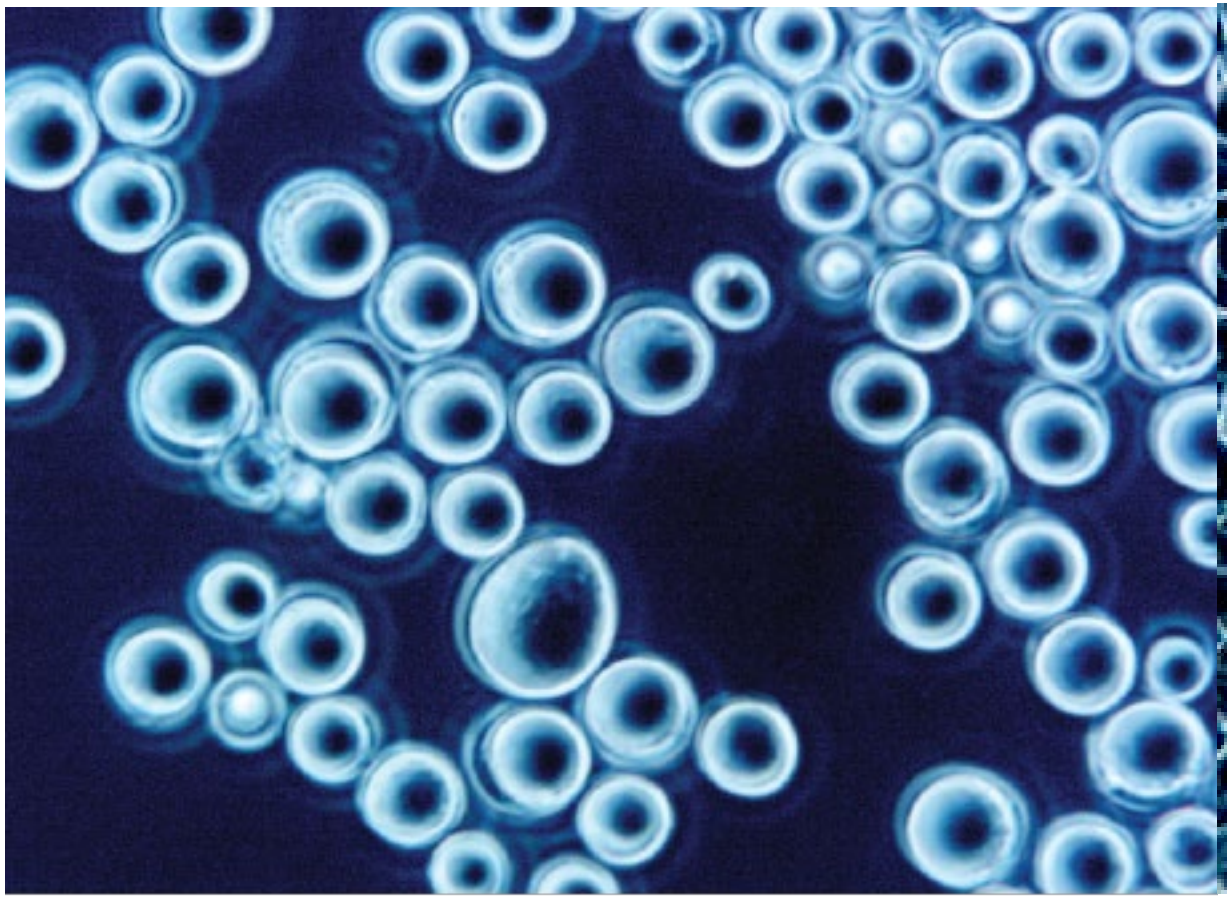


Early stage transformant

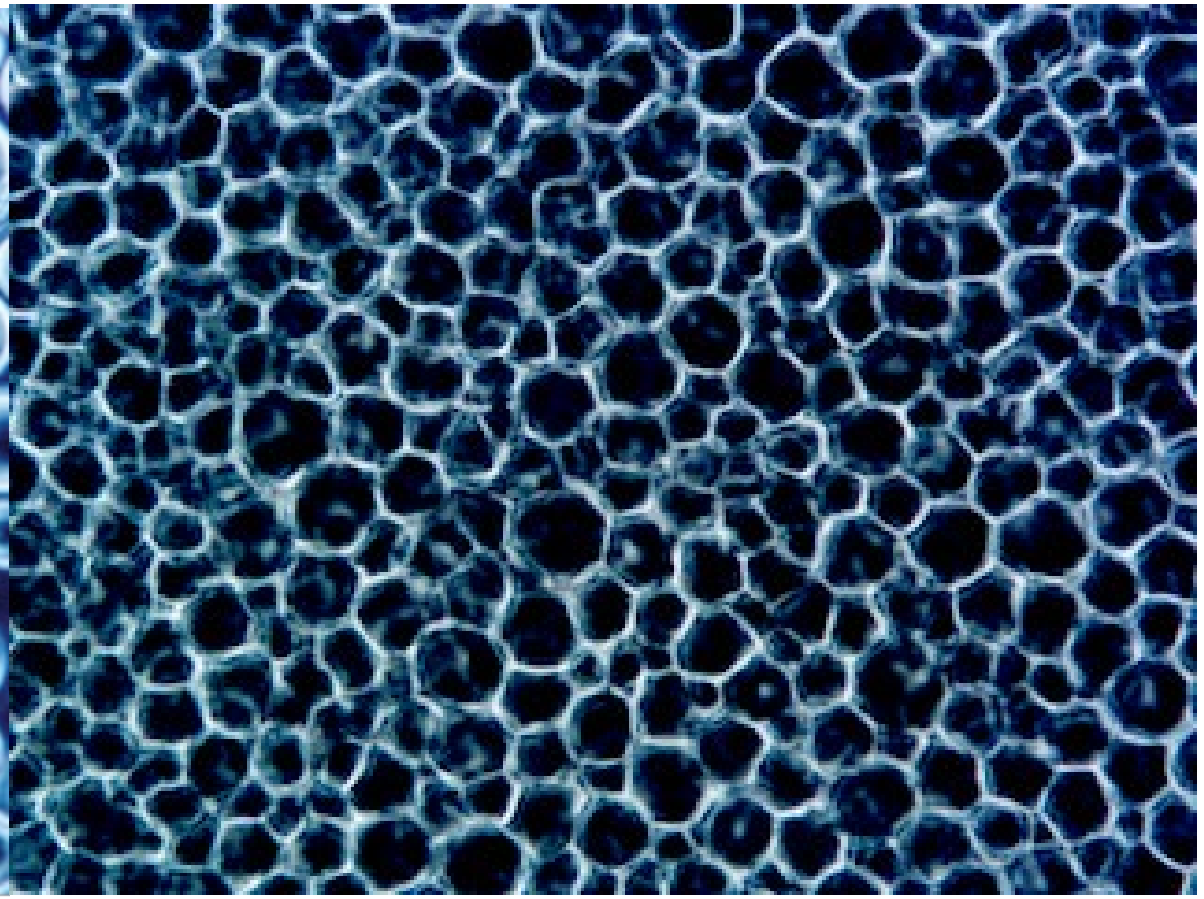


# Strain used for cellulase/xylanase expression

Hyperlipogenic strain Xyl-403 (oil)



Hyperlipogenic strain Xyl-403 post-hydrolysis



# Lipid harvested by hydrolysis

- Lipid bodies rise to the top
- Followed by washing

Wild-Type Y11557 Hyper-lipogenic

## Management Approach

- R&D objectives set by consensus of leadership.
- Twice-weekly formal and periodic informal video conferences:
  - set priorities, communicate problems, assess risks
  - review progress and discuss upcoming deadlines.
- Staff members write periodic summaries of activities and findings.
- Electronic timesheets track hours spent on different projects.
- Xylome interacts with partner organizations through calls and on-line meetings.

## Project leadership, staff and duties:

- **T. W. Jeffries, PhD – President and PI**: Manages internal affairs, accounts, proposals, and reports
- **T. J. Kelleher, PhD – CEO and CoPI**: Manages external affairs, commercialization, partnerships and investors
- **D. Z. Mokry, PhD – Director R&D**: Develops strategies, assigns tasks, establishes priorities, writes reports
- **D. T. Doering, PhD – Senior Scientist**: Molecular biology, metabolic engineering, strain development, computational and bioinformatic analysis
- **R. J. Taylor, MS – Fermentation Specialist**: Shake flask and bioreactor experiments, lipid extraction, GC and HPLC analyses
- **A. T. Zitzow, BS – Professional Support**: Clones gene targets, prepares media and reagents, curates strains, maintains supplies

## Project Goals

**Objective 1**: Genetically modify *Lipomyces starkeyi* by introducing cellulases and xylanases that can be secreted extracellularly

**Objective 2**: Demonstrate growth and yeast oil production on cellulose, hemicellulose and hydrolysates from at least three sources

**Objective 3**: Validate trials at Ethanol Producer's laboratory

**Objective 4**: Run demonstration at Ethanol Producer's pilot plant

## Research tasks (Budget Period 2)

Clone and transform into *L. starkeyi* at least

- one endoglucanase
- one endoxylanase
- one cellobiohydrolase I
- one cellobiohydrolase II

Assay the secretome of >10 strains from each transformation

Select top strains (high-throughput approaches)

Create combinations of genes by mating the top transformants from various cassettes (each with different selection marker)

Select progeny resistant to both selection markers and screen

Create subsequent yeast strains capable of liberating sugars from cellulose and hemicellulose.

Select top strains based on solubilization of a cellulose-hemicellulose substrate (at least 10%).

## Research Task

- Clone and transform into *L. starkeyi* at least
  - one endoglucanase
  - one endoxylanase
  - one cellobiohydrolase I
  - one cellobiohydrolase II
- Assay the secretome of >10 transformants from each gene
- Select top strains using high-throughput approaches
- Create combinations of genes by mating top transformants
- Mate 1st gen transformants to obtain additional improvements

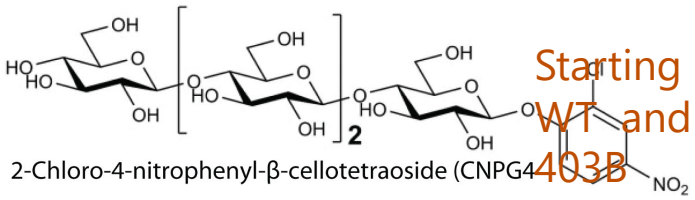
## Approach

- ***L. starkeyi* has amylases but not cellulases or xylanases**
  - Selected enzymes with known activities and performance
  - Screened and used *L. starkeyi* known secretion signals
  - Used *L. starkeyi* promoters known to work well
  - Tested constructs with fluorescent protein markers
- **Developed sensitive high-throughput micro-titer assays**
  - Cultivated individual transformants in small volumes
  - Employed 96 well fluorescent and absorbance screening
- **Used proprietary mating and screening techniques**
  - Identified and selected 1st generation transformants
  - Identified and selected 2nd and higher generation hybrids
- **Repeat process starting with highly lipogenic strains**

Transformation of highly lipogenic XYL403 with cellulase yields a range of activities

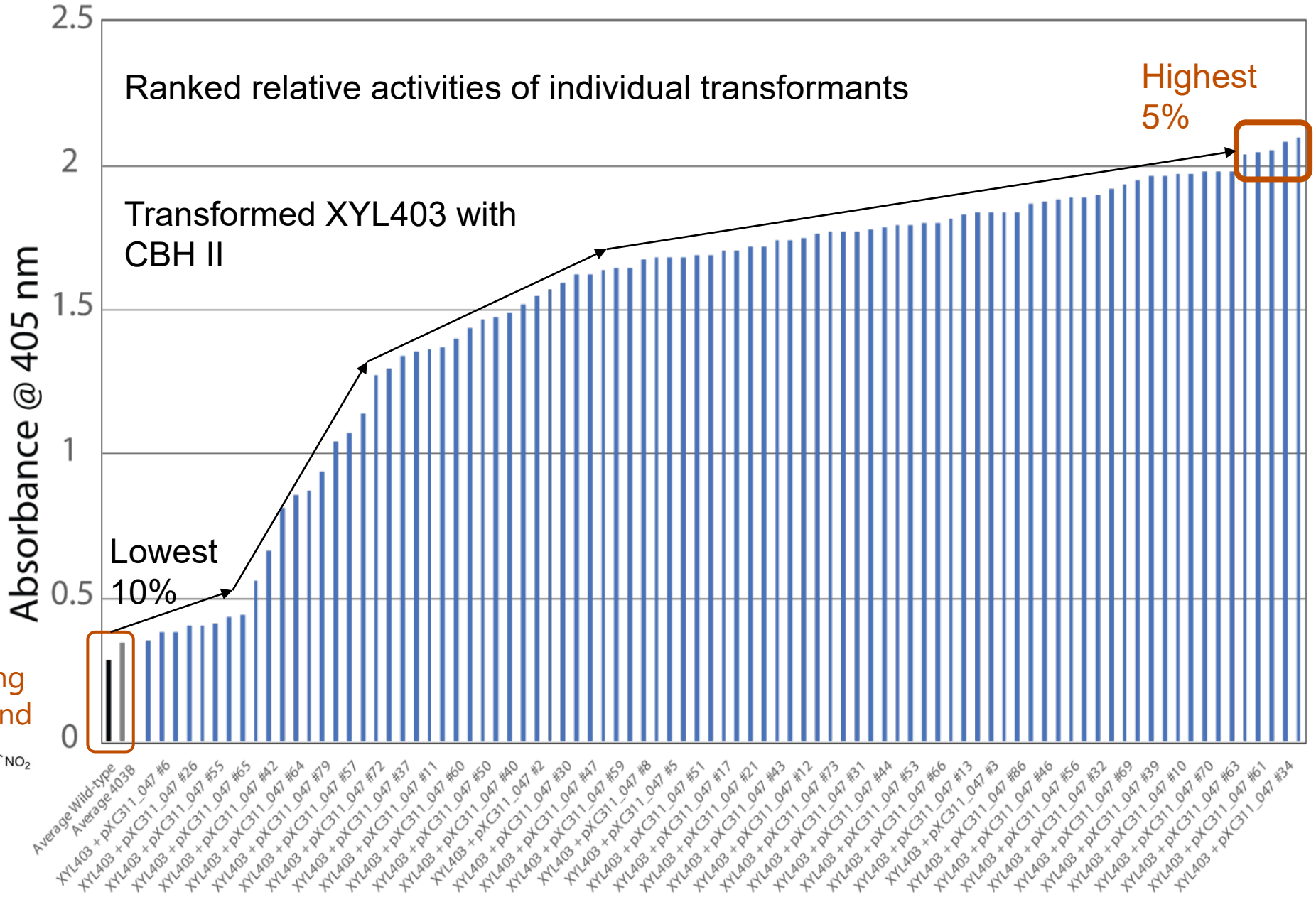
We take the best for our next round of strain development

We screened 88 transformants with CNPG4




2-Chloro-4-nitrophenyl-β-cellotetraoside (CNPG4

Starting WT and 403B



# Hyper-lipogenic XYL403 strain yields higher cellulase activities than Y-11557 (WT) when transformed with CBH2

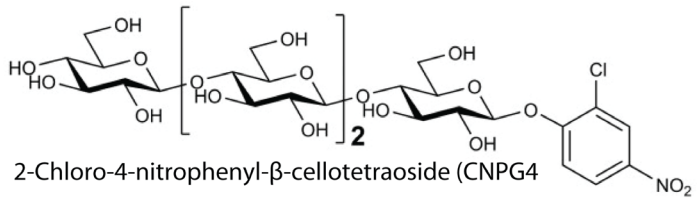
Y11557 (WT)

+CBH2: 93 transformants

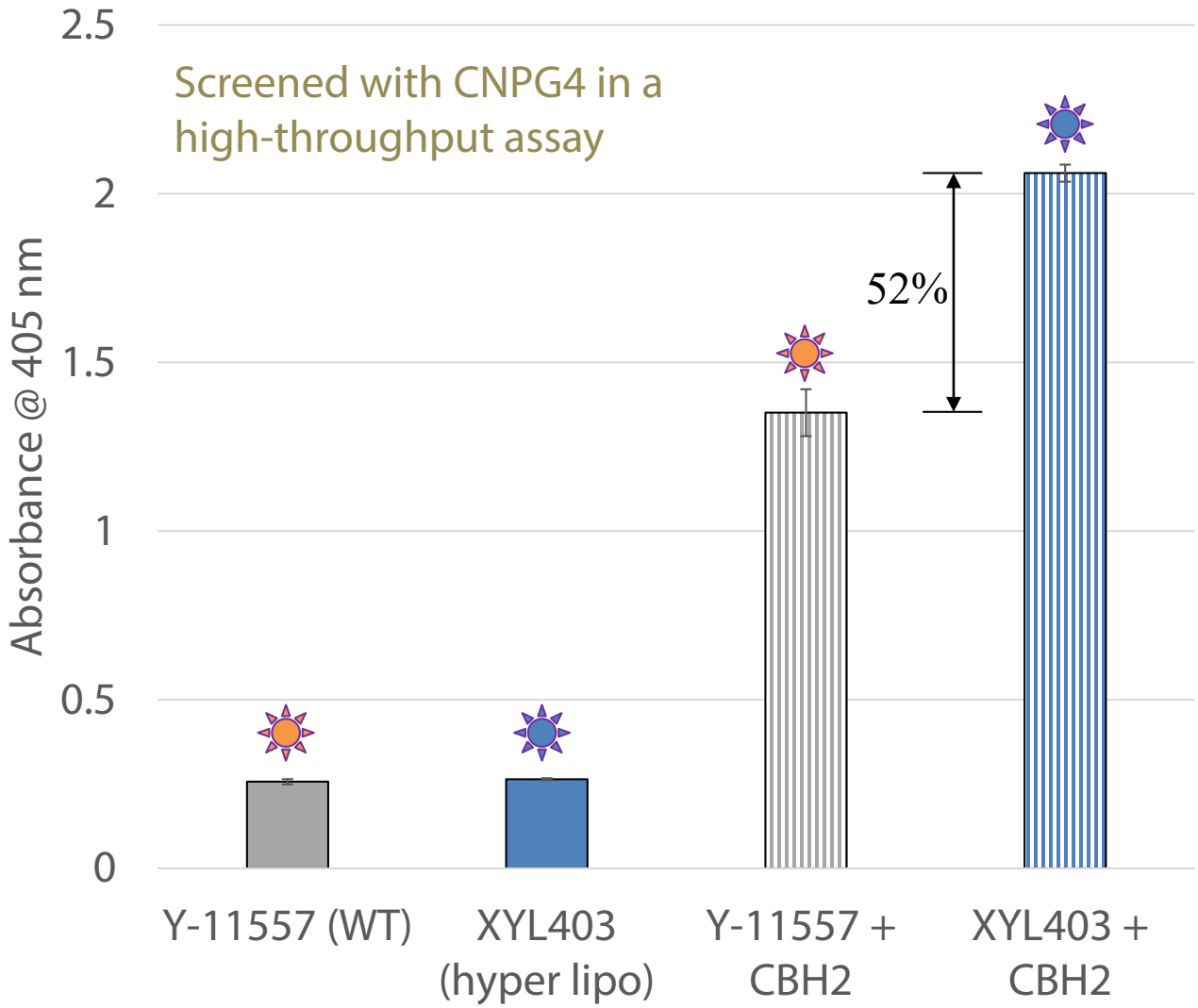
Xyl403 (hyper-lipogenic)

+CBH2: 88 transformants

Average activity observed with the top 5 transformants from each



2-Chloro-4-nitrophenyl-β-cellotetraoside (CNPG4



# **Objective 2**: Demonstrate growth and yeast oil production on cellulose, hemicellulose and hydrolysates from at least three sources

## Task

- Select top strains that can solubilize >10% of a cellulose or hemicellulose substrate

## Potential Challenges Approach

- **Model substrates**
  - Don't necessarily predict activity on actual substrates
  - Kinetics and yields with single enzymes often differ from those of enzyme cocktails

## Strategy

- **Select top strains that will solubilize >10%**
  - Primary screens based on model substrates
  - Secondary screens based on pretreated lignocellulosics
  - Final screens based on industrial substrates
- **Quantitative saccharification trials require**
  - Better control over culture conditions
  - Larger volumes of enzyme preps
- **Quantitative saccharification assays require**
  - A range of dilutions for each prep
  - Timed samples for each prep
  - Quantitative assays based on sugar release

## Research Tasks

- Clone and transform into *L. starkeyi* at least
  - 1 endoglucanase
  - 1 endoxylanase
  - 1 cellobiohydrolase I
  - 1 cellobiohydrolase II
- Assay the secretome of >10 transformants from each gene
- Select top strains using high-throughput approaches
- Create combinations of genes by mating top transformants
- Mate 1st gen transformants to obtain additional improvements

# Xylome has exceeded its goals for cloning, transforming and screening

- Cloned, transformed and expressed
  - 4 endoglucanases (GH 5 and 12 from three fungi)
  - 3 exo-cellobiohydrolase I (GH 7 from two fungi)
  - 2 exo-cellobiohydrolase II (GH 6)
  - 5 endo xylanases (GH 10 and 11)

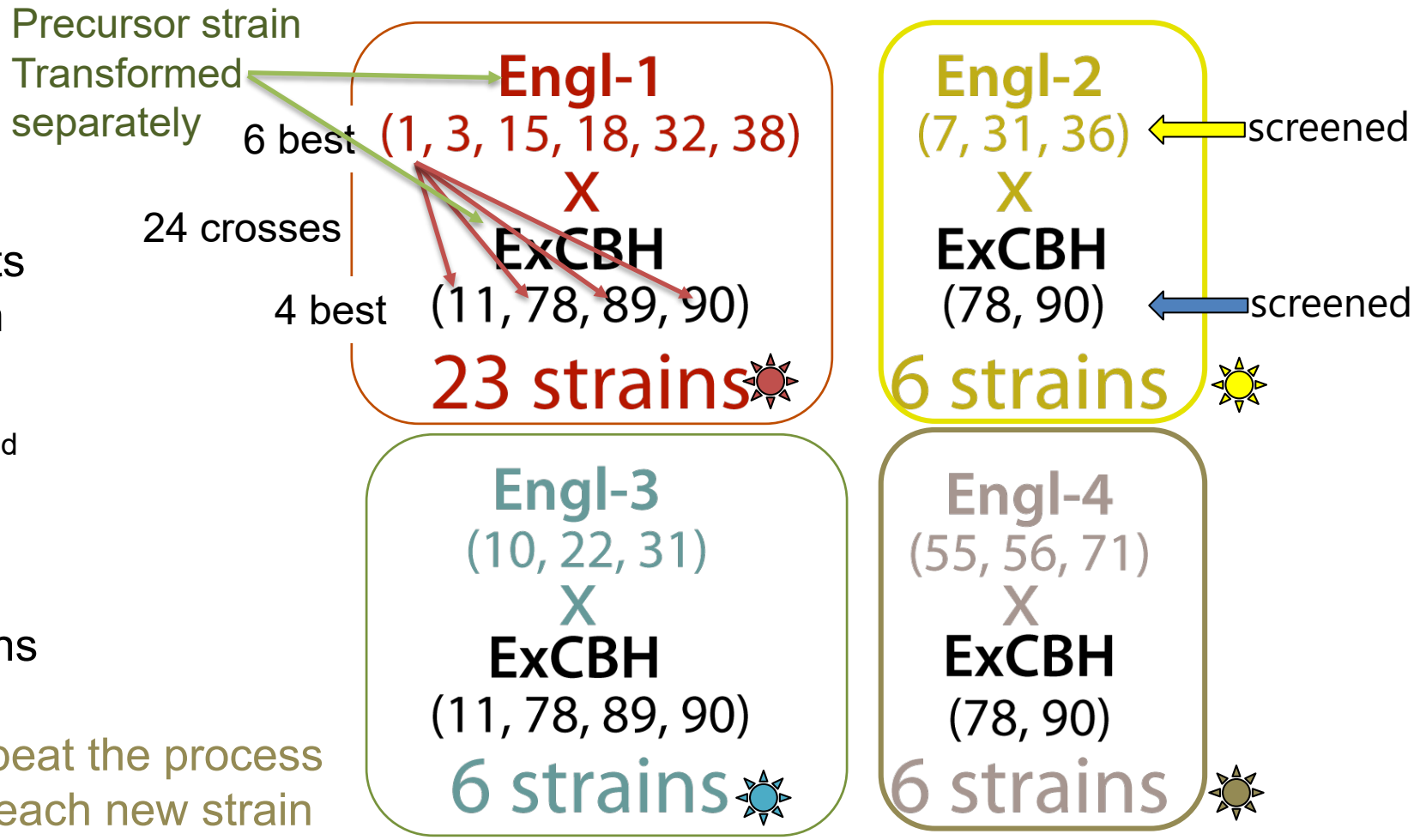
**More genes to come**

- Typically screened 40 to 100 transformants with gene
- Selected the top strains for mating using both
  - High throughput and
  - Cellulosic and hemicellulosic substrates

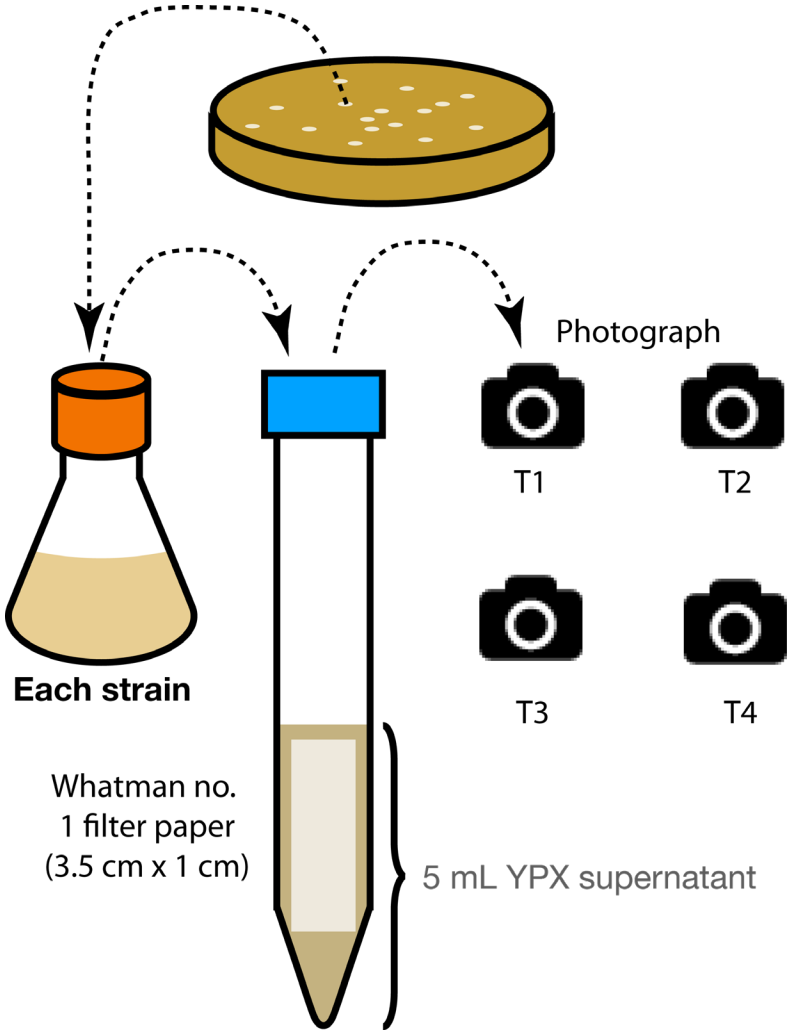
# Research Tasks

- Create combinations of genes by mating top transformants
- Mate 1st gen transformants to obtain improvements in 2nd gen hybrid strains
- Screen and select best 2nd gen hybrids
- Cross with the best from other 2nd gen hybridizations

# Xylome has made numerous crosses among top transformants



# We have carried out qualitative filter paper assays




**Engl-1**
(1, 3, 15, 18, 32, 38)
X
**ExCBH**
(11, 78, 89, 90)
23 strains

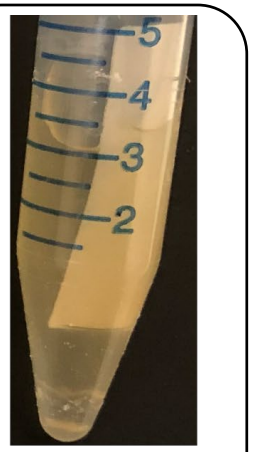
**Engl-2**
(7, 31, 36)
X
**ExCBH**
(78, 90)
6 strains

**Engl-3**
(10, 22, 31)
X
**ExCBH**
(11, 78, 89, 90)
6 strains

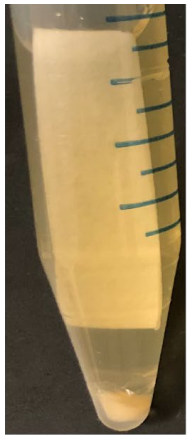
**Engl-4**
(55, 56, 71)
X
**ExCBH**
(78, 90)
6 strains

# Filter paper results with crossed strains (2nd Gen examples)

- 2nd Gen strains were cultivated
- Photos show fragmentation observed with enzyme from specific strain crosses
- The Wild-Type (Y-11557) was inactive
- Various crosses showed a range of filter paper activities
- Some crosses broke down the filter paper extensively

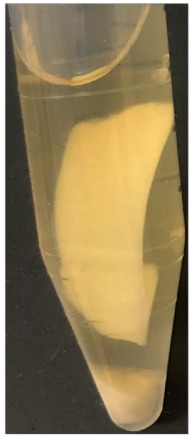



*L. starkeyi*
NRRL Y-11557

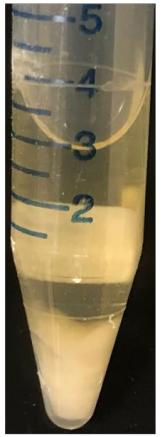


Engl2
x
ExoCBH4

Engl4
x
ExoCBH4



Engl3
x
ExoCBH4

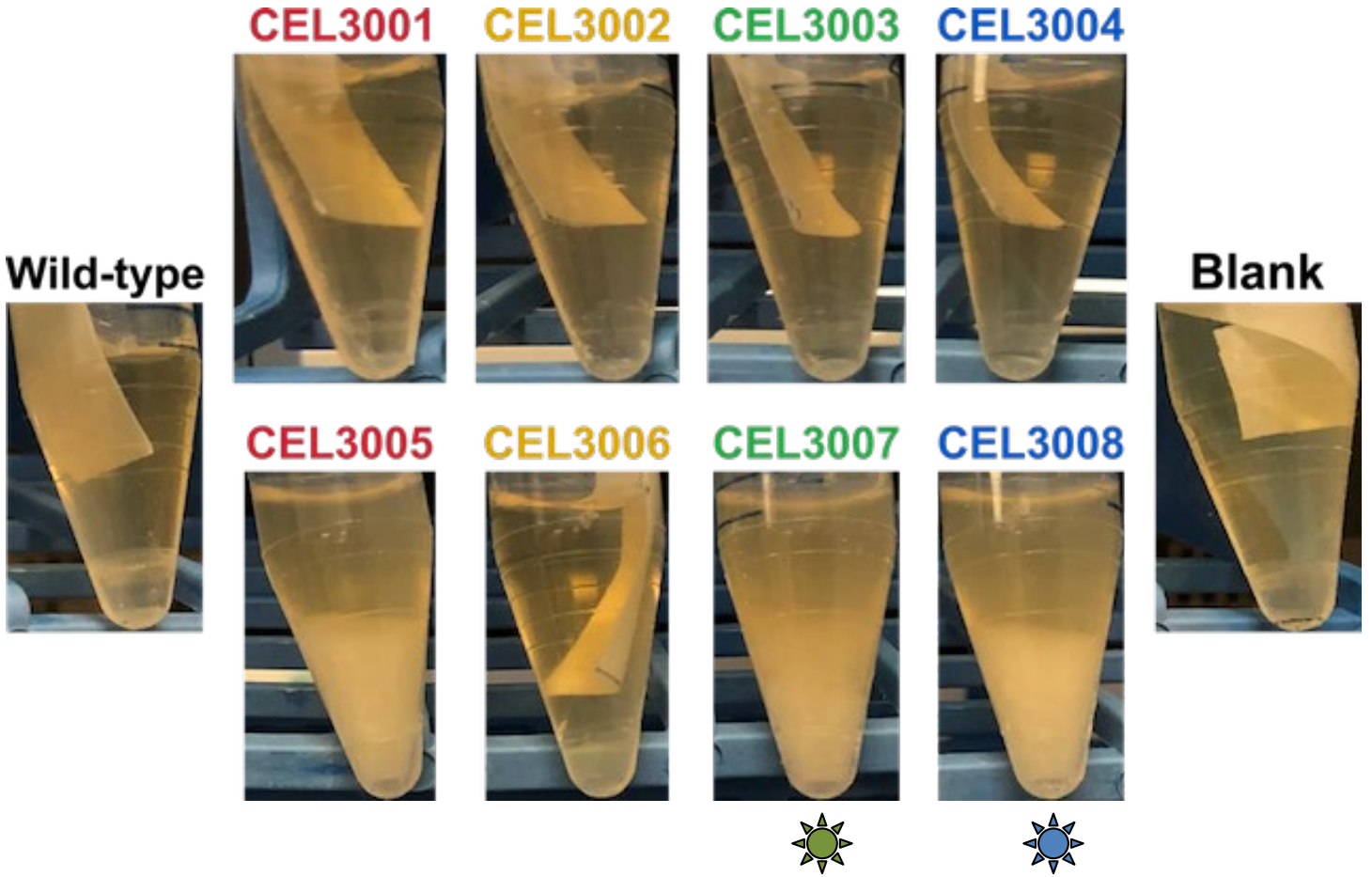



Engl1.1
x
ExoCBH4.1

Engl1.1
x
ExoCBH4.2

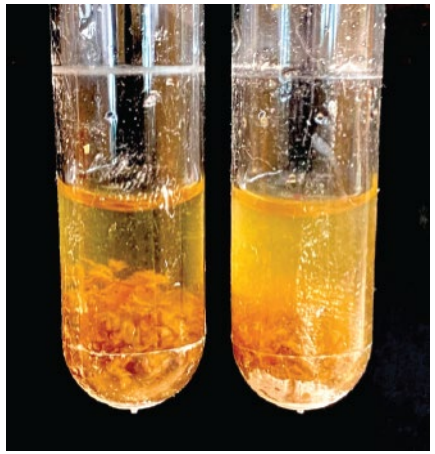
# The effectiveness of 3rd gen transformants depended on the combination of enzymes used

- Activities of the best strains transformed with CBHII plus a hybrid CBH1 and an endo-glucanase varied with the enzyme source.
- CEL3007 and CEL3008 performed the best with filter paper
- We introduced an endoxylanase into CEL3008 and screened for the best transformants

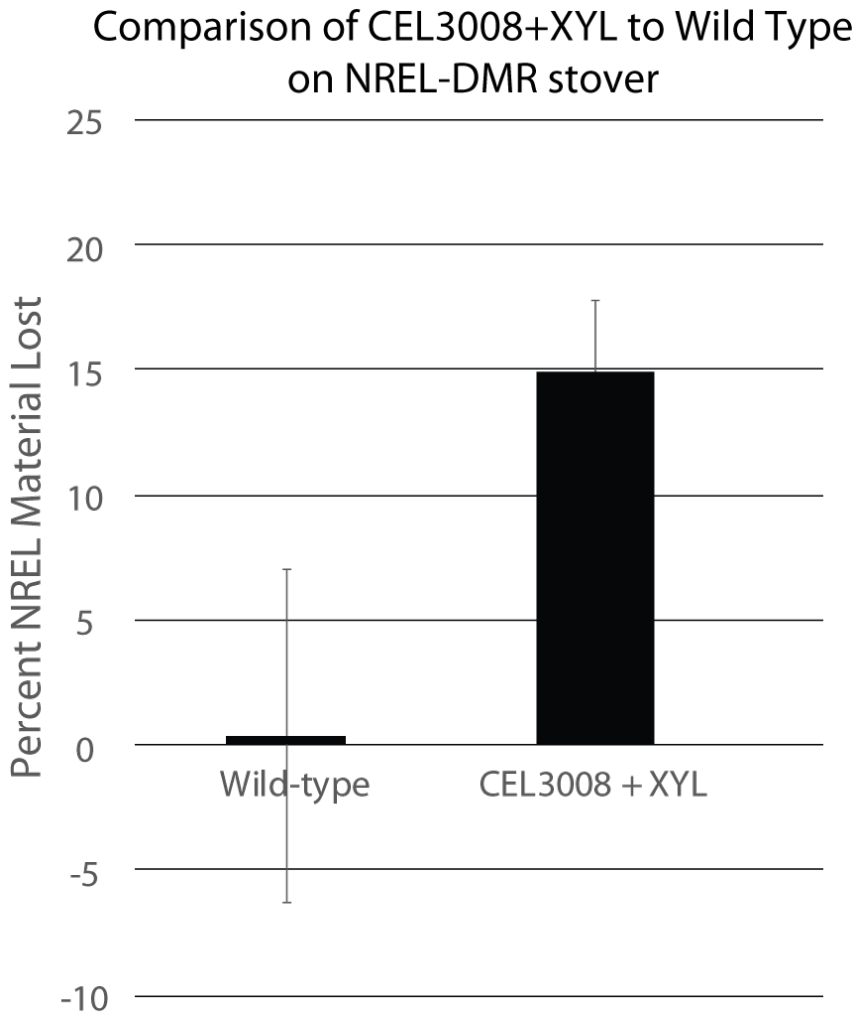


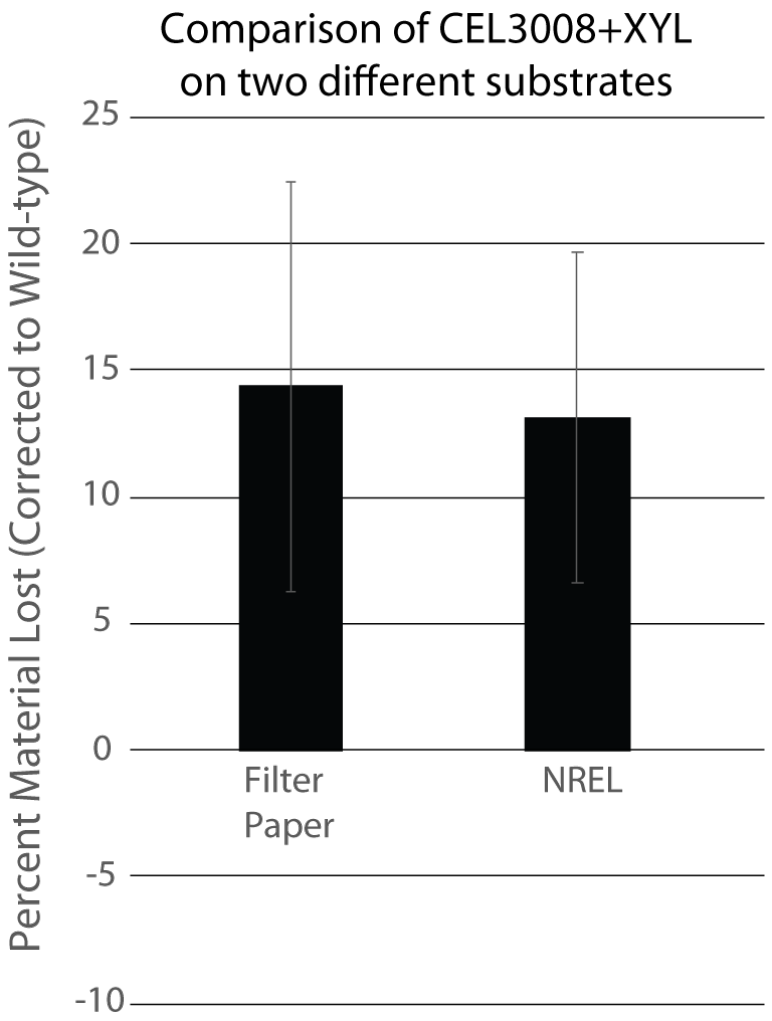
# We compared our best cellulolytic strain to the wild-type and assayed for ability to solubilize two different substrates

Wild-type | CEL3008 +XYL



NREL-DMR

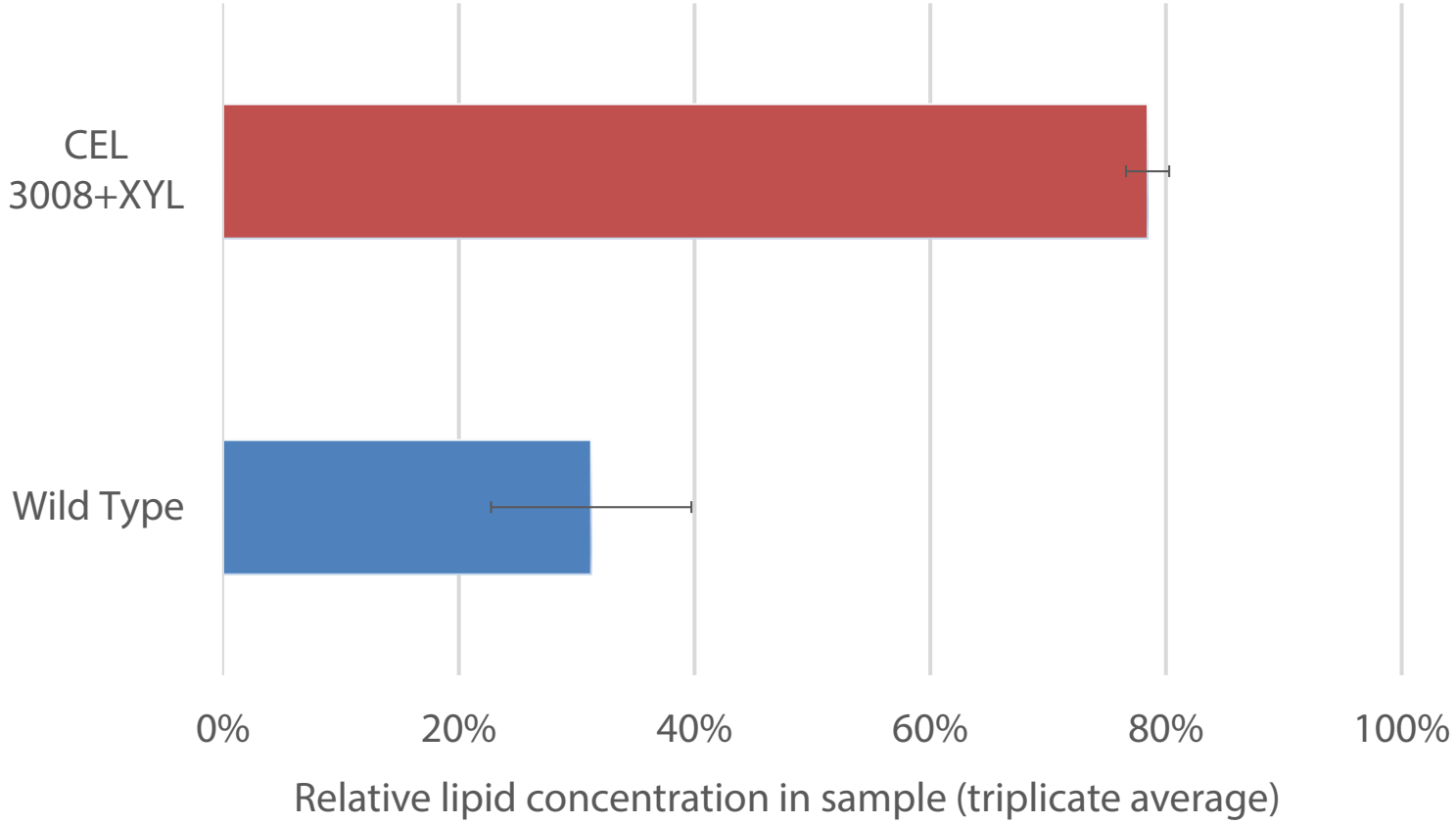


# We compared the Y-11557 strain with CEL3008+Xyl on stillage fiber in shake flasks

- Compared lipid accumulation on stillage fiber between
  - *L. starkeyi* Y-11557 (WT)

  and
  - *L. starkeyi* CEL3008 (cellulolytic)
- Subtracted oil in the substrate
- Observed increase in oil production of 47% when cultivated on stillage fiber solids



# Summary

- Xylome is nearly through Phase 2 and half-way through its overall budget
- We have exceeded our planned targets for strain construction
- We have achieved Phase-2 Go / No-Go goal for
  - Lipid accumulation from fiber
  - Solubilization of cellulose and hemicellulose
- In the next few months, we expect to
  - Transfer additional cellulases, xylanases and LPMO's into our best lipogenic strains
  - Re-transfer enzymes that have worked well
  - Further enhance secretion
  - Run 3-L bench trials in the lab
  - Begin to plan for commercial scale up with partners

# Quad Chart Overview: Biodiesel and higher value products from stillage fiber

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We plan to accomplish this by engineering hyper-lipogenic strains of *L. starkeyi* to express and secrete cellulases and xylanases.

### End of Project Milestone

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We are currently in Phase 2

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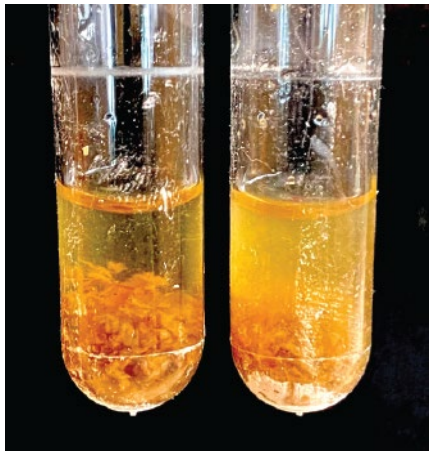


NREL-DMR