

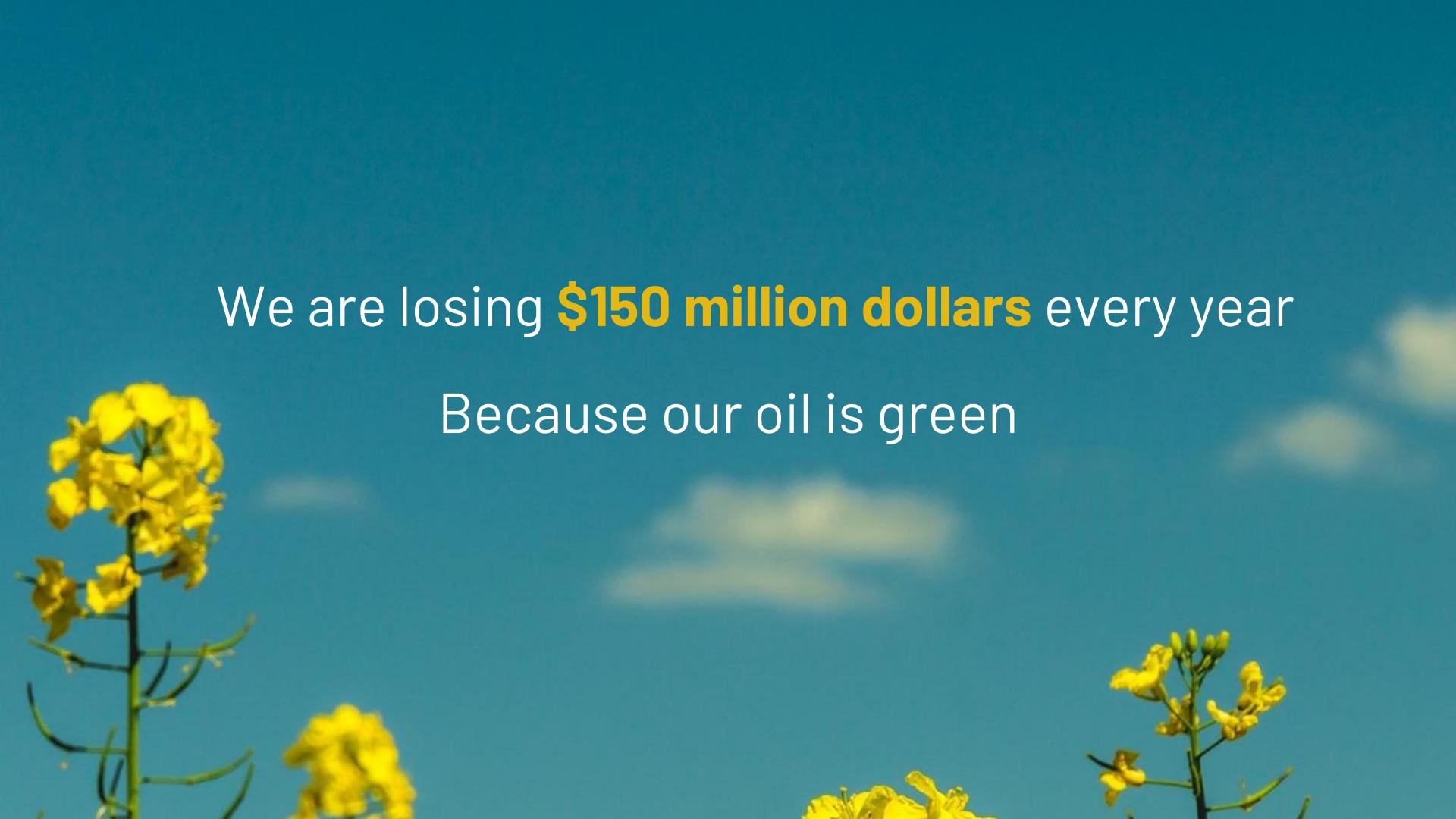
iGEM  
2019  Calgary

yOIL

An all-encompassing solution to the green seed  
problem

A photograph of a field of yellow canola flowers against a clear blue sky. In the foreground, several green stems with clusters of bright yellow flowers are visible. The background is a vast, clear blue sky with a few wispy white clouds.

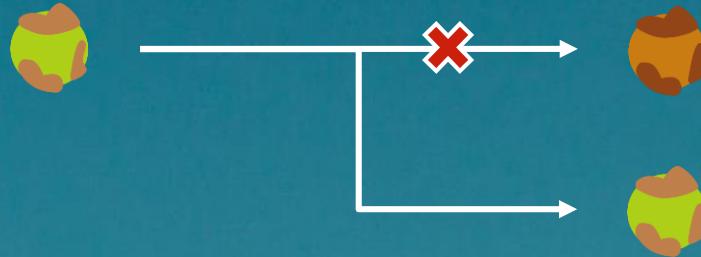
Canada is the world's largest producer and  
exporter of canola oil

The background of the image is a photograph of a field of yellow canola flowers (rapeseed) under a clear, bright blue sky. The flowers are in sharp focus in the foreground and middle ground, while the sky is a soft, out-of-focus teal.

We are losing **\$150 million dollars** every year

Because our oil is green

# The Green Seed Problem



Chlorophyll in Oil

Faster  
Spoilage

Bitter  
Taste

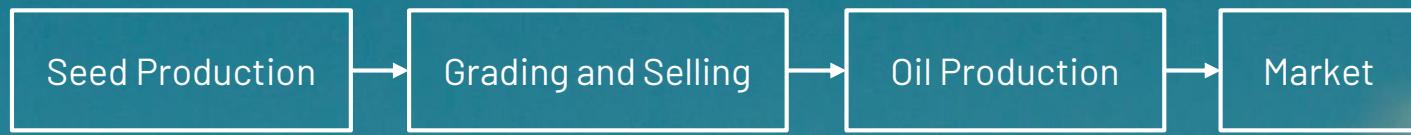
Lower Smoke  
Point



An all-encompassing solution to the green seed  
problem



# The Green Seed Problem



# The Green Seed Problem

Oil Production

# Oil Producers

Extra Processing: Acid Activated Clay



Green      Clay  
oil            method

# Oil Production

**Up to 20% of Oil  
Lost**



**Negative  
Environmental  
Consequences**

"Clay is the most **expensive** input cost"

-Dallas Gade  
Project Manager, Richardson Oilseed

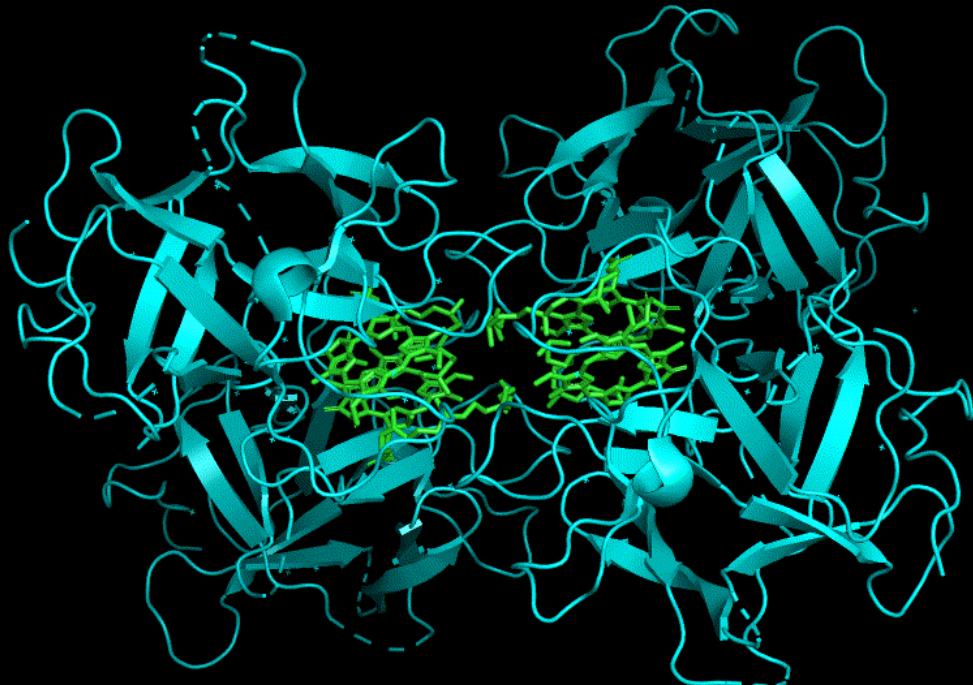
A photograph of a vast field of yellow rapeseed plants under a clear blue sky with scattered white clouds. The plants are in full bloom, creating a dense sea of yellow against the blue.

**Synthetic biology** can improve green oil processing

# Our Solution

6GIX: Water Soluble Chlorophyll Binding Protein

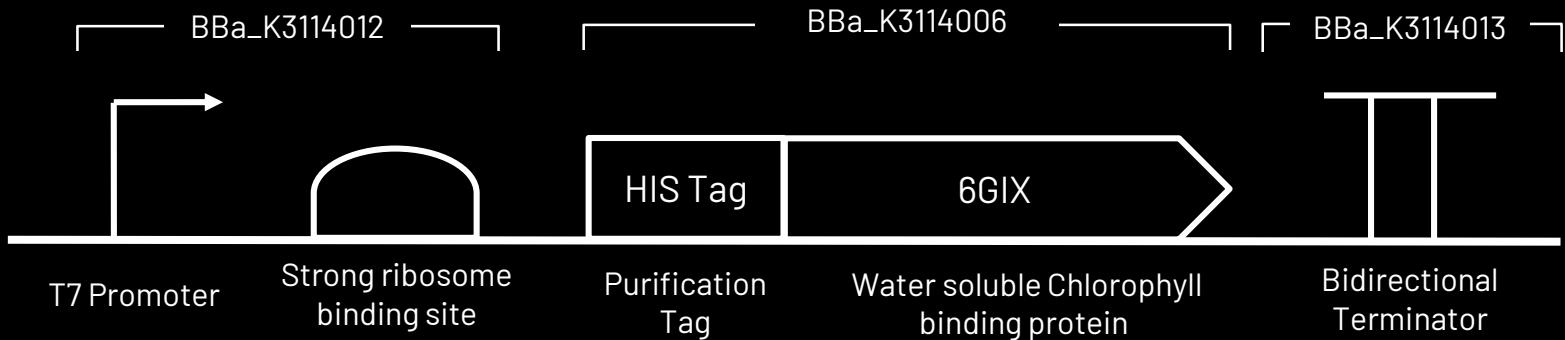
- 1) Binds four chlorophyll molecules
- 2) Protein-chlorophyll complex removed
- 3) Pure yellow oil



Bednarczyk and Noy, 2016

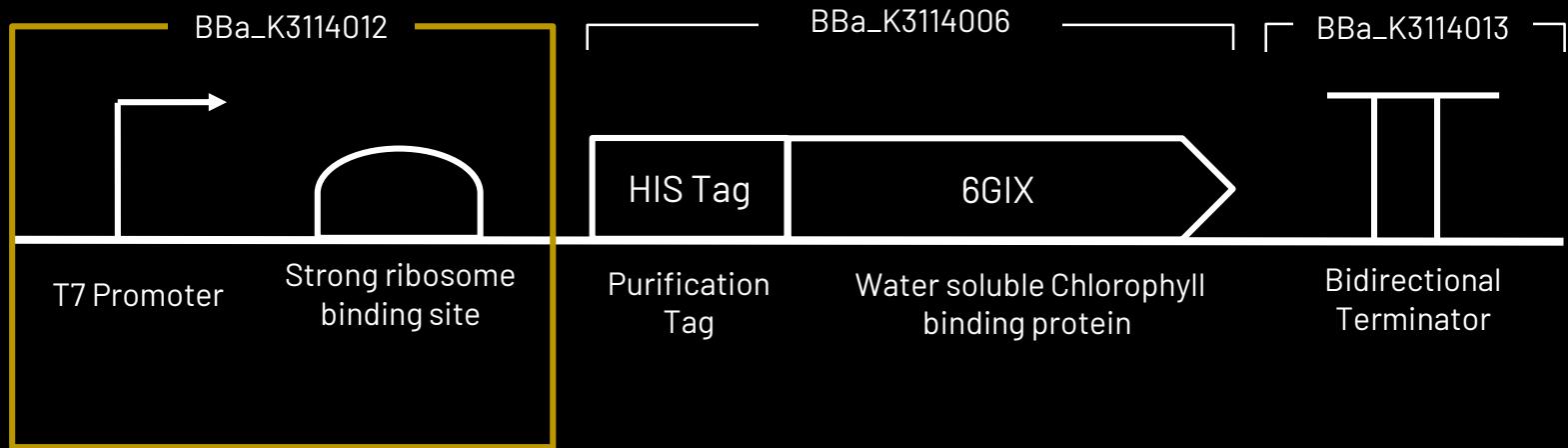
10.2210/pdb6GIX/pdb

# Synbio for Green Seed Processing

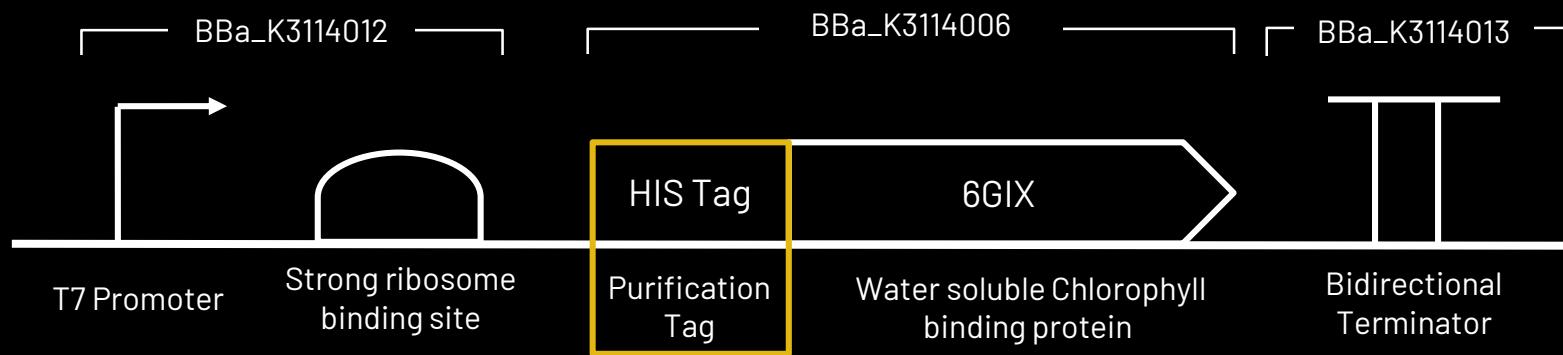


BBa\_K3114015 allows golden gate cloning  
(Improved BBa\_K1467400)

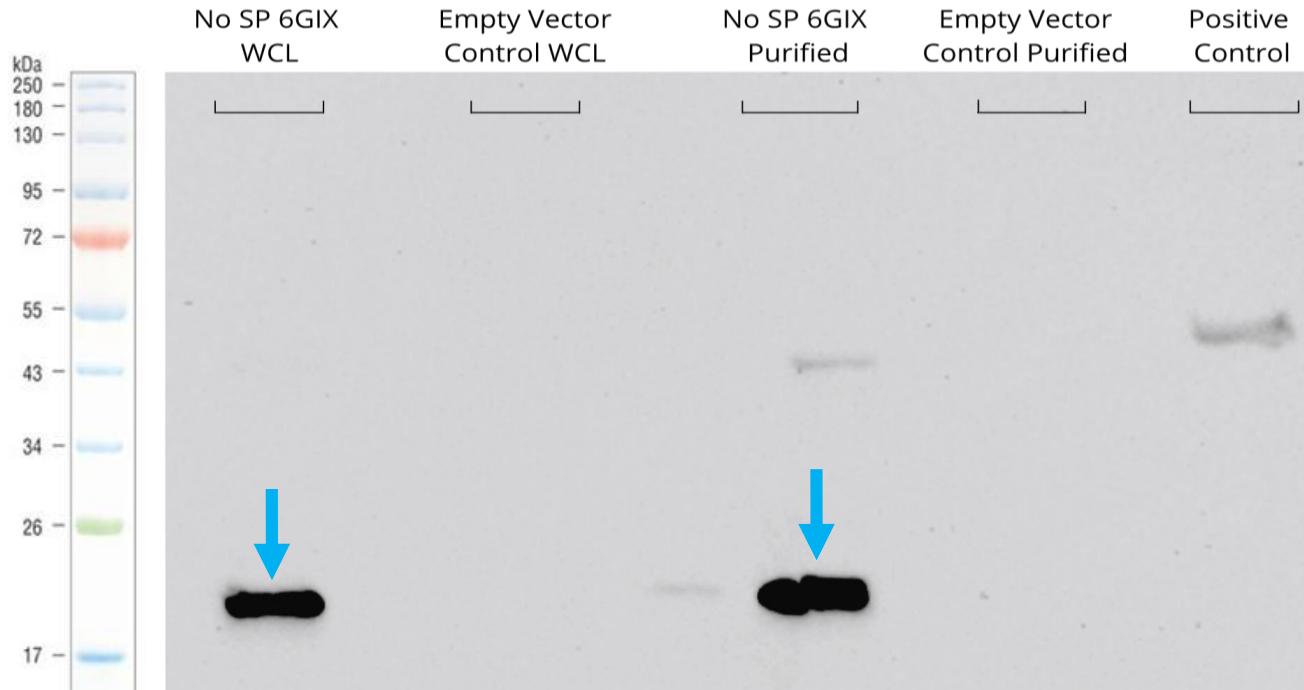
# Synbio for Green Seed Processing



# Synbio for Green Seed Processing



# Purification of 6GIX



We SUCCESSFULLY expressed and purified 6GIX from the Cell Lysate

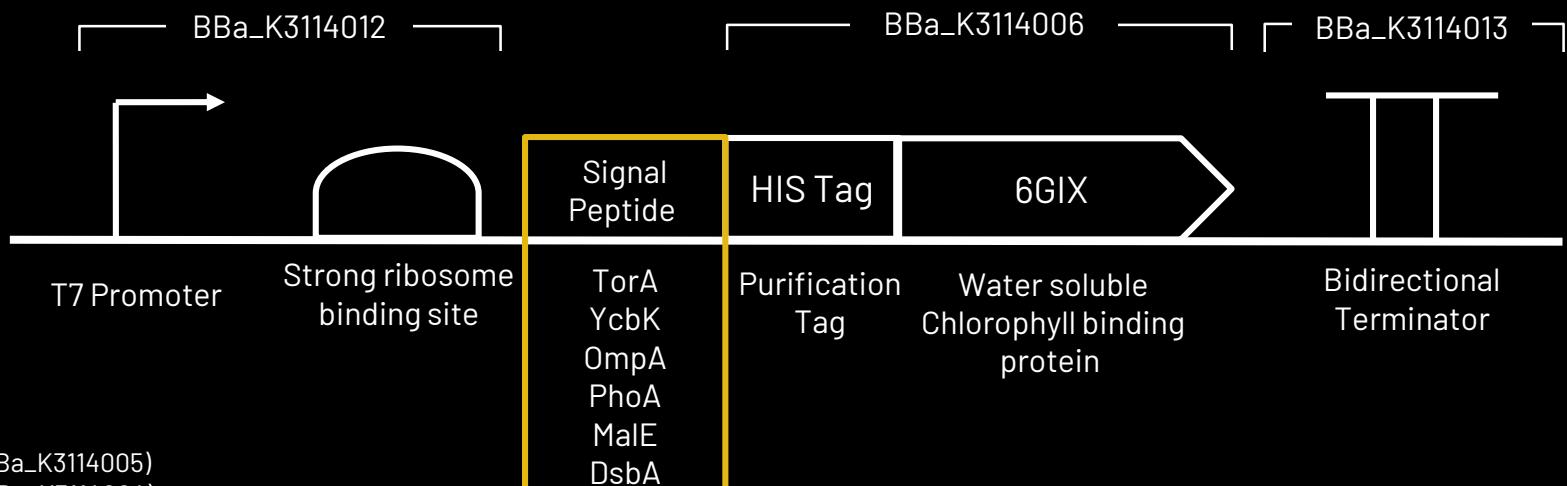
# How can we **minimize costs** spent on purification?



Dr. Raymond Turner

University of Calgary  
Biochemistry Faculty  
Professor

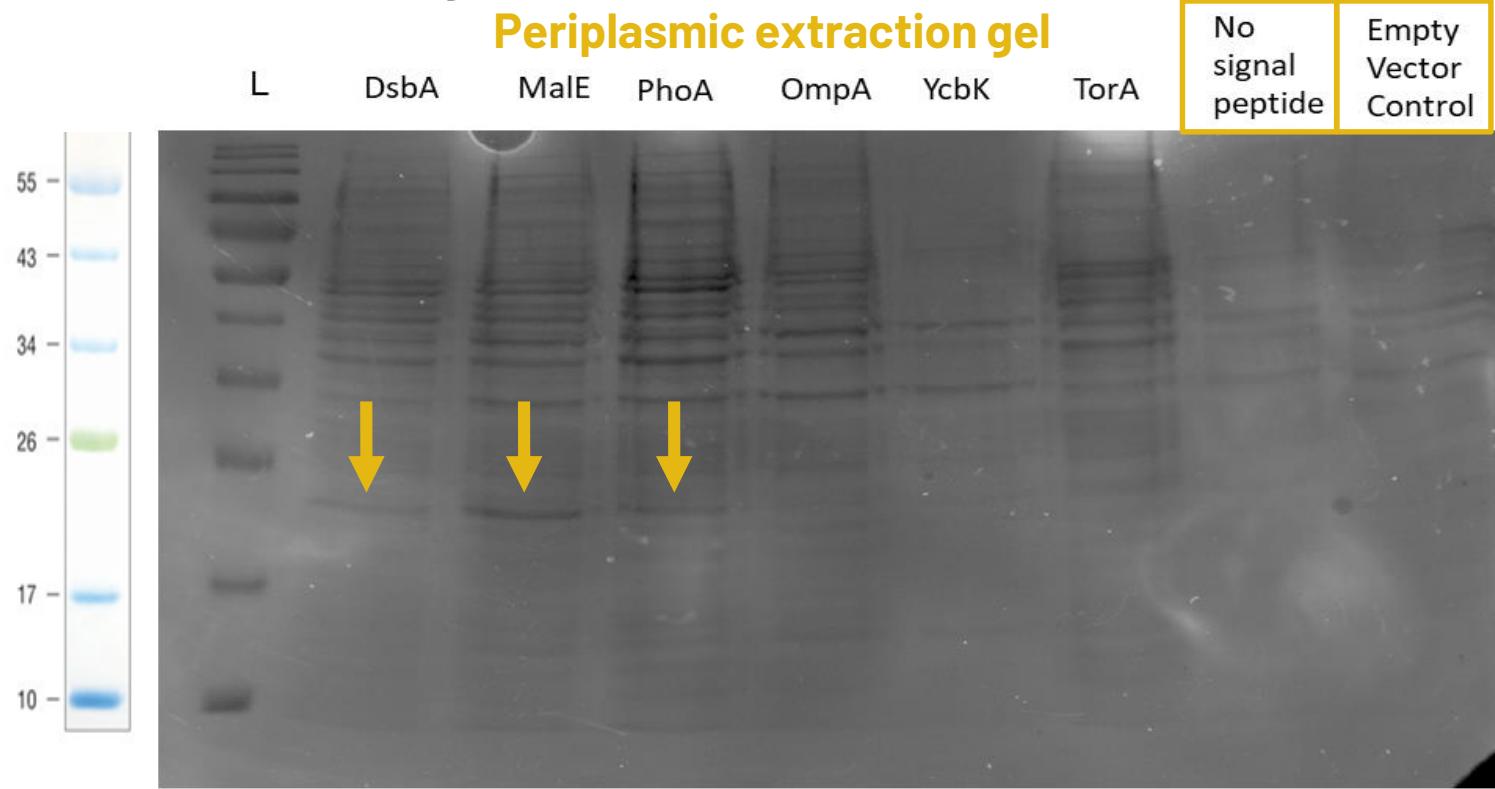
# Synbio for Green Seed Processing



TorA: (BBa\_K3114005)  
YcbK: (BBa\_K3114004)  
OmpA: (BBa\_K3114002)  
PhoA: (BBa\_K3114003)  
MalE: (BBa\_3114001)  
DsbA: (BBa\_K3114000)

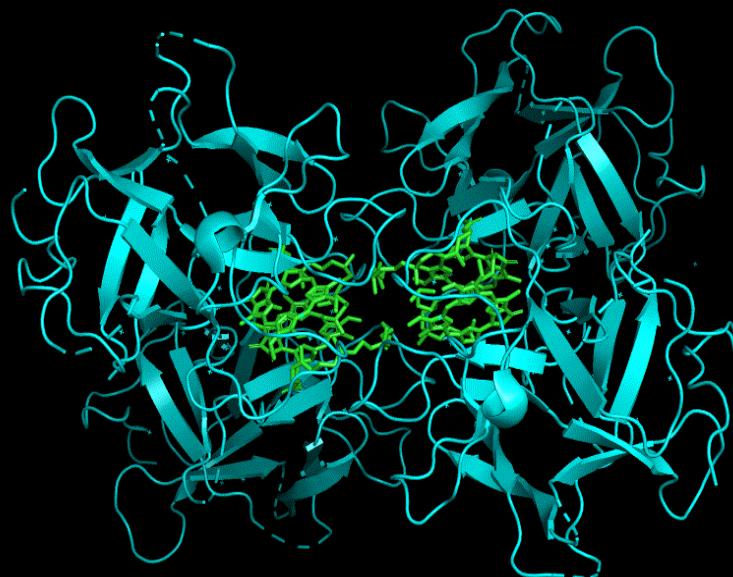
# Signal Peptide Results

Periplasmic extraction gel



PhoA, MaIE, and DsbA signal peptides SECRETE 6GIX into periplasm

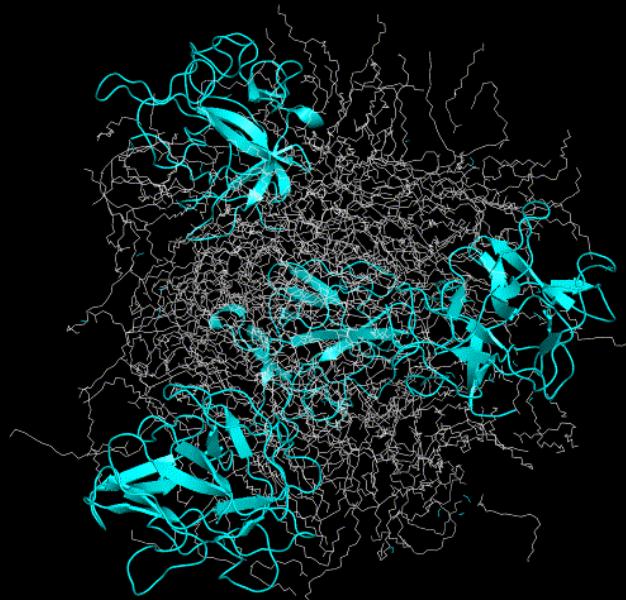
# Hydrophobic Effect



6GIX normal structure

# Hydrophobic Effect

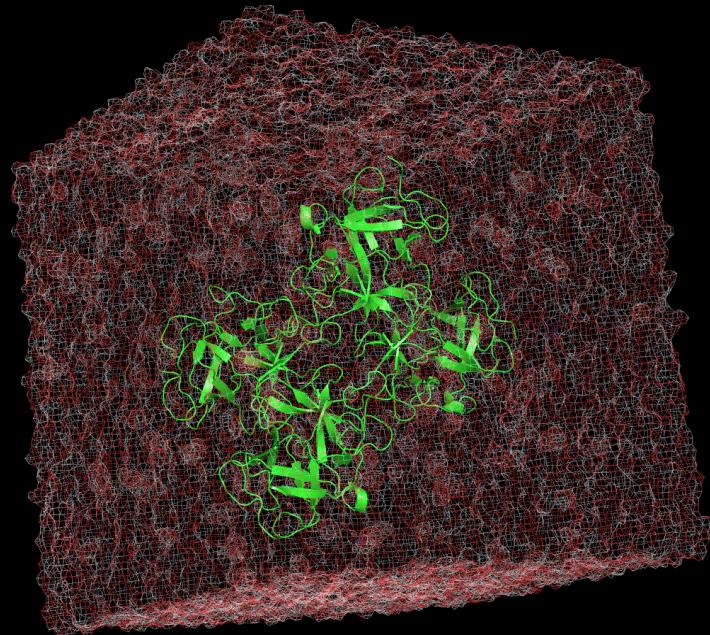
Water soluble proteins don't work in oil!!!!!!



6GIX denatured structure

# Our Solution

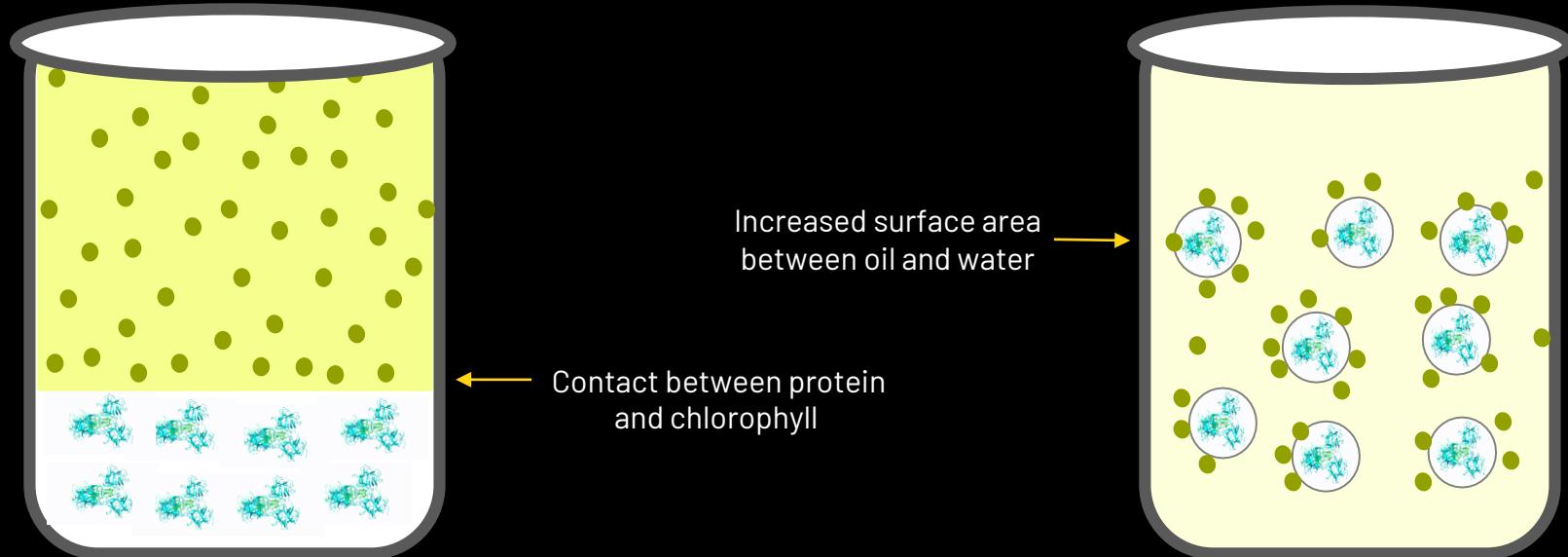
6GIX in Aqueous Phase



6GIX in water droplet

# Our Solution

Oil-in-Water Emulsion

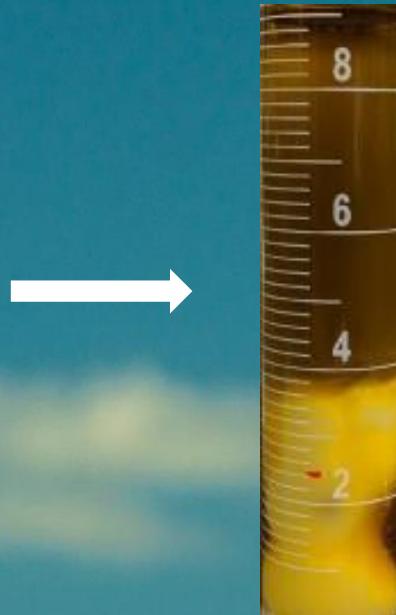


# Emulsion Types

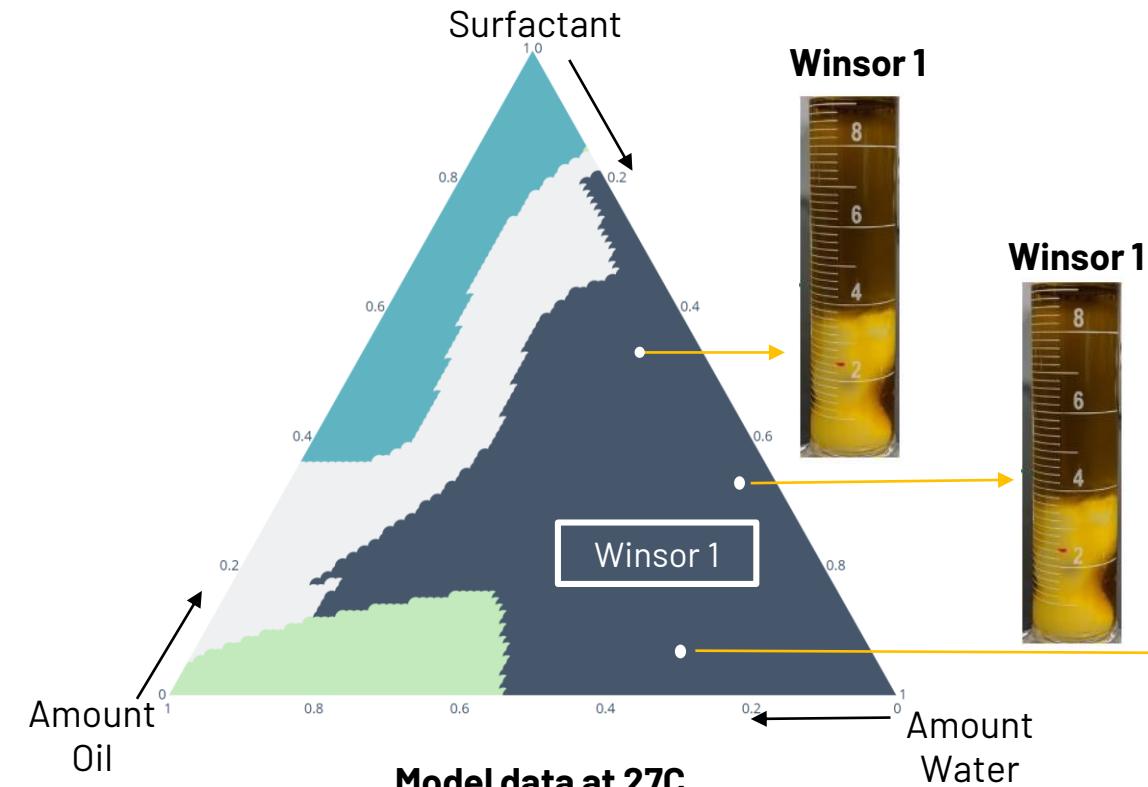
Different combinations of phases give different emulsions



Winsor 1



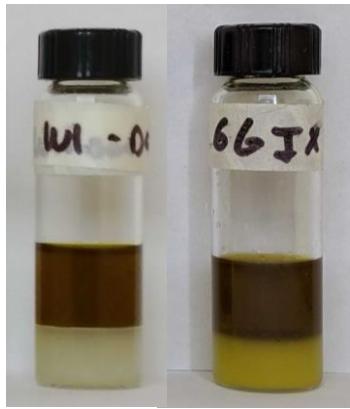
# Phase Diagram Models



Proof of Concept:  
BSA Experiments

# 6GIX Emulsion Results

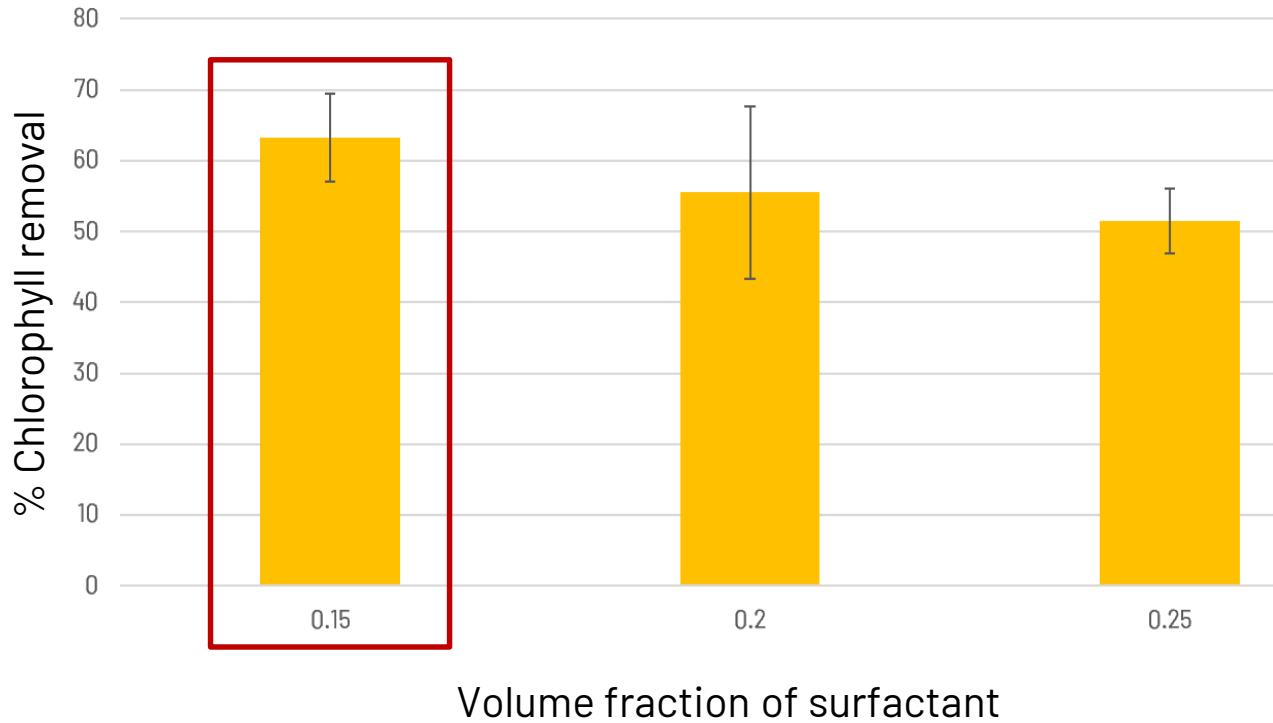
## Three different emulsion compositions



No protein  
Emulsion

6GIX  
Emulsion

6GIX Concentration= ~5uM

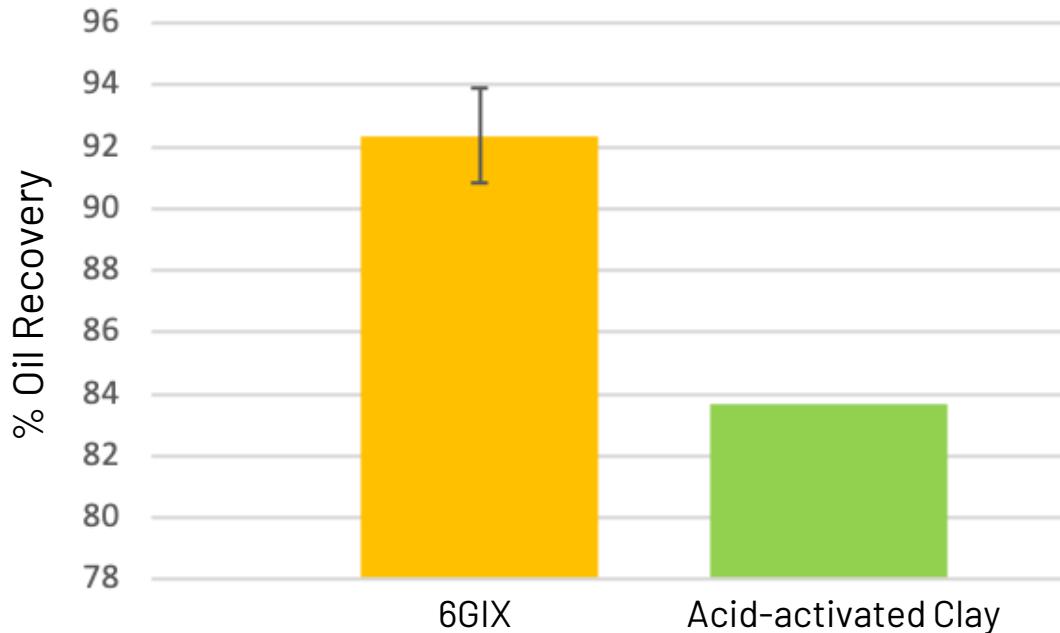


# Oil Recovery

## How much oil is lost through processing



6GIX  
Emulsion

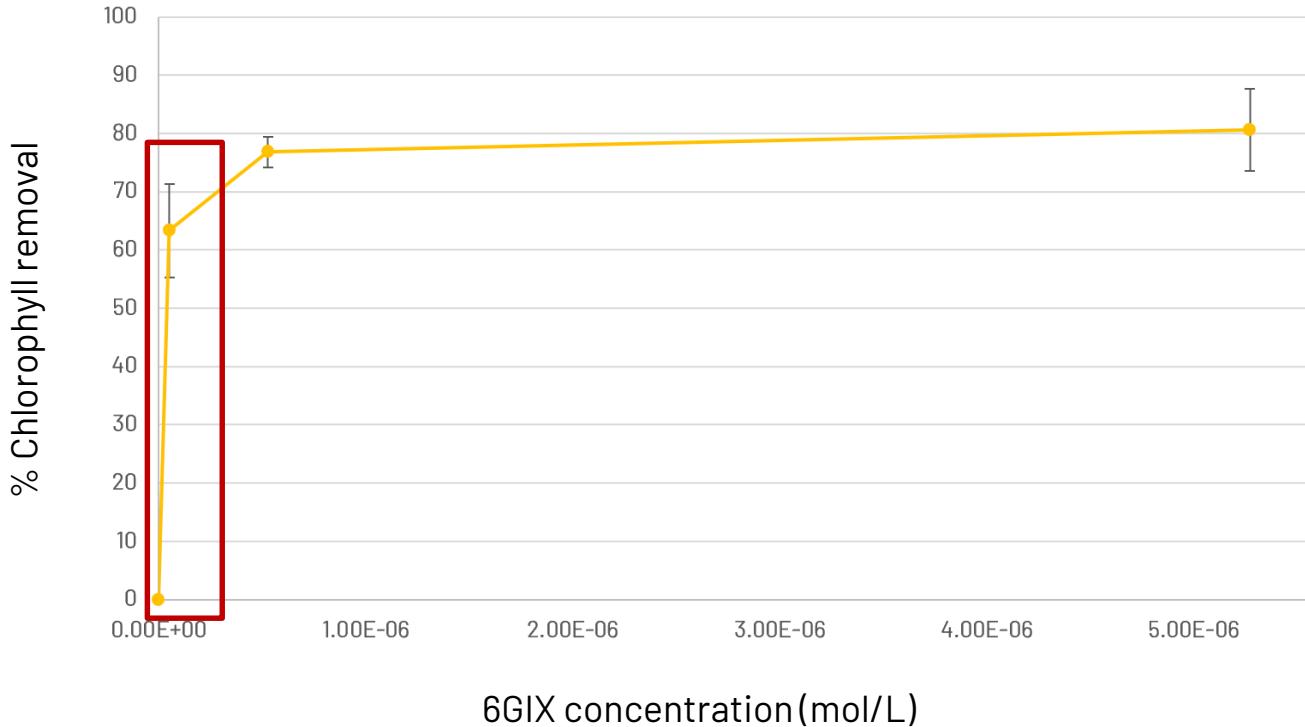


# 6GIX Emulsions Results

## Protein concentration vs. Chlorophyll removal



Aqueous  
phase  
with 6GIX



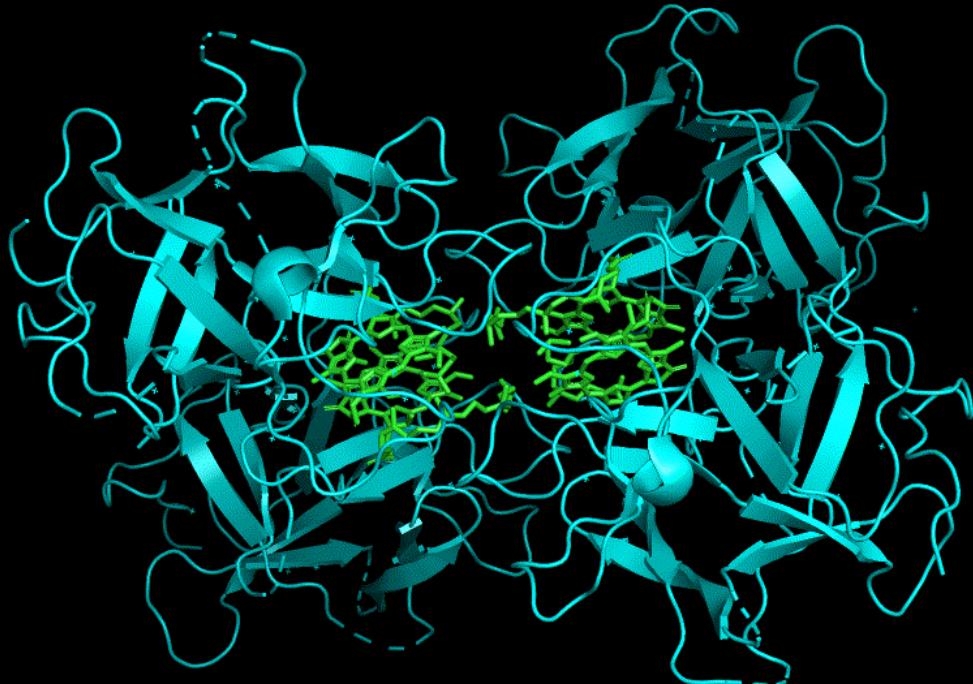
# 6GIX Instability

How to improve function and stability

12 amino acids cause variance

Denaturing at interface

iGAM - Genetic algorithm and PCA



# MODGIX

Modified 6GIX Protein

Modified for enhanced Chlorophyll Binding

Designed for stability

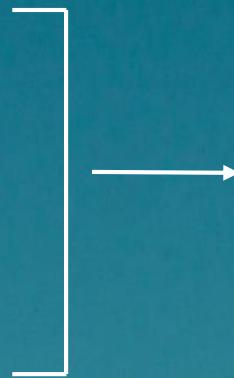
Higher binding potential

Less stabilizing agents required



# Accomplishments

- ✓ Produce 6GIX
- ✓ Secrete 6GIX
- ✓ Purify 6GIX



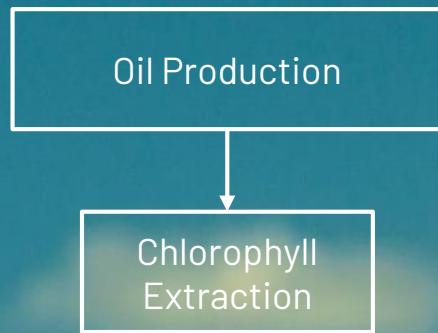
→ Produce 6GIX on  
industrial level

- ✓ Determine best emulsion  
compositions
- ✓ Prove 6GIX's function in  
purifying green oil



→ Optimize for industry

# Canola Oil Industry Pipeline



# Canola Oil Industry Pipeline





Craig Shand



Ward Toma



John Mayko

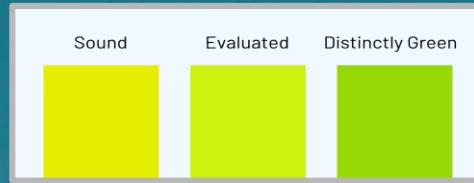


"Green is the difference between **profit** and **loss.**"

-Craig Shand  
Co-Owner/Operator, Shand Farms Ltd.

# Current Grading System

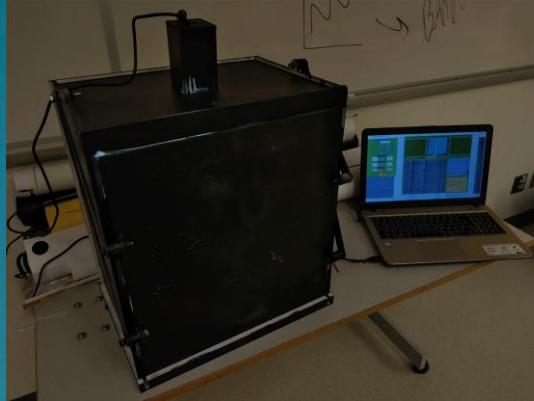
Seed Sample Assessed Against Colour Guide



Canadian Grain  
Commission



# Mean Green Machine



Consistent Lighting



Accurate Capture



Colour Determination  
Algorithm

# Can we empower farmers with the ability to produce **better quality seeds?**



Angela Brackenreed  
Agronomist



Autumn Barnes  
Agronomist

# Sunny Days

A Precise Predictive Algorithm to Inform Effective  
Agronomy

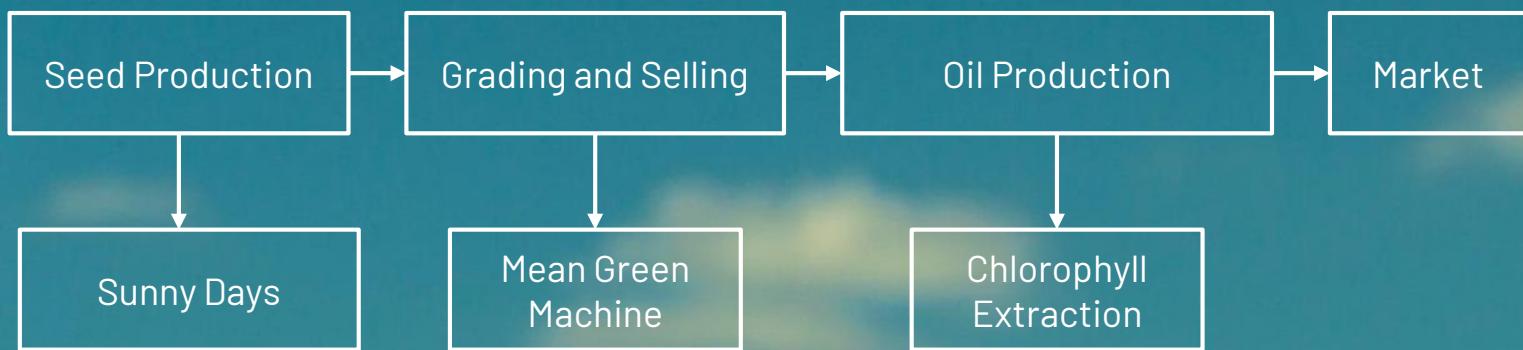


Within 2.5 degrees on  
average

Prediction 5 months in  
advance

Informed Management

# Canola Oil Industry Pipeline



Check out our poster and wiki!



# canolaPALOOZA



A photograph of a field filled with vibrant yellow rapeseed flowers. The flowers are densely packed along tall green stems. The background is a clear blue sky with a few wispy white clouds.

Green seed is a **financial problem.**

# Canola Oil Industry Pipeline

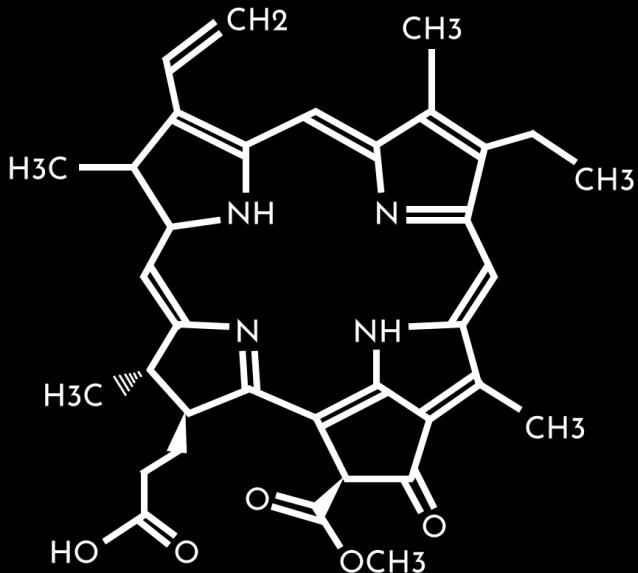




"Byproducts are valuable if they **offset** production costs"

- Dallas Gade  
General Manager, Richardson Oilseed

# Pheophorbide a



High value

Research: cancer & anti-fungal treatment

Canola suffers from fungal diseases

# Sclerotinia

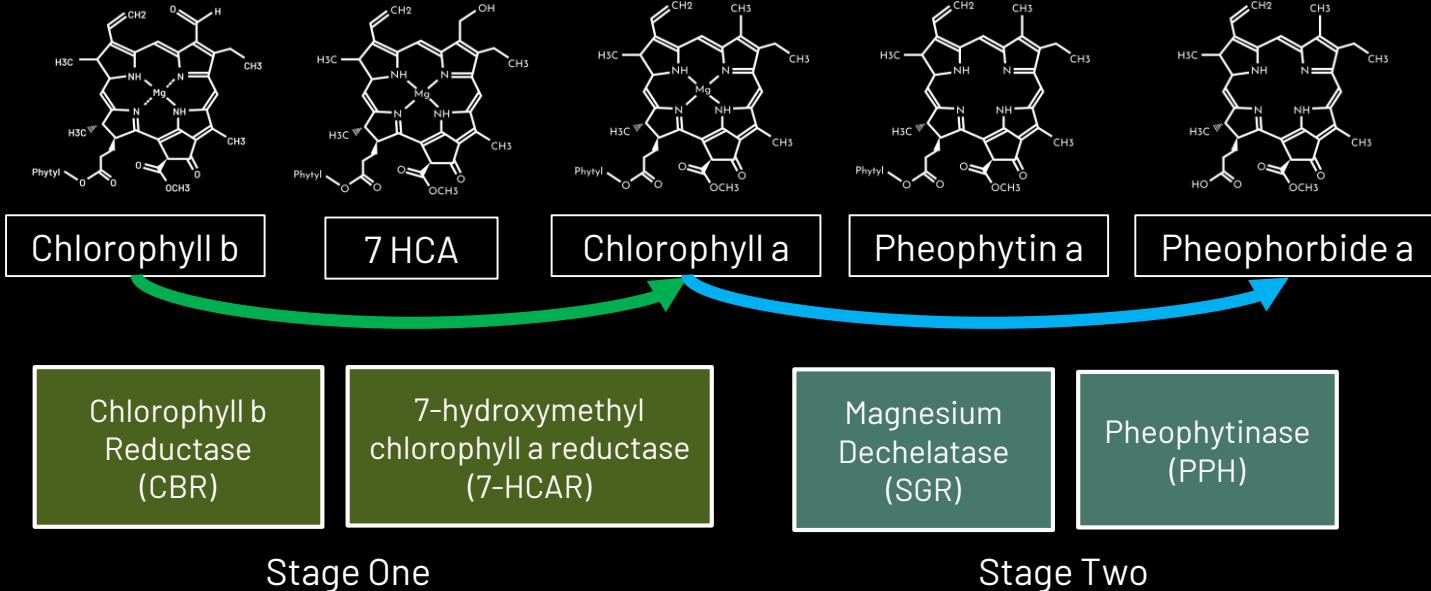


**"Anybody growing canola** in Alberta will  
have to deal with [fungus]."

John Mayko

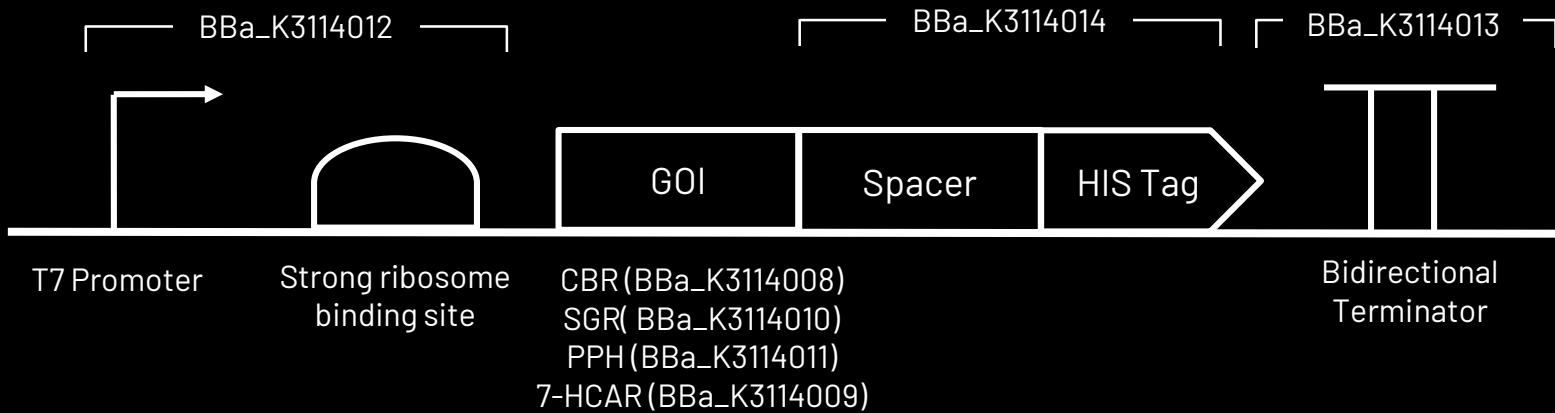
Farmer, The Alberta Canola Producers Commission

# Chlorophyll to Pheophorbide: Steps



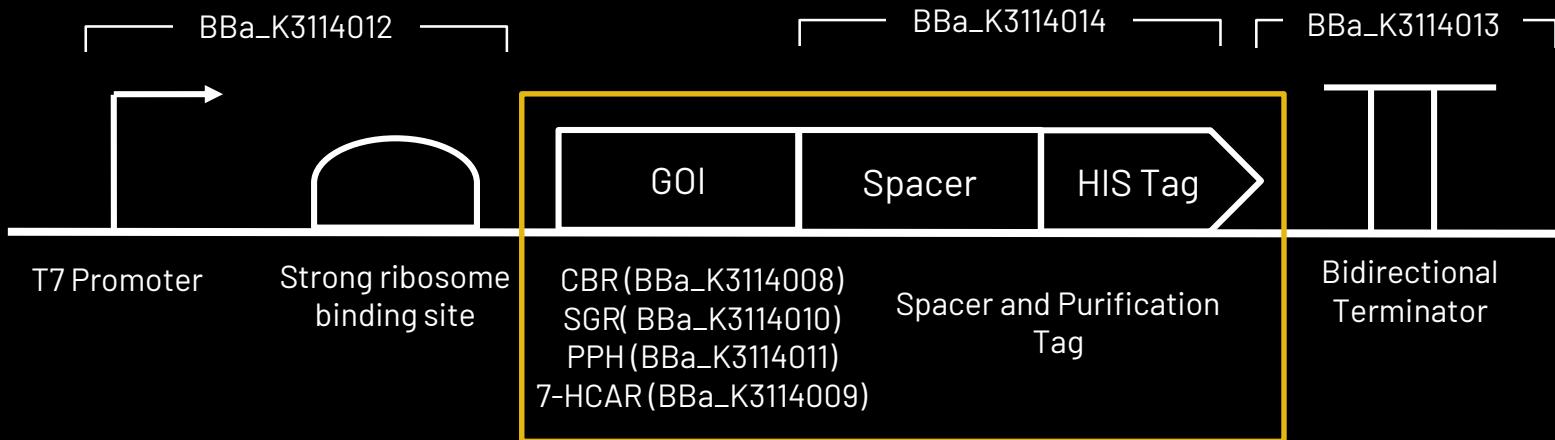
# Synbio for Green Seed

## Chlorophyll Degradation Proteins



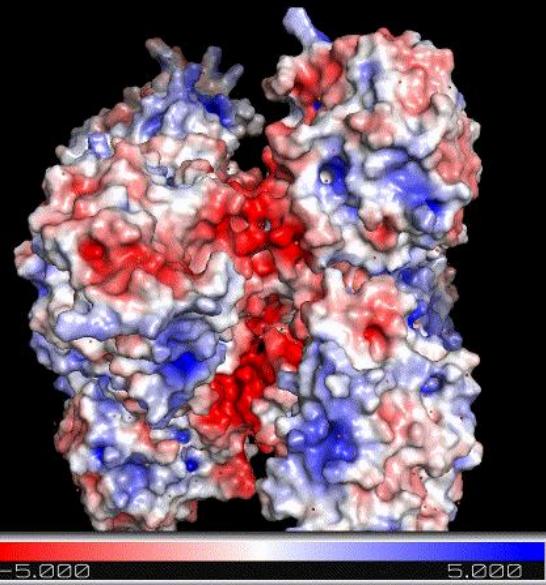
# Synbio for Green Seed

## Chlorophyll Degradation Proteins



# Synbio for Green Seed

Protein-Spacer-His Tag

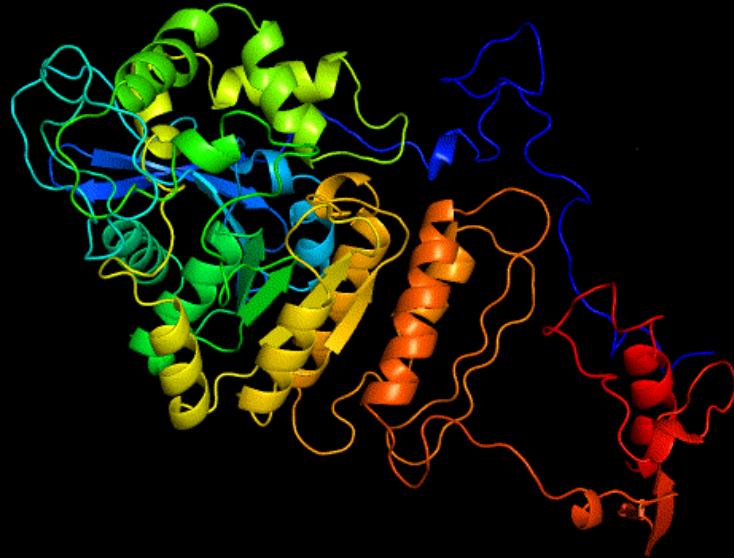


7-HCAR

(Meguro et al., 2011)

# Synbio for Green Seed

Protein-Spacer-His Tag

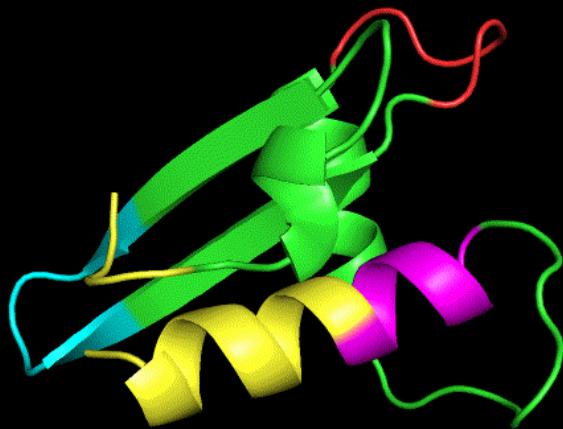


(Guyer, Salinger, Krügel,  
& Hörtensteiner, 2017)

PPH (Predictive Homology Model)

# ICARUS Universal Spacer

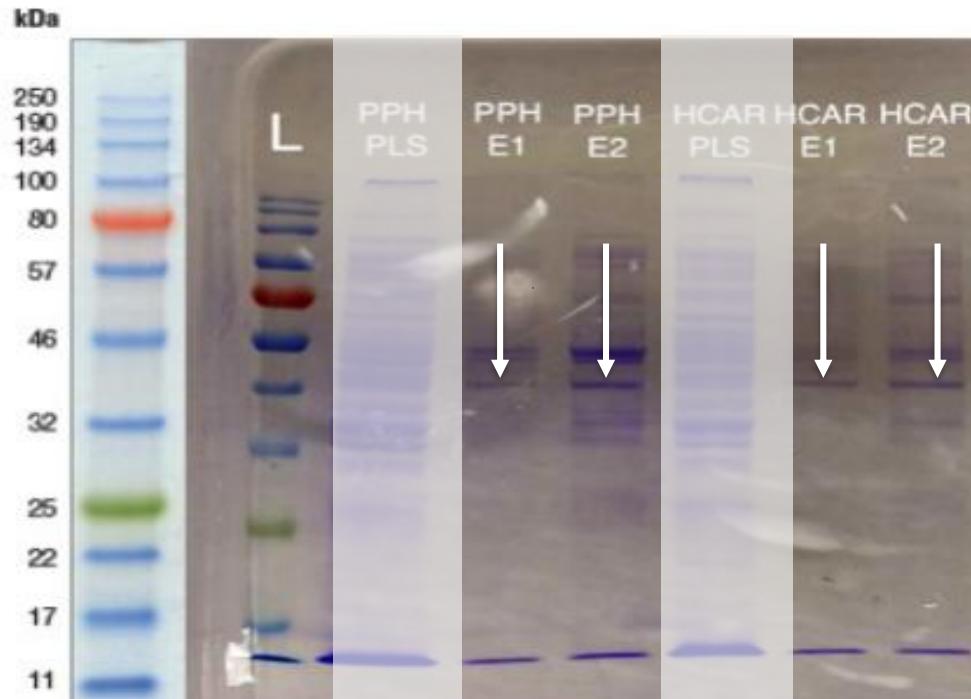
BBa\_K3114014



ICARUS (Predictive Homology Model)

# Protein Purification

Protein-Spacer-His Tag



Both HCAR and PPH SUCCESSFULLY purified.

Pheophytin



Pheophytin + Pheophorbide



Pheophytin + PPH = Pheophorbide



Pheophytin - Red

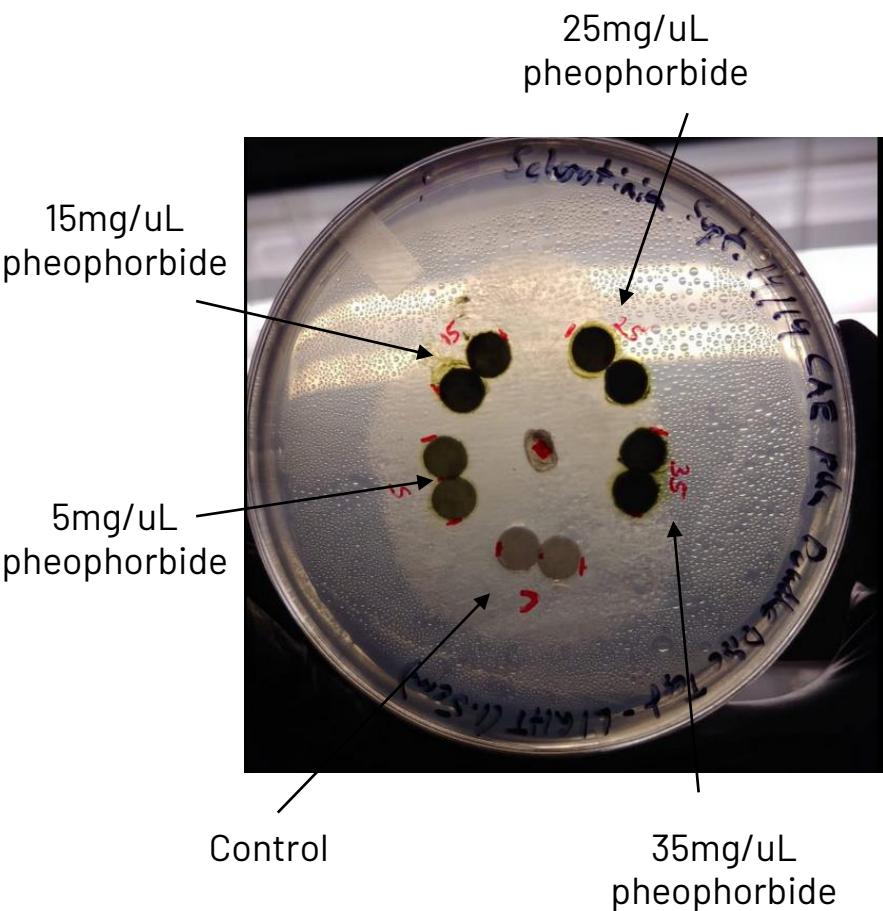
Pheophorbide - Pink

## Thin Layer Chromatography Showing PPH Function

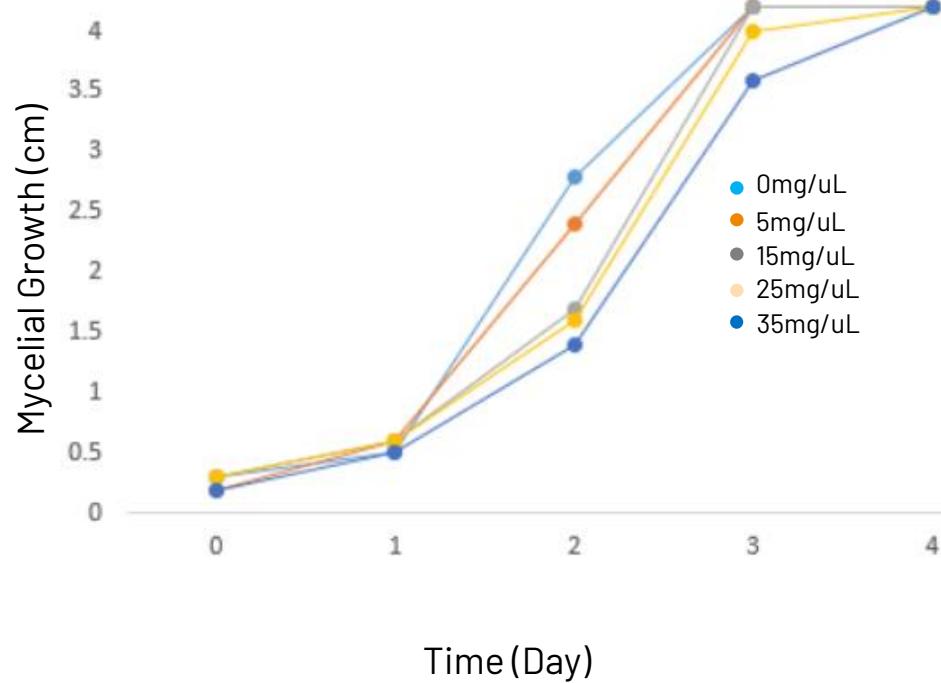
Pheophytinase (PPH) converts  
Pheophytin a into Pheophorbide

# Pheophorbide Testing

## Disc Test



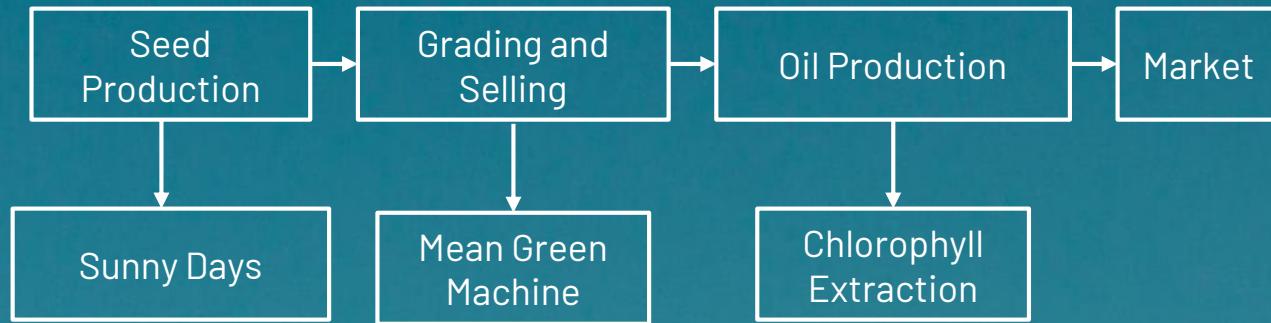
Mycelial growth in pheophorbide a presence



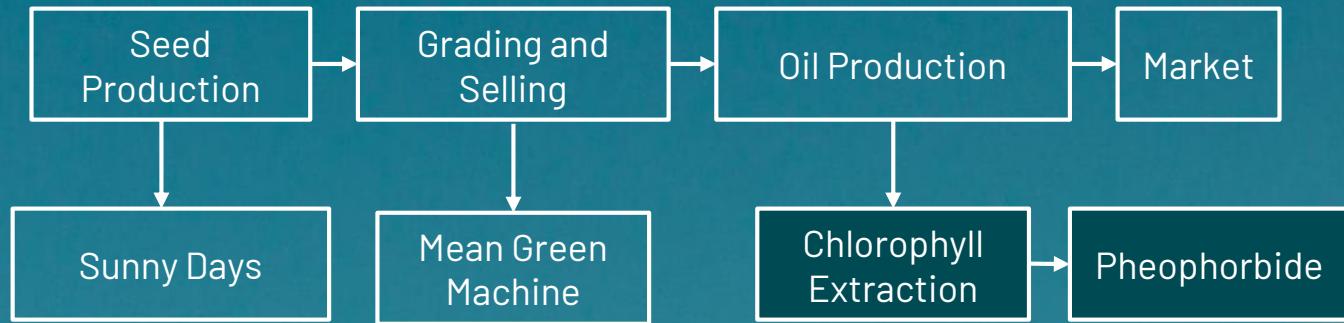
# Accomplishments

- ✓ Produced PPH and HCAR
  - ✓ Purified PPH and HCAR with ICARUS
  - ✓ Showed Pheophorbide Inhibited sclerotinia
- Produce chlorophyll degradation enzymes
- Field test Pheophorbide

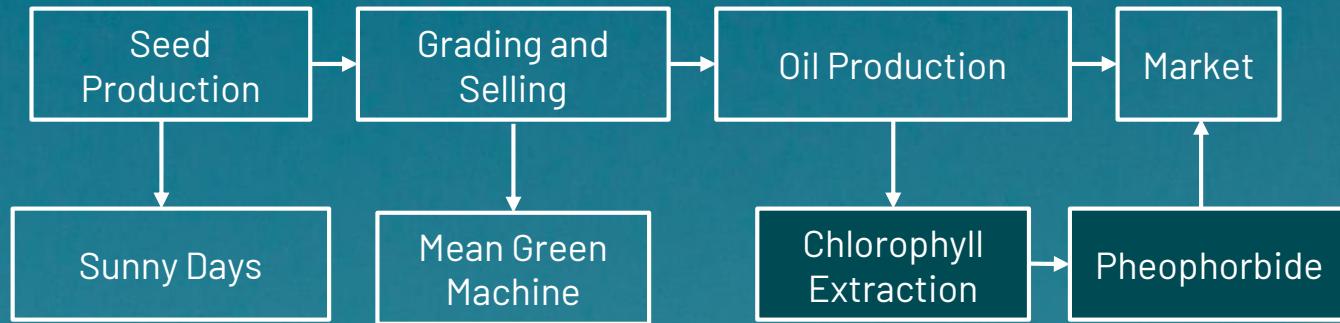
# Canola Oil Industry Pipeline



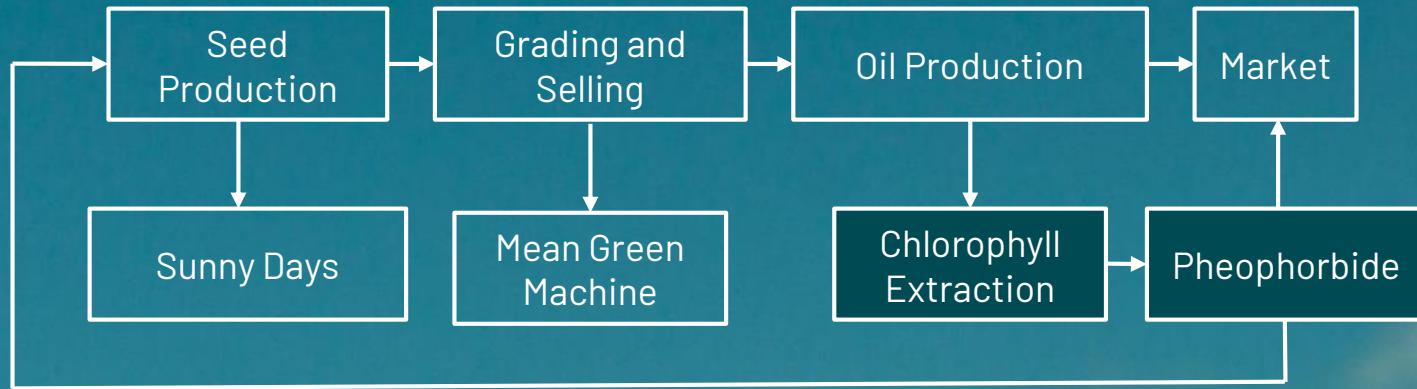
# Canola Oil Industry Pipeline



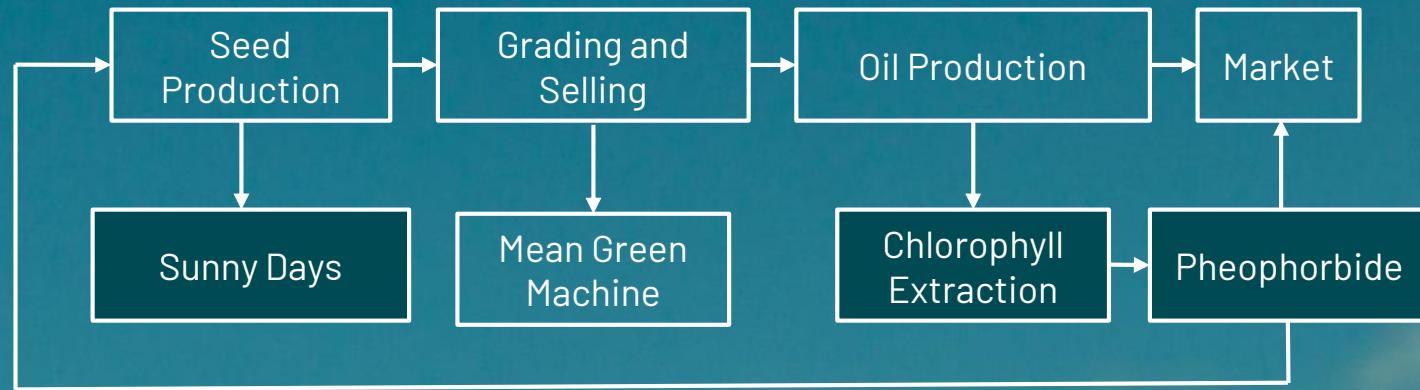
# Canola Oil Industry Pipeline



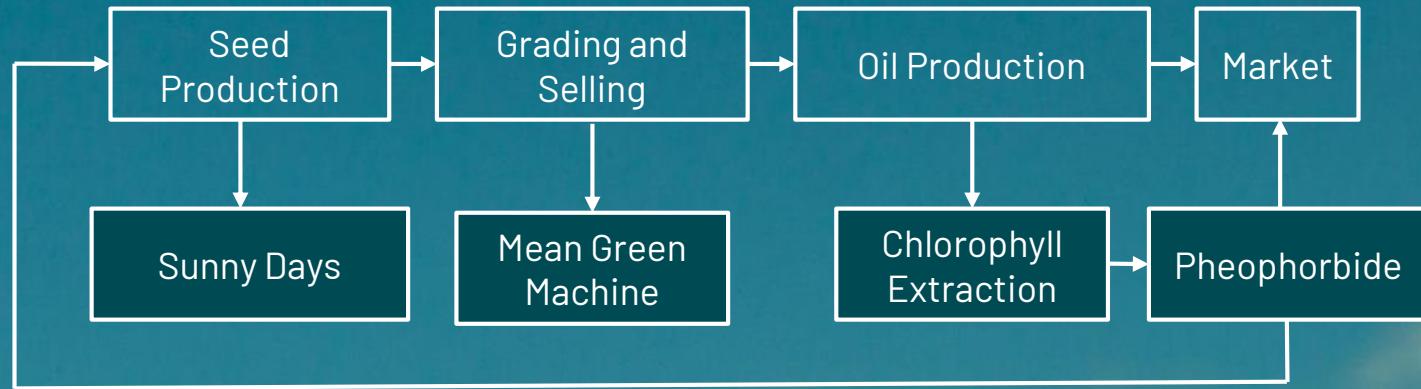
# Canola Oil Industry Pipeline



# Canola Oil Industry Pipeline



# Canola Oil Industry Pipeline



Building  
Partnerships



IP Protection

The background of the slide features a photograph of a field of yellow rapeseed flowers (canola) under a clear blue sky with a few wispy white clouds.

28 New Parts

7 Models that Informed Project Design

37 Stakeholder Meetings

Unquantifiable Number of Hours in the Lab



An all-encompassing solution to the green seed  
problem

Sunny Days

Standardized  
Grading

Chlorophyll  
Extraction

Pheophorbide



General Support:

David Bailey  
Anita Ludwar  
Patrick Wu  
Dr. Gijs van Rooijen  
Emily Hicks  
Robert Mayall  
Dr. Peter Facchini  
Swapan Kakumanu  
Dr. John Baker  
Dr. Gavin Cameron  
Deirdre Lobb

General Wet Lab Support:

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Dr. Vanina Zaremburg  
Dr. Isabelle-Barette-Ng  
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Dr. Marie Elizabeth Fraser  
Dr. Marcus Samuel  
Dr. Ian Lewis  
Dr. Gordon Chua  
Dr. Joe Harrison

Emulsions:

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Dr. Giovanniantonio Natale  
Dr. Hector Siegler  
Dr. Jianxun He  
Dr. Kazi Sumon

Chlorophyll Repurposing:

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Dr. Heather Addy  
Fran Cusack  
John Mayko  
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HP:

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Randall Weselake  
Dr. Veronique Barthet  
Angela Brackenreed  
Autumn Barnes  
Pleasant Valley Oil Mills  
Dallas Gade  
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Entrepreneurship:

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Noren Howg  
Erin Kulhawy



An all-encompassing solution to the green seed  
problem



Sunny Days



Standardized  
Grading



Chlorophyll  
Extraction



Pheophorbide



An all-encompassing solution to the green seed  
problem

Sunny Days

Standardized  
Grading

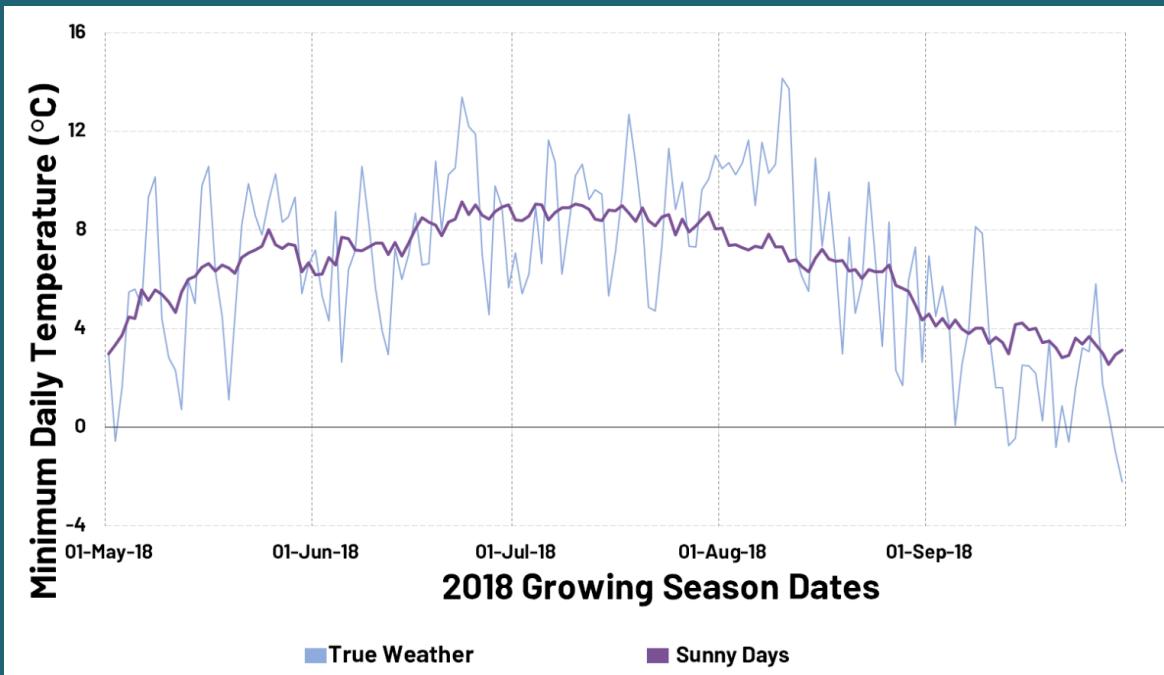
Chlorophyll  
Extraction

Pheophorbide

Sunny  
Days

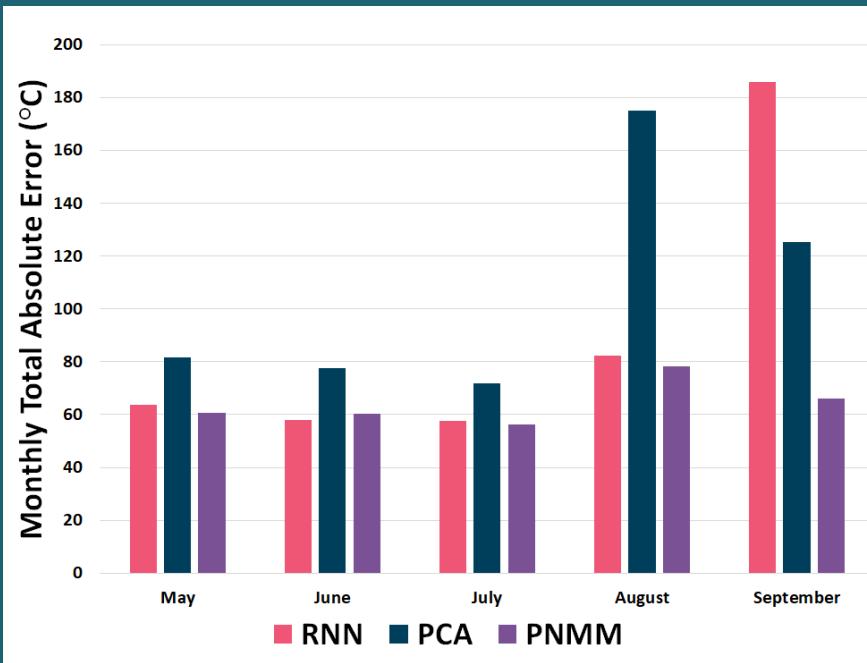
# Sunny Days

A Precise Predictive Algorithm to Inform Timing of Seeding



# Sunny Days

A Precise Predictive Algorithm to Inform Timing of Seeding



Prediction 5 months in advance

Within 2.1 degrees on average

In Peer Review at Alberta Academic Review

# Air temperature forecasts' accuracy of selected short-term and long-term numerical weather prediction models over Poland

Sebastian Kendzierski<sup>1</sup>, Bartosz Czernecki<sup>1</sup>, Leszek Kolendowicz<sup>1</sup> and Adam Jaczewski<sup>2</sup>

<sup>1</sup> Department of Climatology, Adam Mickiewicz University, Poznań, Poland

<sup>2</sup> Institute of Meteorology and Water Management – National Research Institute, Warszawa, Poland

Sunny Days  
accomplished  
estimates for  
180 days with a  
Mean Absolute  
Error of 2.109 C

# Sunny Days

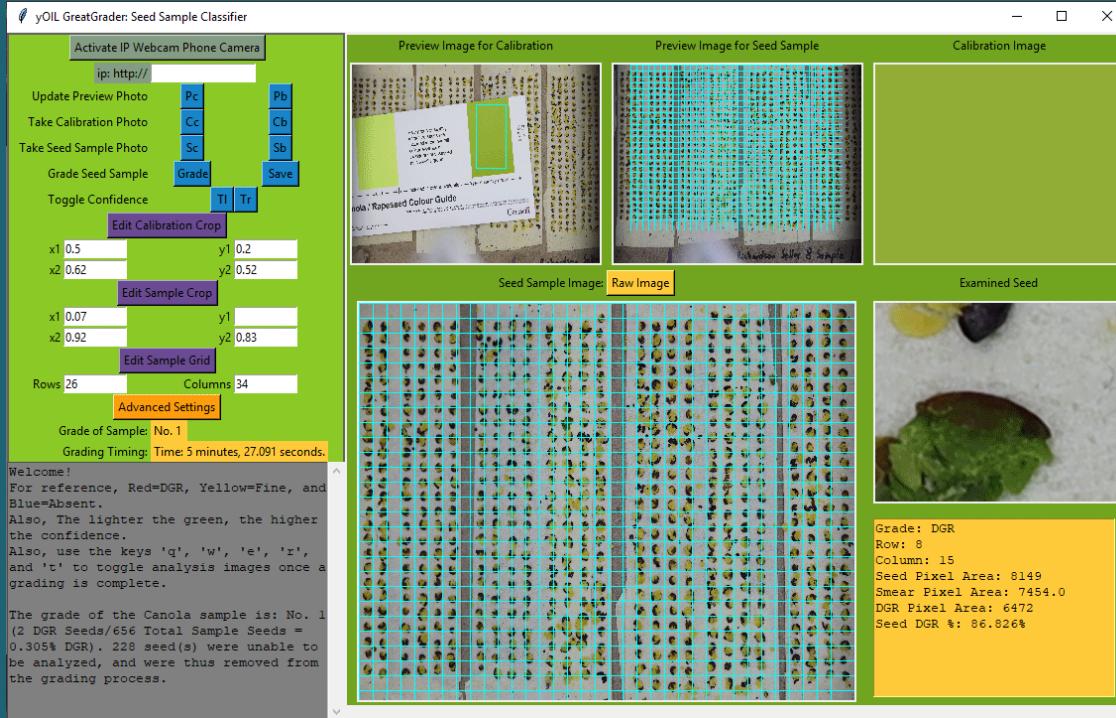
Comparison With  
Contemporary Methods

Table 3. Statistical results of long-term forecast in different time horizons.

| MODEL  | <i>t</i> (hs) | ME    | MAE  | RMSE | MSE   | BIAS | <i>r</i> |
|--------|---------------|-------|------|------|-------|------|----------|
| GFS    | 0–48          | -0.09 | 1.63 | 2.24 | 5.00  | 0.97 | 0.94     |
|        | 49–96         | -0.07 | 1.91 | 2.56 | 6.53  | 0.96 | 0.95     |
|        | 97–144        | -0.17 | 2.38 | 3.17 | 10.02 | 0.93 | 0.93     |
|        | 145–192       | -0.23 | 2.93 | 3.87 | 14.94 | 0.90 | 0.93     |
|        | 193–240       | -0.25 | 3.52 | 4.60 | 21.85 | 0.86 | 0.92     |
| HIRLAM | 0–48          | 0.11  | 1.53 | 2.06 | 4.25  | 0.97 | 0.98     |
|        | 49–96         | 0.09  | 1.82 | 2.42 | 5.84  | 0.96 | 0.98     |
|        | 97–144        | -0.04 | 2.31 | 3.07 | 9.42  | 0.94 | 0.97     |
|        | 145–192       | -0.18 | 2.97 | 3.92 | 15.34 | 0.90 | 0.95     |
|        | 193–240       | -0.15 | 3.40 | 4.46 | 19.91 | 0.87 | 0.94     |

# Standardized Seed Grading

# GreatGrader



A software tool for standardizing seed grading:

- Calibrate the system using a colour chip
- Take a seed sample picture
- Crop and divide seeds
- Grade
- Examine individual seeds
- See confidence

GitHub links:

Python Script Version:

<https://github.com/iGEMCalgary/DGRClassification>

Standalone Version for Windows 10 64-bit: <https://github.com/iGEMCalgary/GreatGrader>

# GreatGrader Grading Process



1) Seed Sample



2) Individual Seed



3) Watershed Marking



4)

Watershed Mask



5)

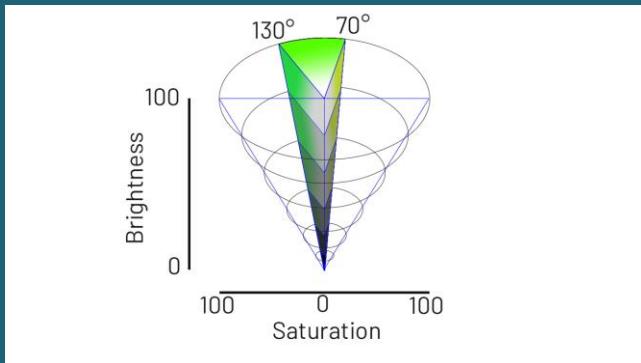
Modified Flood Fill



6)

Final Seed Mask

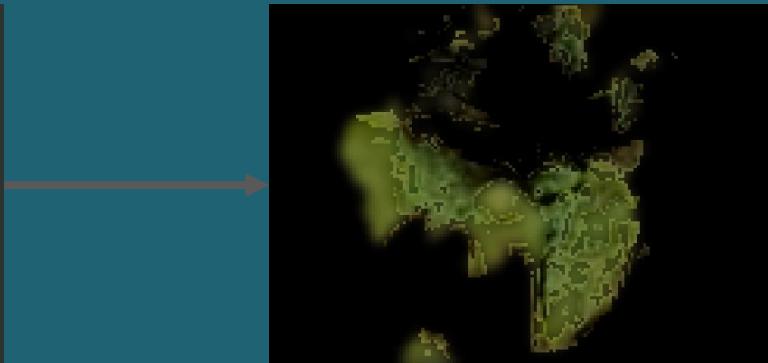
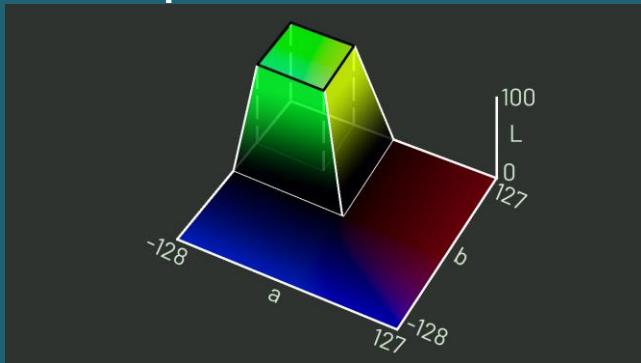
# GreatGrader Grading Process



HSV colour space distance calculations used

7)

Smear Parse



Lab colour space distance calculations used

8)

DGR pixel grading

# GreatGrader Performance

| Sample            | Human Grading<br>DGR% | MGM & GG<br>DGR% | Absolute<br>Error (DGR%) | Time to Grade<br>(seconds) |
|-------------------|-----------------------|------------------|--------------------------|----------------------------|
| <b>CGC #1</b>     | 6.400%                | 7.444%           | 1.044%                   | 164.287                    |
| <b>CGC #2</b>     | 7.000%                | 7.739%           | 0.739%                   | 189.261                    |
| <b>Grainger</b>   | 4.143%                | 5.734%           | 1.591%                   | 289.408                    |
| <b>Richardson</b> | 0.308%                | 0.305%           | 0.003%                   | 267.353                    |

# GreatGrader Performance

**CGC Sample 1 Overall (GreatGrader ):**

**145/1948 = 7.444% Distinctly Green Seed**

**1**

$$68/(612-105) =$$

$$68/507 =$$

$$13.4\%$$

**2**

$$11/(612-135) =$$

$$11/477 =$$

$$2.3\%$$

**3**

$$22/(612-137) =$$

$$22/475 =$$

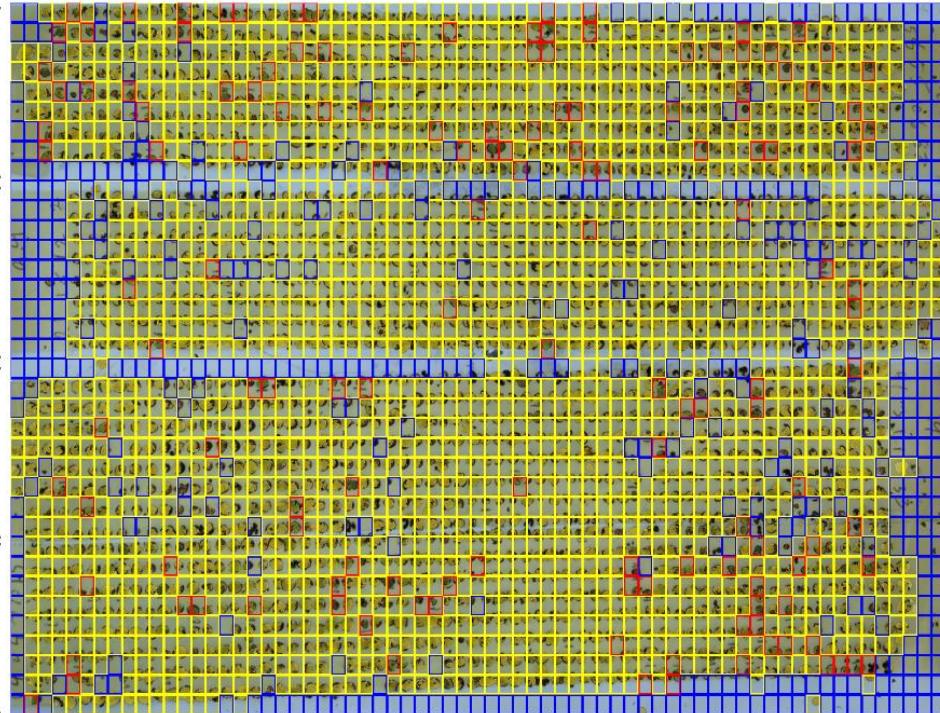
$$4.6\%$$

**4**

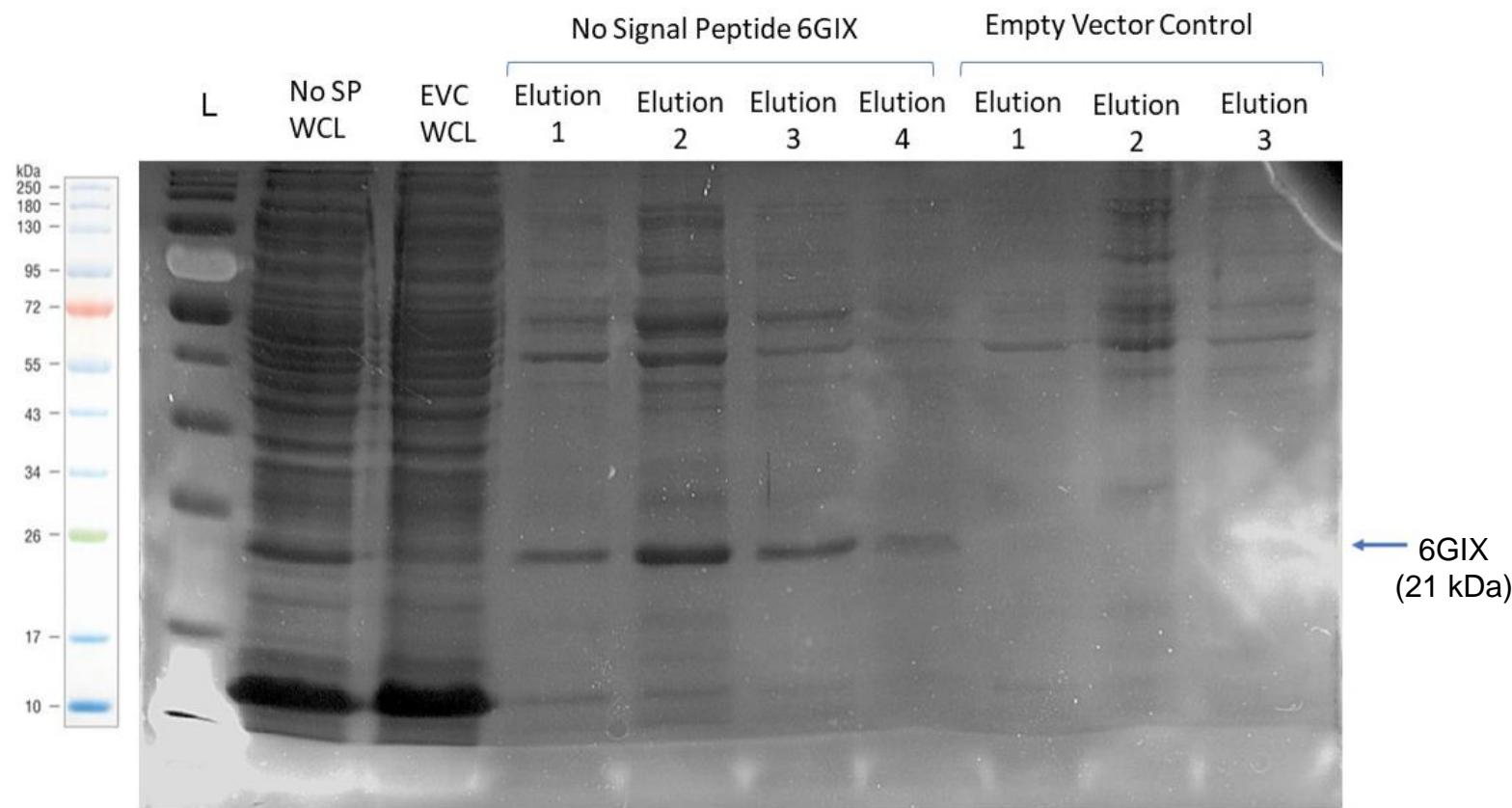
$$44/(612-127) =$$

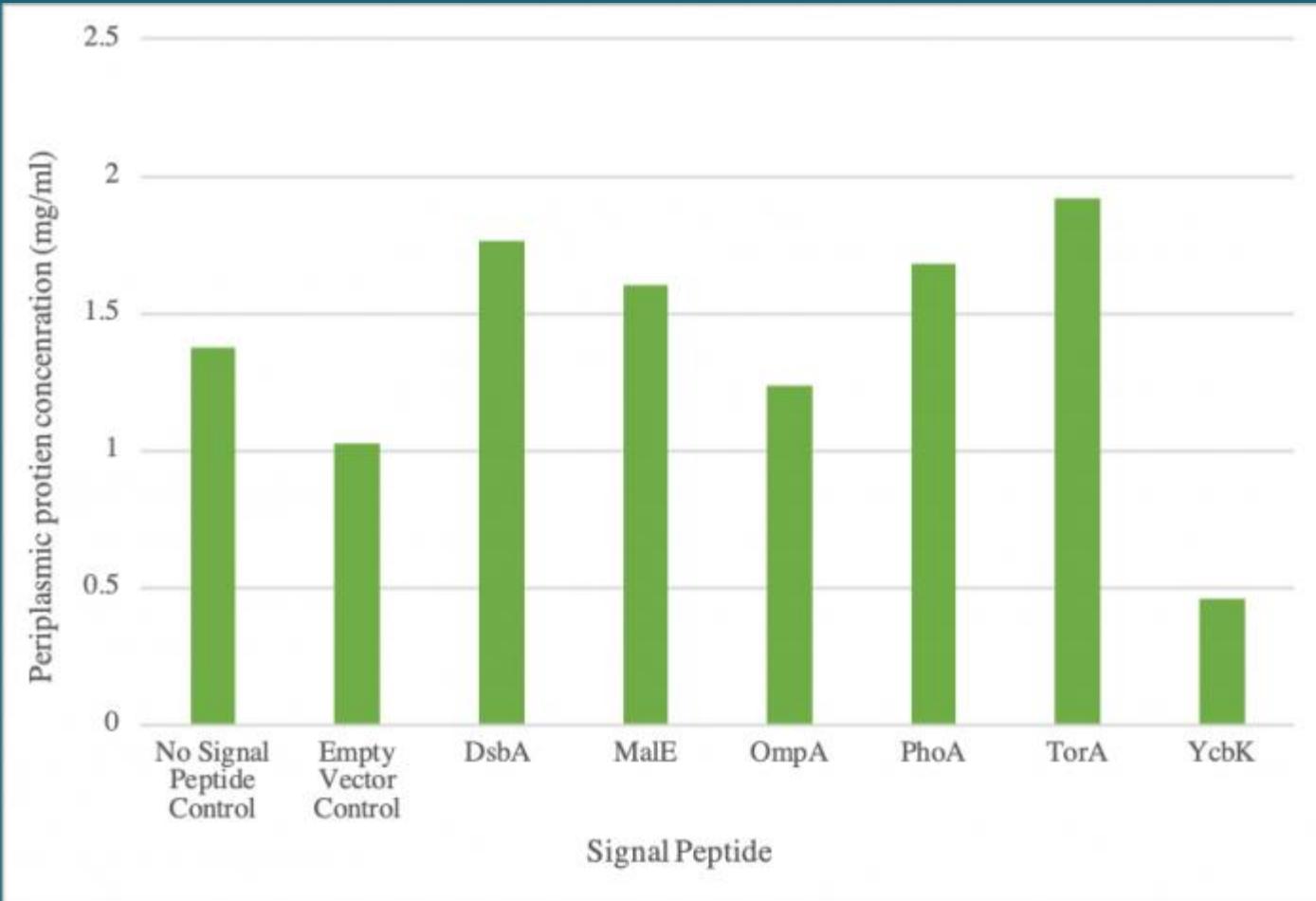
$$44/485 =$$

$$9.1\%$$

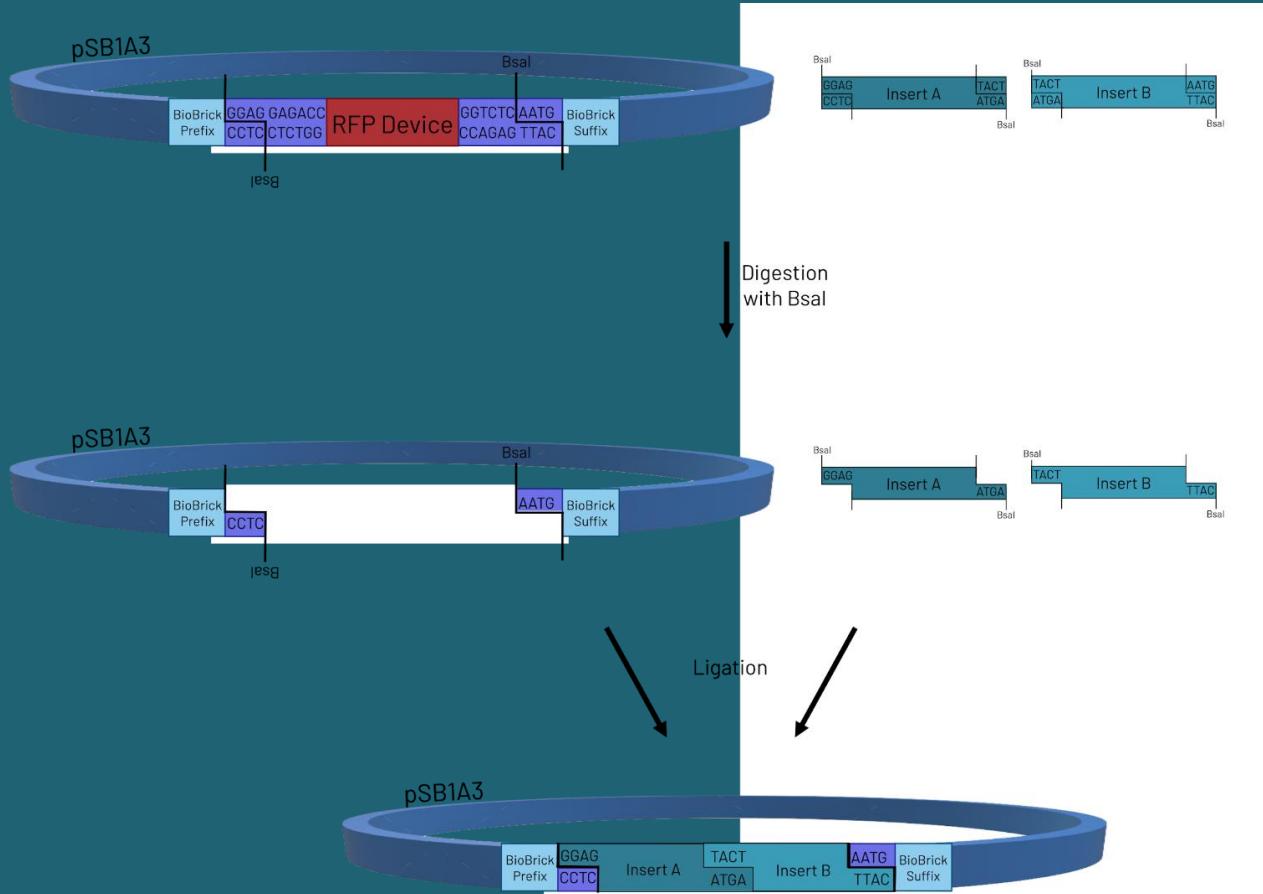


# 6GIX Production

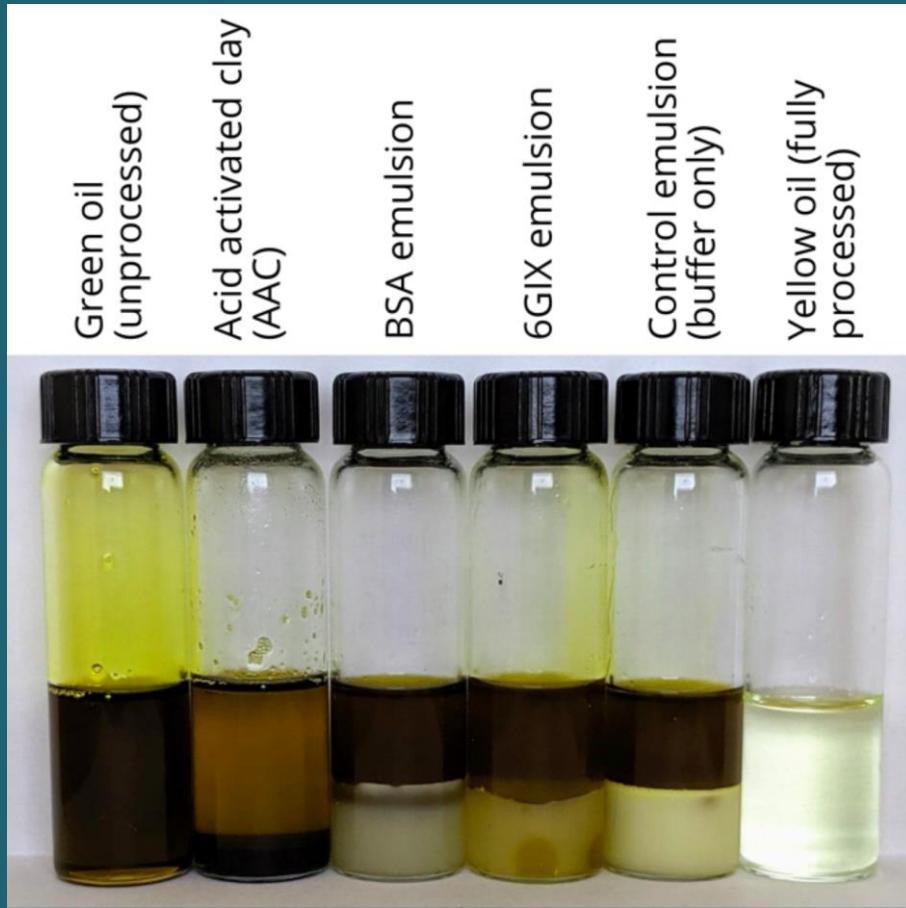


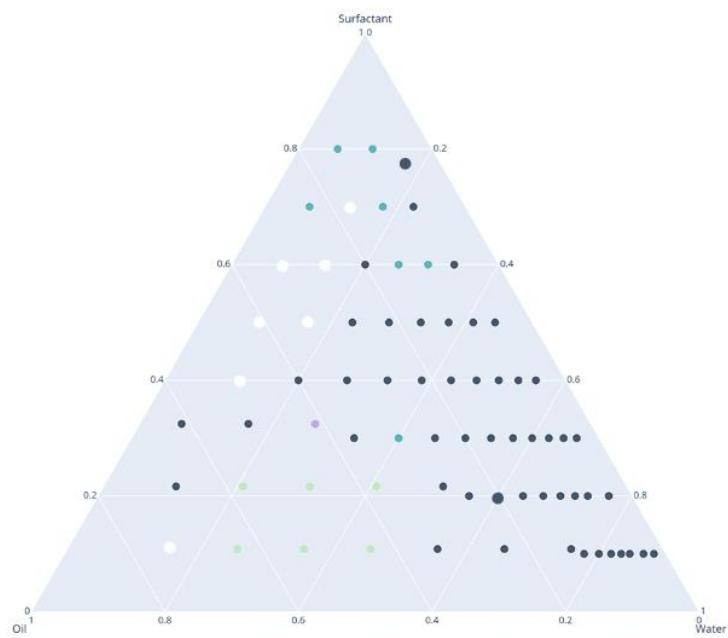


# RFP Flipper Devices for Golden Gate Assembly

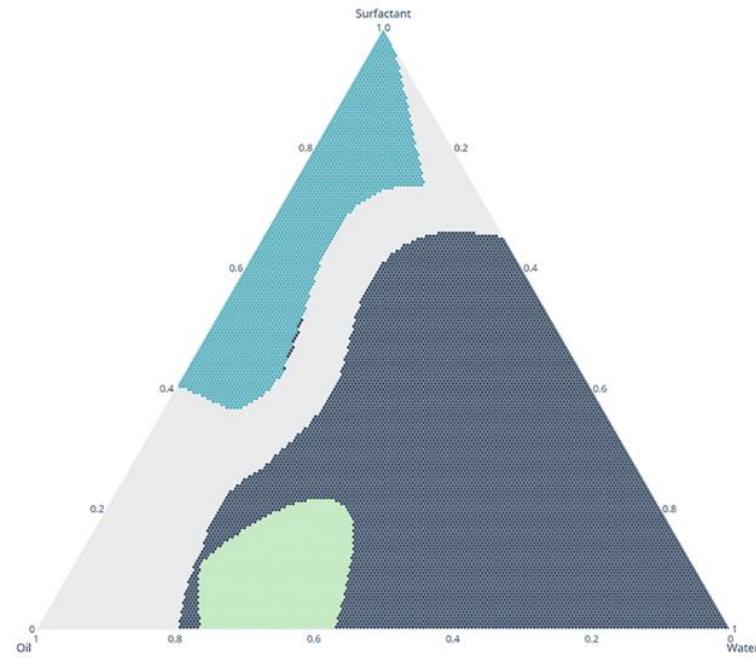


# Emulsions





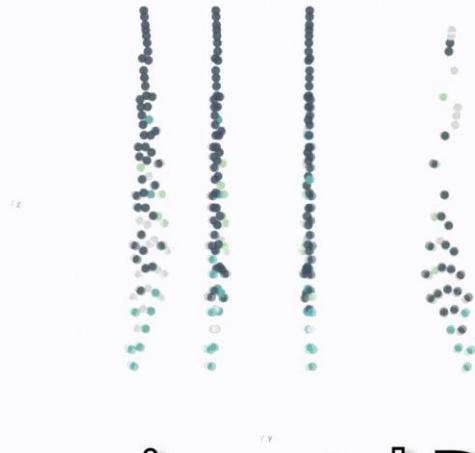
Collected Lab Data



Classification Model

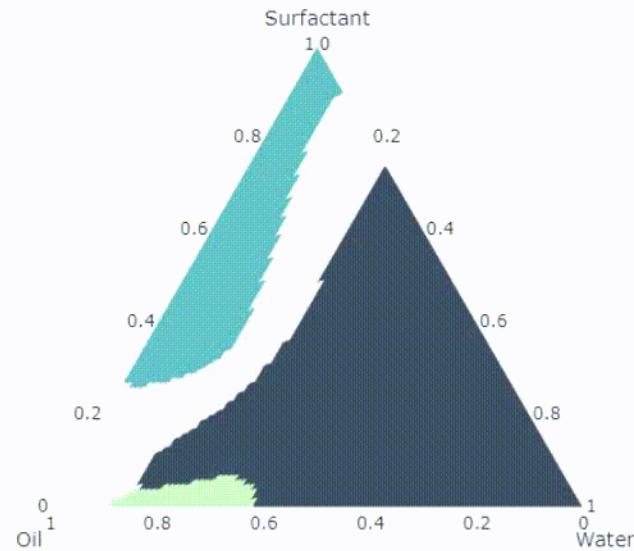
# Phase Modelling Process

1. Experimental Data
2. SVM with RBF Kernel models phase data for the experimental temperatures
3. MLP models phase data for any temperature



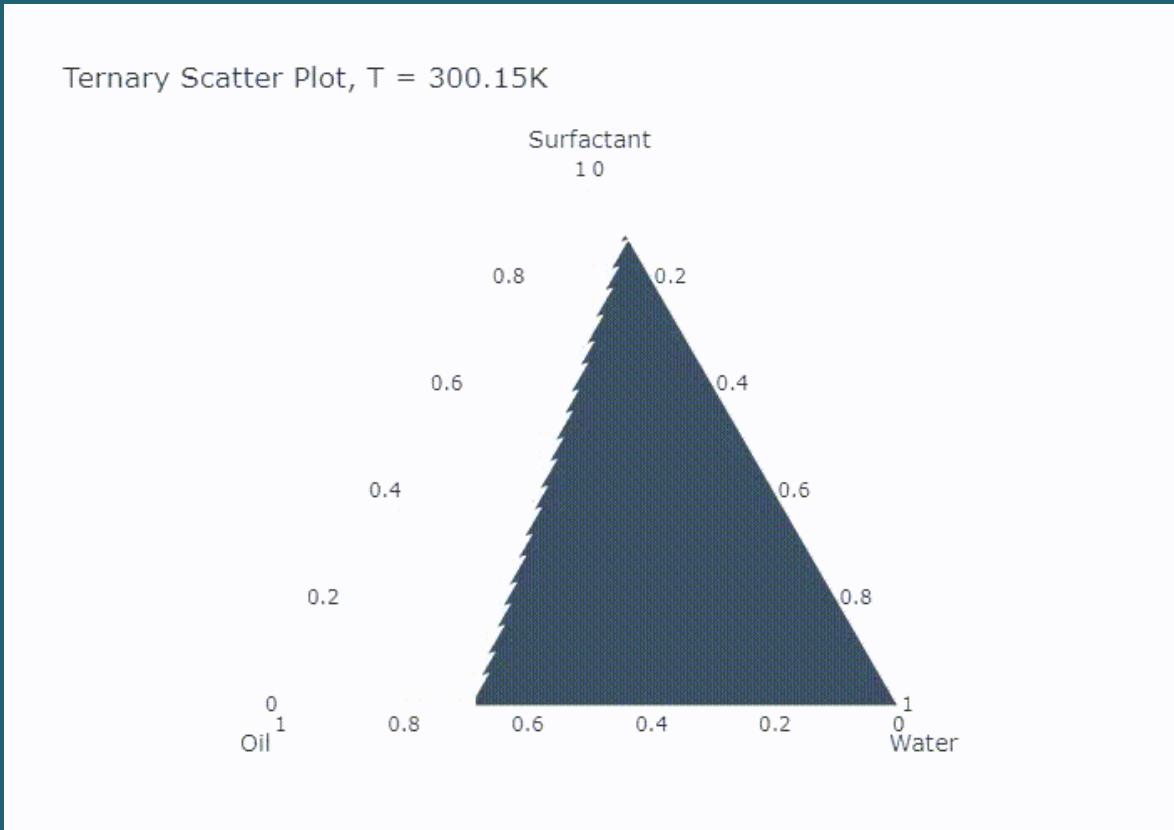
**Experimental Data**

Ternary Scatter Plot, T = 295.15K



# Validation using Confidence Map

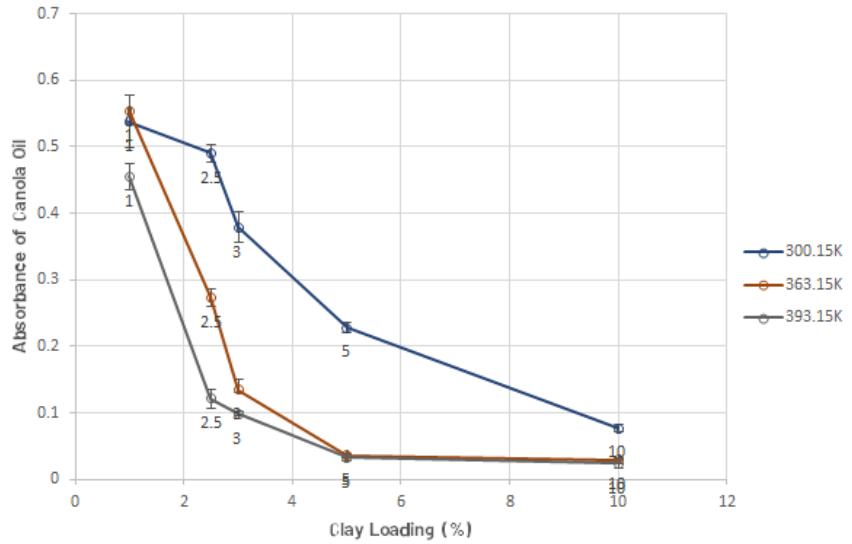
High confidence areas do  
**not change in predicted  
phase** for underfit to  
overfit models



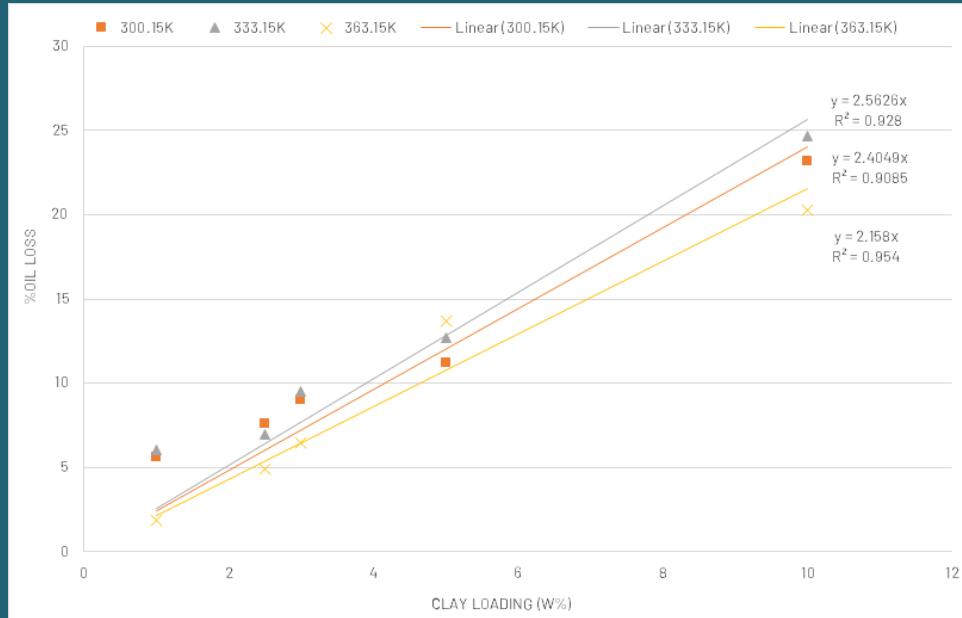
# Acid-Activated Clay Performance

## Chlorophyll Removal and Oil Loss

Chlorophyll Removal with Acid-Activated Clay

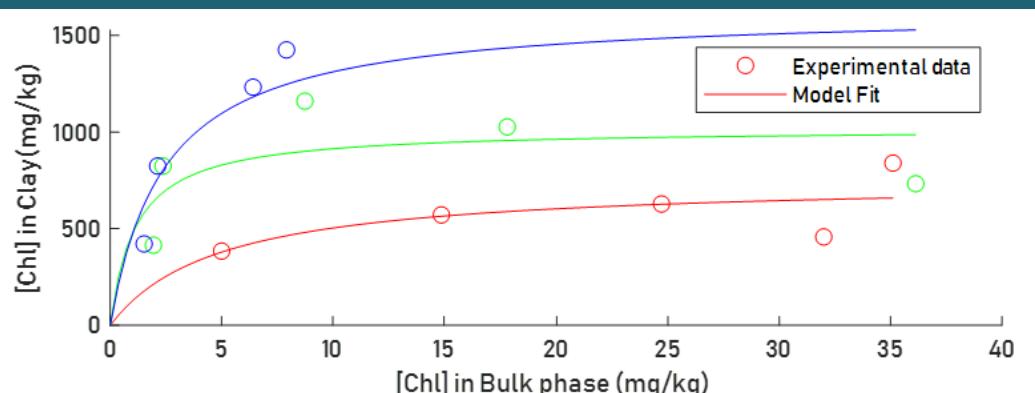


Chlorophyll Removal Experimental Results



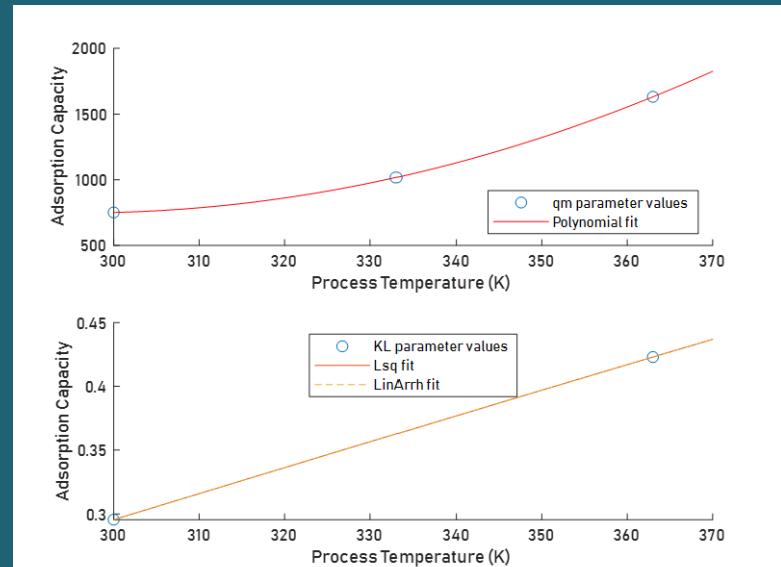
Oil Loss Experimental Results

# Acid-Activated Clay Performance Chlorophyll Removal and Oil Loss



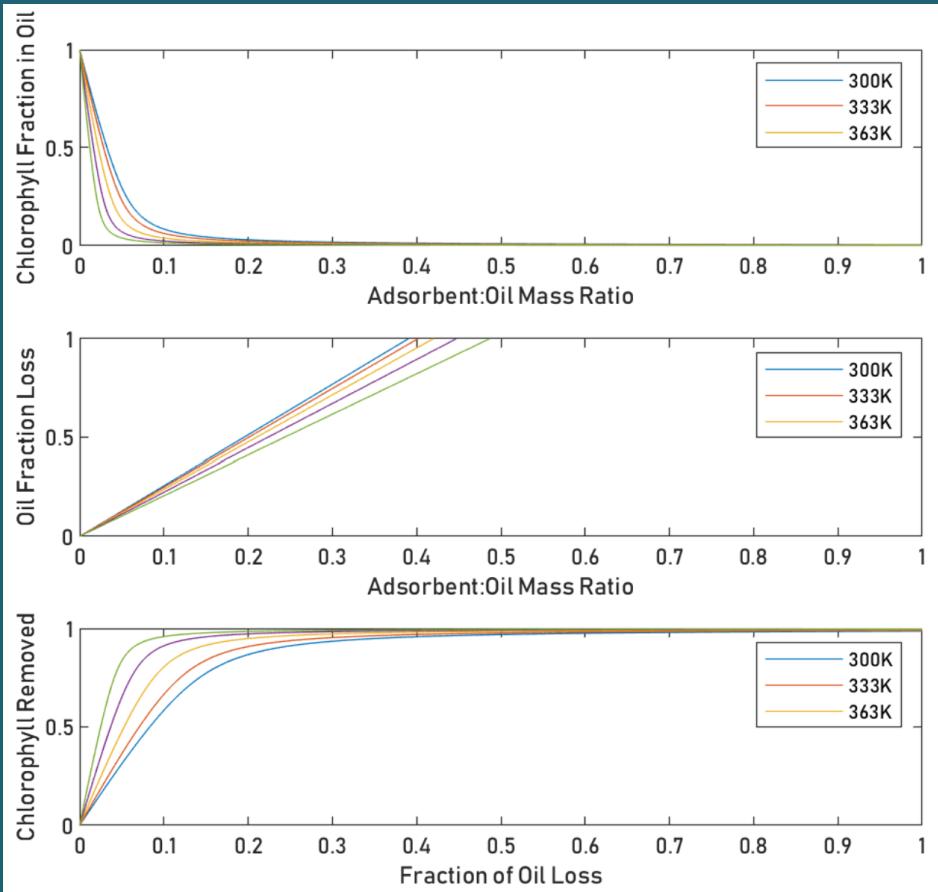
Langmuir Isotherm Model Fitting

$R =$

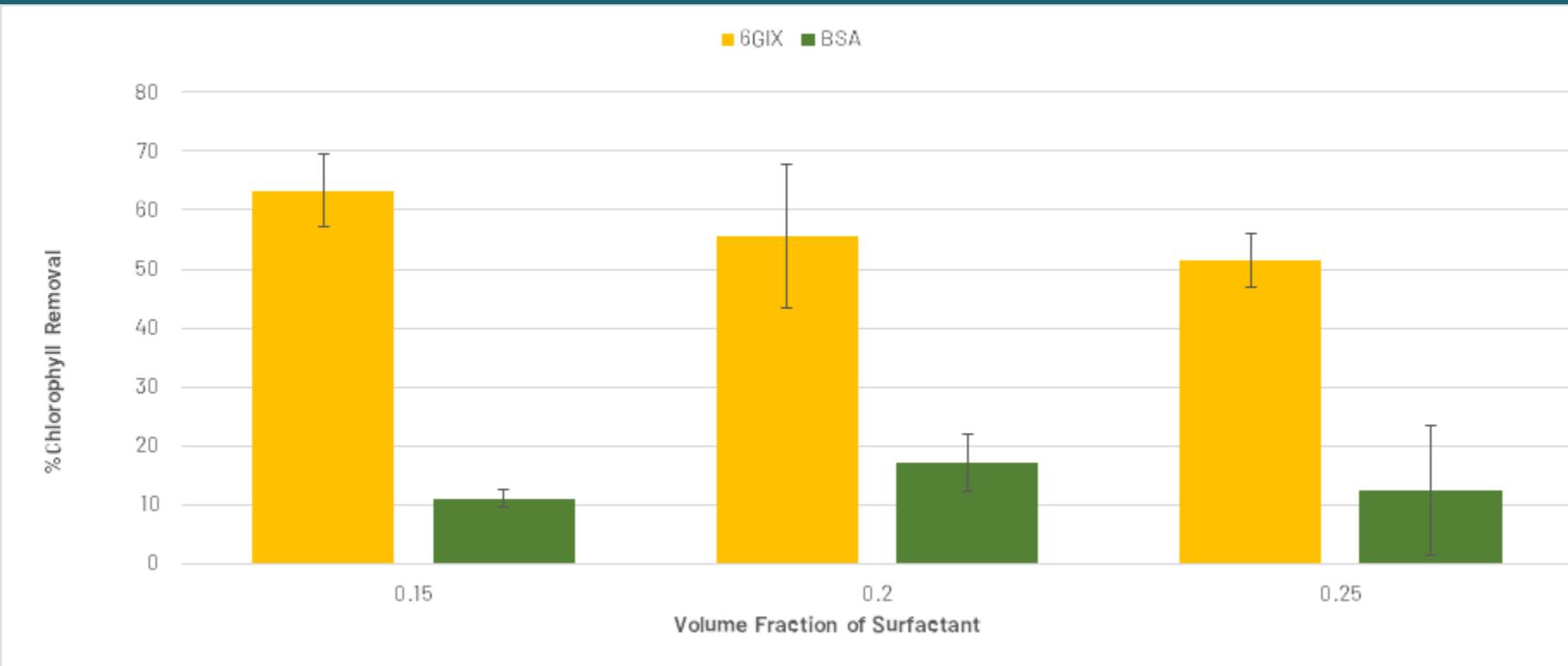


Temperature Dependence Fitting

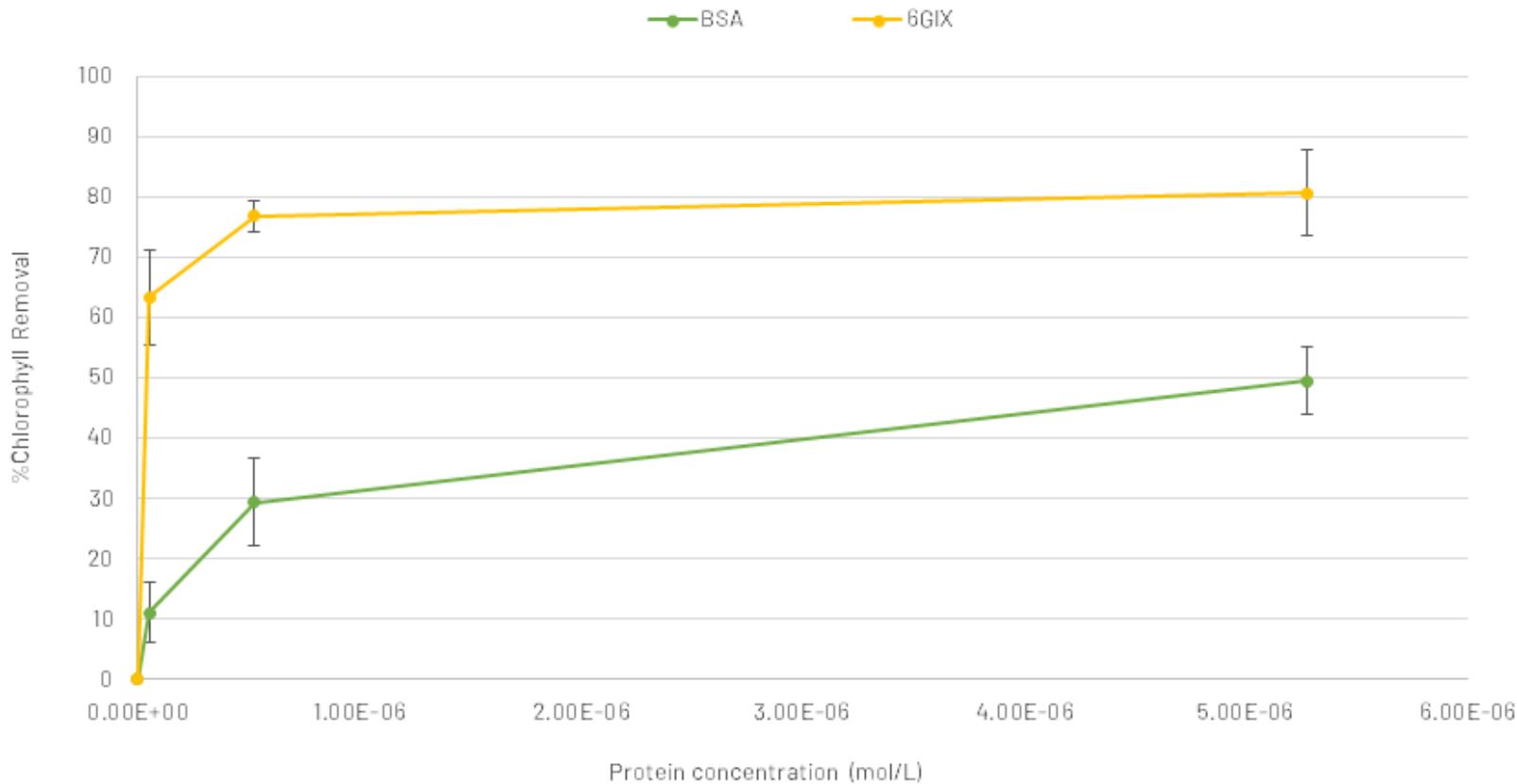
# Acid Activated Clay Performance Model



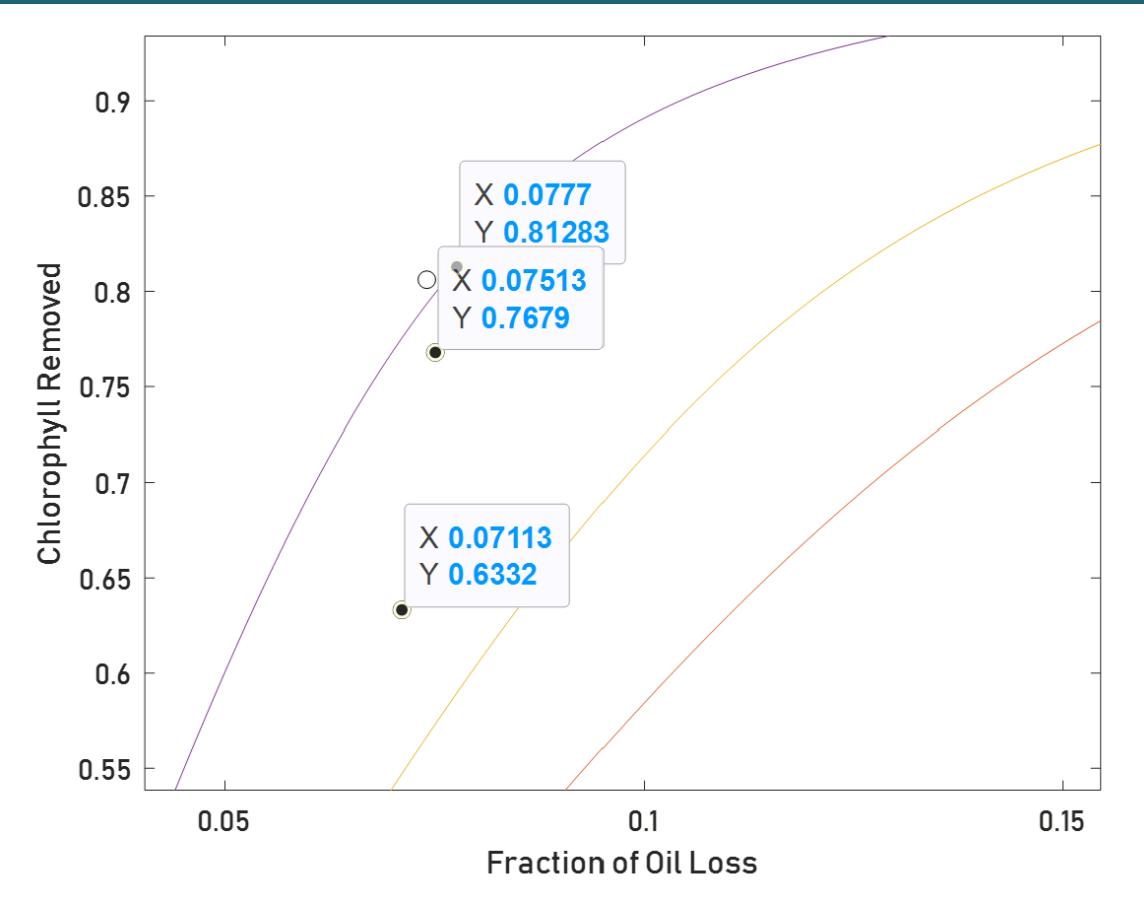
# Surfactant Composition Experiments



# Emulsified-Protein Chlorophyll Removal

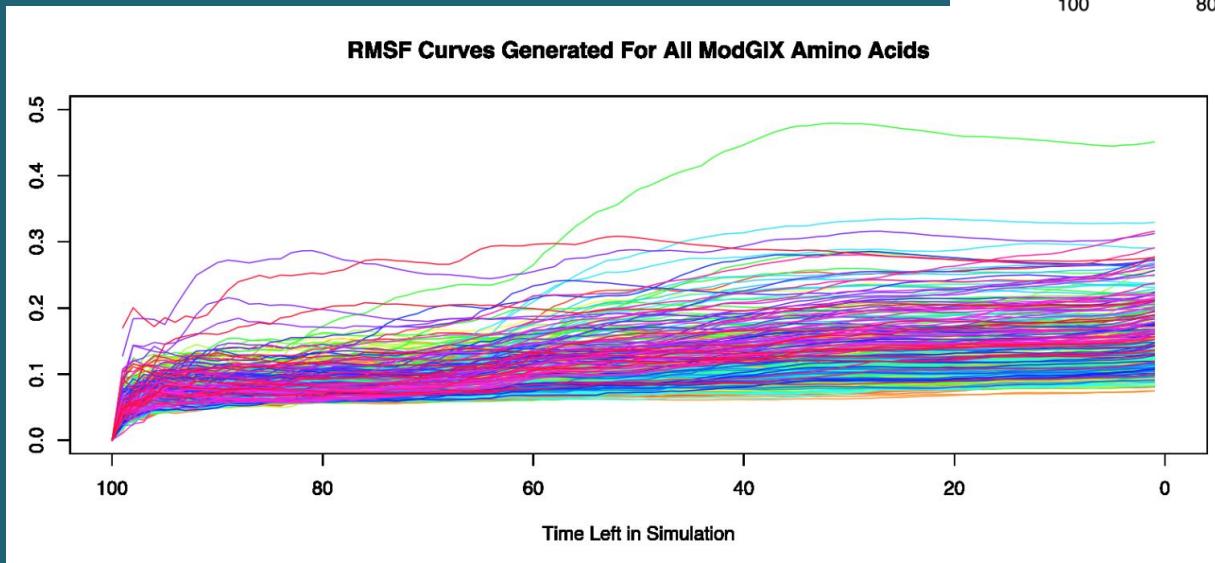
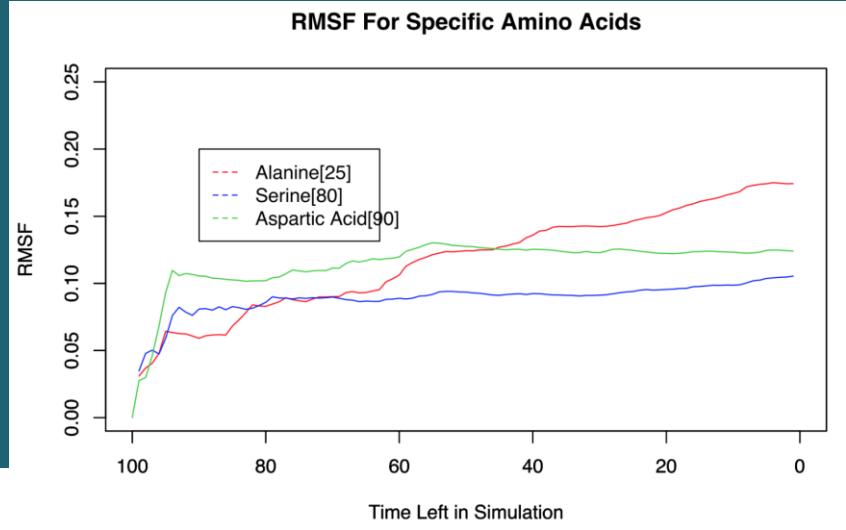


# Performance Comparison



MODGIX

# Measurement





# Pheophorbide Production

## CHLOROPHYLL REPURPOSING ACHIEVEMENTS

1. Universal spacer (ICARUS) for large proteins with a binding pocket that has strong electrostatic interactions

First to:

2. Test pheophorbide a on *Sclerotinia sclerotiorum*
3. Test pheophorbide a on *Pestalotiopsis microspora*
4. Execute pheophorbide a disc test
5. Purify Pheophytinase
6. Submit these proteins to the registry, characterized.

TO BE

1. Pheophorbide minimum inhibitory concentration
1. First in-vitro system to degrade chlorophyll a and b → pheophorbide a enzymatically
1. Quantitative characterization of pheophytinase

## Chlorophyll Repurposing Achievements

1. Recombinant PPH converted pheophytin into pheophorbide, our desired product, not seen in prior literature.

## ICARUS

1. ICARUS, a novel contribution, functionally allowed for purification of HCAR and PPH using a 6xHis-tag, despite problematic electrostatic interactions

## Anti-Fungal

1. The harmful canola pathogen *S. sclerotiorum*'s mycelium was inhibited by pheophorbide a treatment, not seen in prior literature
2. Comparative analysis of *P. microspora* suggests pheophorbide a specificity toward *S. sclerotiorum* inhibition

## TO BE

1. Pheophorbide minimum inhibitory concentration
1. First *in-vitro system* to degrade chlorophyll a and b → pheophorbide a enzymatically
1. Quantitative characterization of pheophytinase

# PHEOPHYTINASE PURIFICATION

Journal of Experimental Botany, Vol. 69, No. 4 pp. 879–889, 2018  
doi:10.1093/jxb/erx326 Advance Access publication 23 September 2017  
This paper is available online free of all access charges (see [http://jxb.oxfordjournals.org/open\\_access.html](http://jxb.oxfordjournals.org/open_access.html) for further details)



## RESEARCH PAPER

### Catalytic and structural properties of pheophytinase, the phytol esterase involved in chlorophyll breakdown

Luzia Guyer, Kathrin Salinger, Undine Krügel and Stefan Hörtensteiner\*

Institute of Plant and Microbial Biology, University of Zurich, Zollikerstrasse 107, CH-8008 Zurich, Switzerland

\* Correspondence: [shorten@botinst.uzh.ch](mailto:shorten@botinst.uzh.ch)

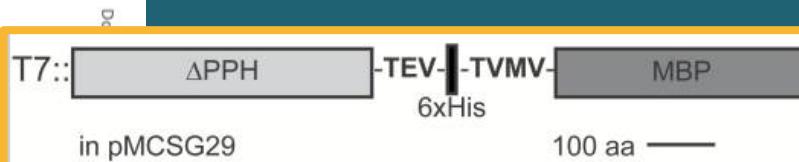
Received 14 July 2017; Editorial decision 21 August 2017; Accepted 21 August 2017

Editor: Christine Foyer, Leeds University, UK

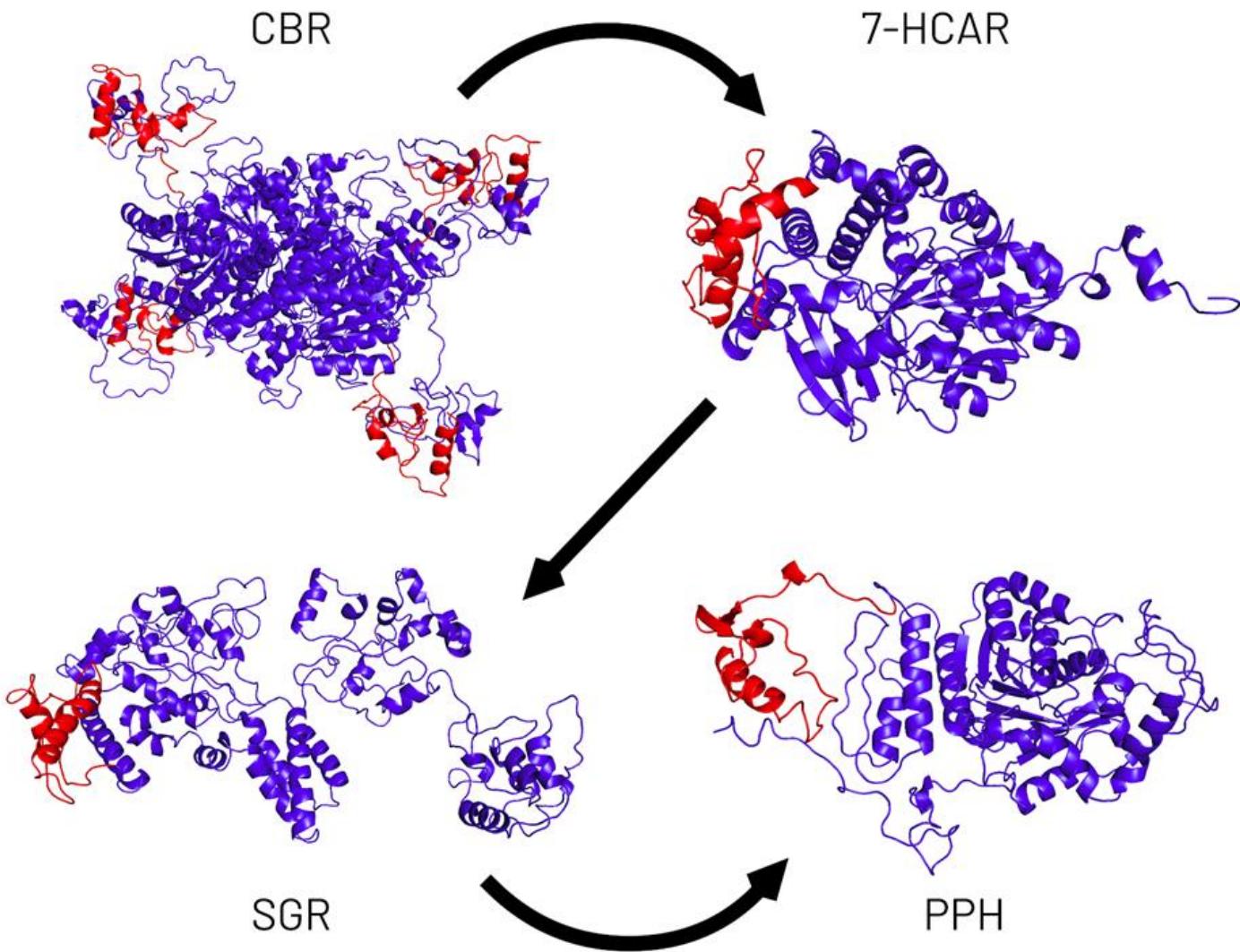
## Abstract

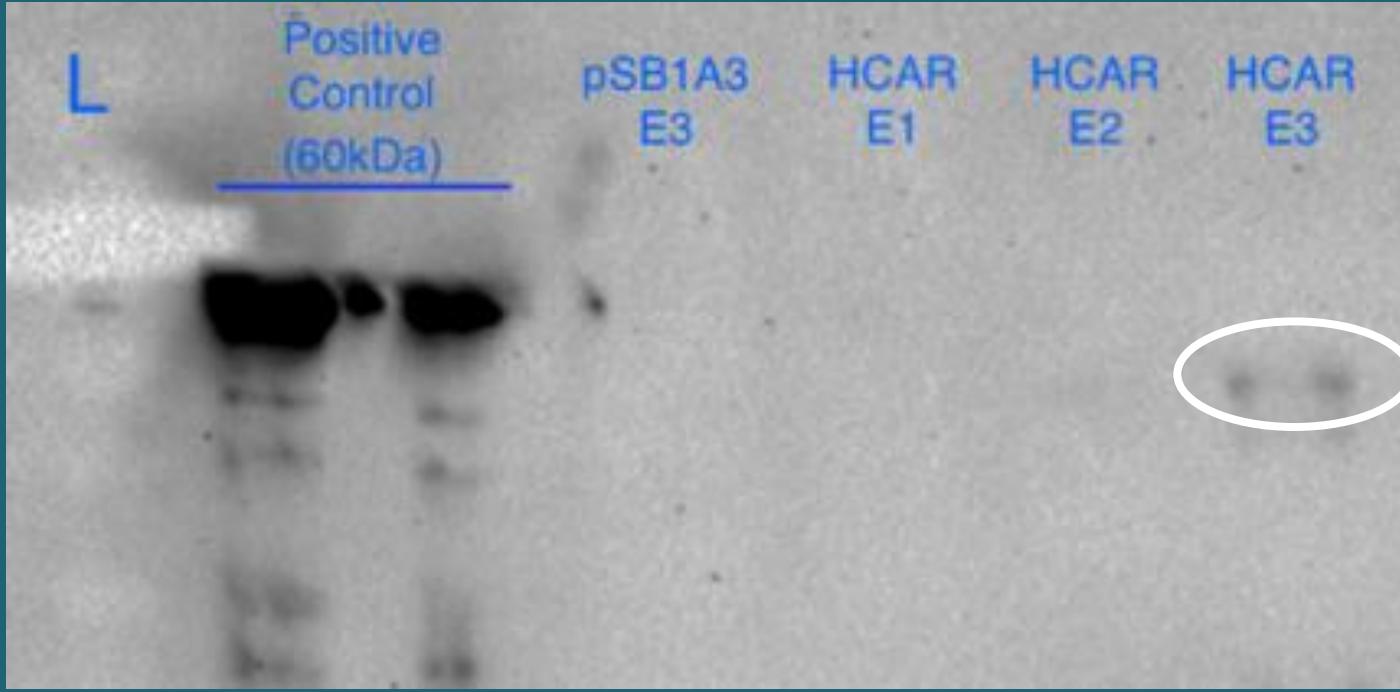
During leaf senescence and fruit ripening, chlorophyll is degraded in a multistep pathway into linear tetrapyrroles called phyllobilins. A key feature of chlorophyll breakdown is the removal of the hydrophobic phytol chain that renders phyllobilins water soluble, an important prerequisite for their ultimate storage in the vacuole of senescent cells. Chlorophyllases had been considered for more than a century to catalyze dephytylation *in vivo*; however, this was recently refuted. Instead, pheophytinase was discovered as a genuine *in vivo* phytol hydrolase. While chlorophyllase acts rather unspecifically towards different porphyrin substrates, pheophytinase was shown to specifically dephytinate pheophytin, namely Mg-free chlorophyll. The aim of this work was to elucidate in detail the biochemical and structural properties of pheophytinase. By testing different porphyrin substrates with recombinant pheophytinase from *Arabidopsis thaliana* we show that pheophytinase has high specificity for the acid moiety of the ester bond, namely the porphyrin ring, while the nature of the alcohol, namely the phytol chain in pheophytin, is irrelevant. *In silico* modelling of the 3-dimensional structure of pheophytinase and subsequent analysis of site-directed pheophytinase mutant forms allowed the identification of the serine, histidine, and aspartic acid residues that compose the catalytic triad, a classical feature of serine-type hydrolases to which both pheophytinase and chlorophyllase 1 belong. Based on substantial structural differences in the models of *Arabidopsis* pheophytinase and chlorophyllase 1, we discuss potential differences in the catalytic properties of these two phytol hydrolases.

Despite intensive attempts to purify and crystallize recombinant PPH, we were unsuccessful. Instead, we modelled the 3D structure of PPH based on publicly available structures of other  $\alpha/\beta$  fold hydrolases. This model (Fig. 5) allowed the identifica-



[academic.oup.com/jxb/article-abstract/69/4/879/4210343](http://academic.oup.com/jxb/article-abstract/69/4/879/4210343) b

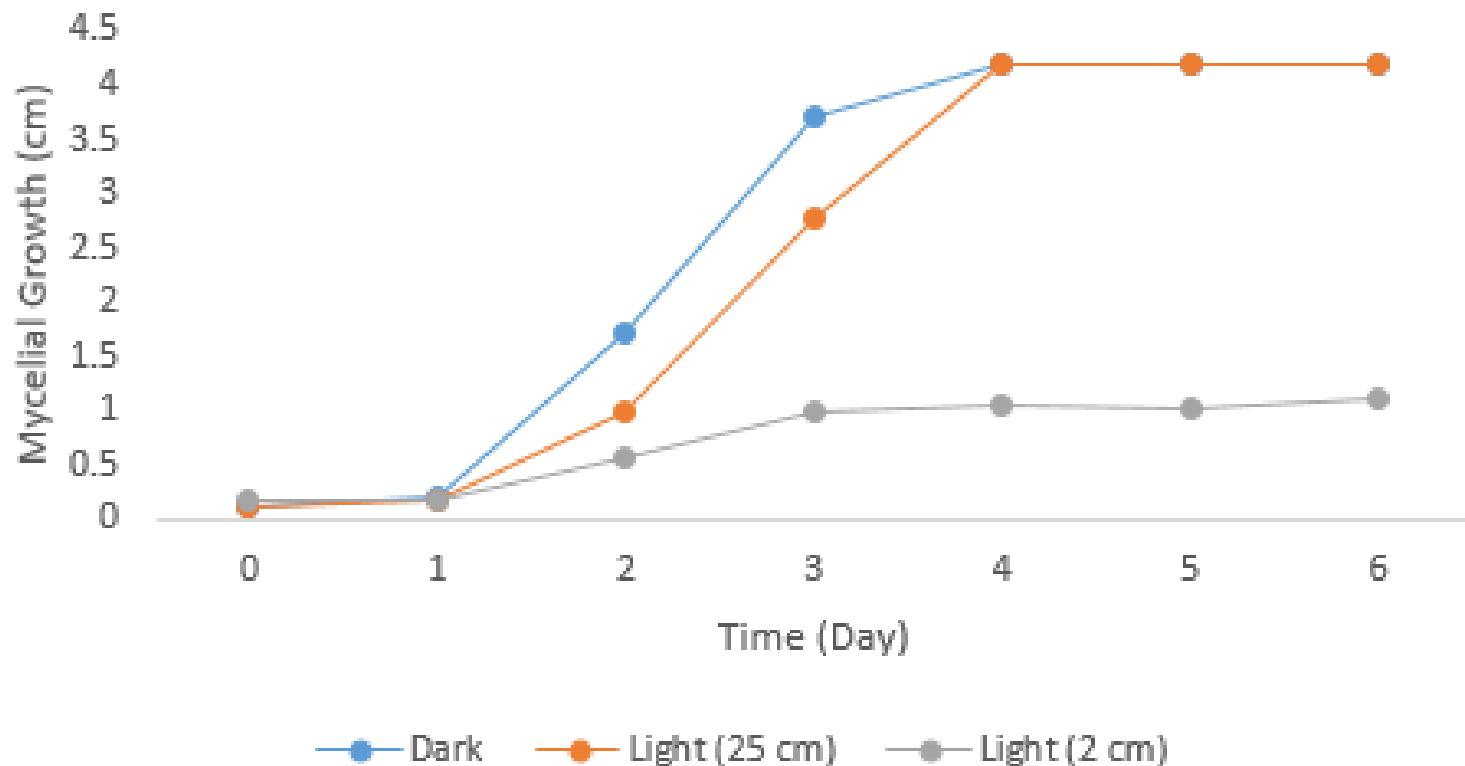




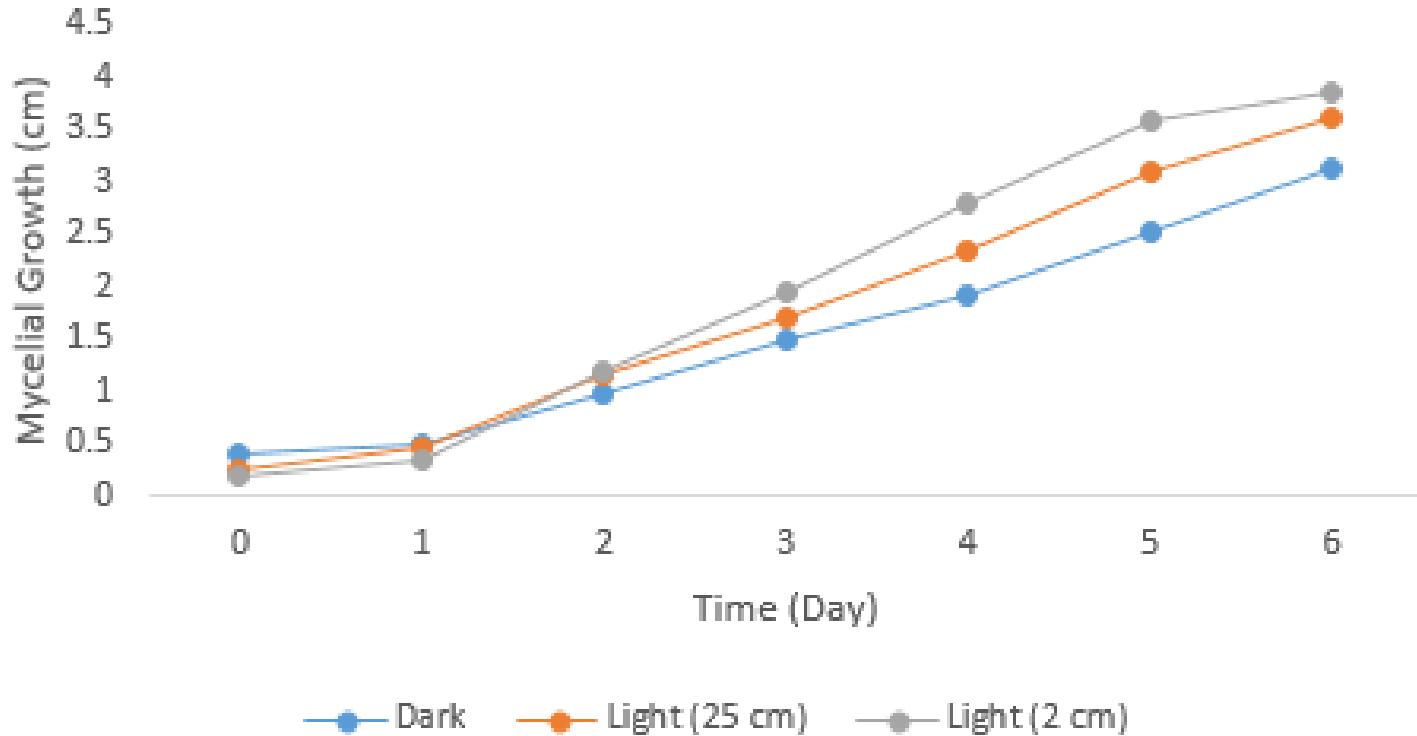
**Figure 6. Western Blot HCAR Purification Confirmation.** 10% SDS-PAGE was run and transferred to a PVDF membrane. An "anti-his-tag MAb" primary antibody was used with an "anti-mouse IgG conjugated with HRP" secondary antibody. ECL was used to visualise. Lanes from left to right on the SDS-PAGE were ladder (Color Prestained Protein Standard, Broad Range (11–245 kDa) (NEB)), ~60 kDa protein (positive control) in two lanes, pSB1A3 (plasmid control in BL21) - elution fraction 3, HCAR - elution fraction 1, HCAR - elution fraction 2, HCAR - elution fraction 3. HCAR is 58 kDa.



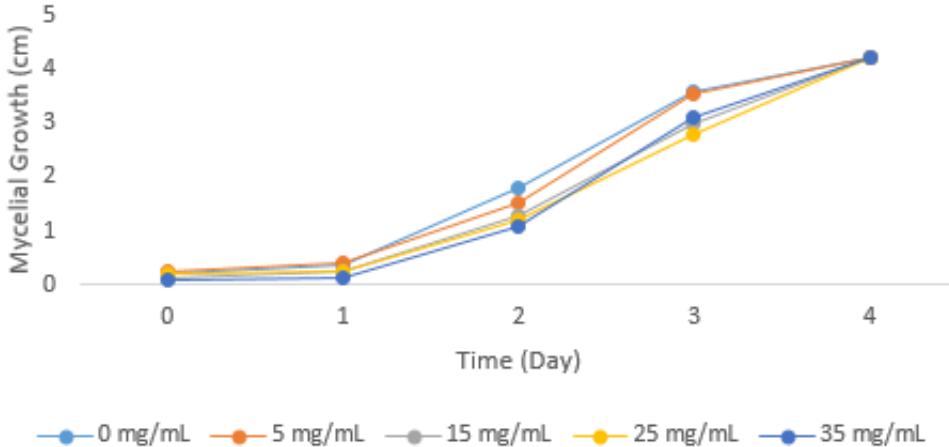
## *Sclerotinia sclerotiorum* Growth Controls



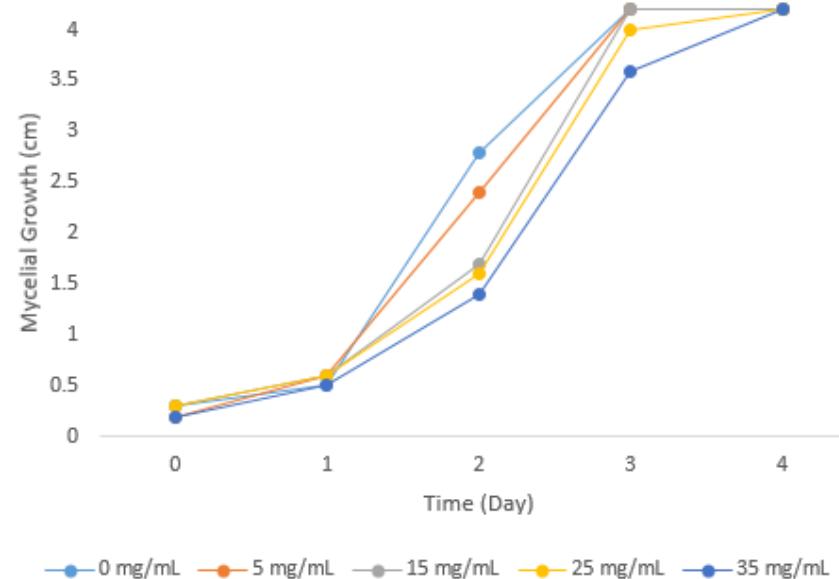
## *Pestalotiopsis microspora* Growth Controls



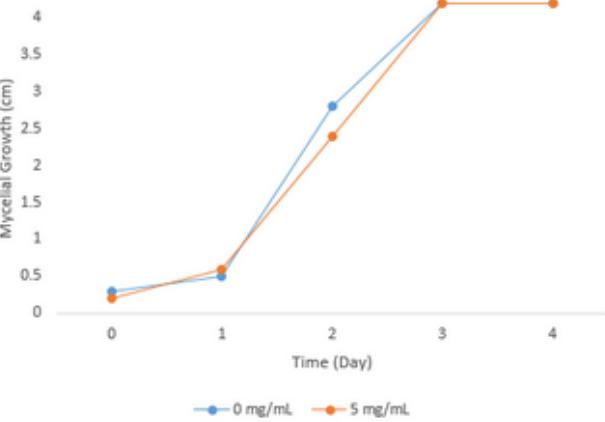
### *Sclerotinia sclerotiorum* Mycelial Growth with Pheophorbide a in Dark



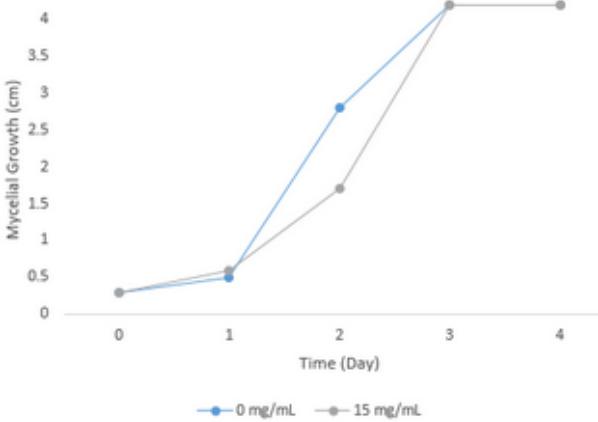
### *Sclerotinia sclerotiorum* Mycelial Growth with Pheophorbide a in Light



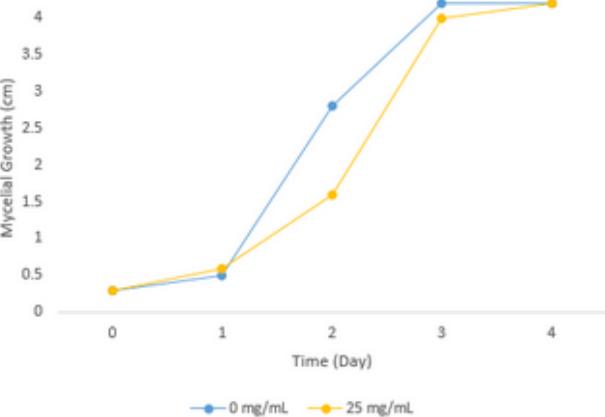
*Sclerotinia sclerotiorum* Mycelial Growth with Pheophorbide a in Light



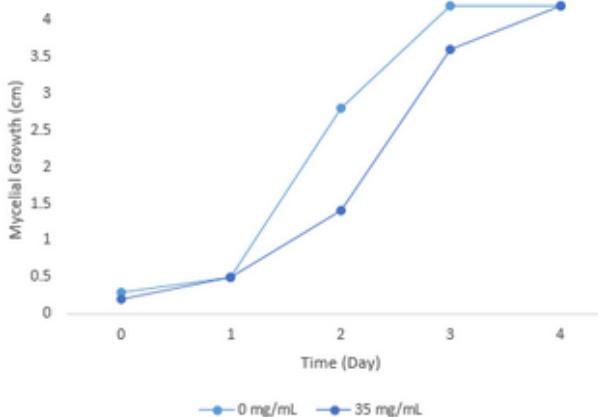
*Sclerotinia sclerotiorum* Mycelial Growth with Pheophorbide a in Light



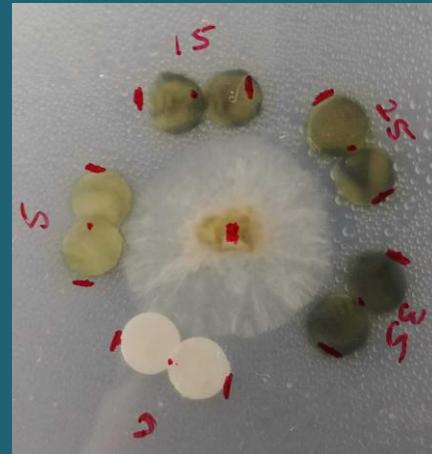
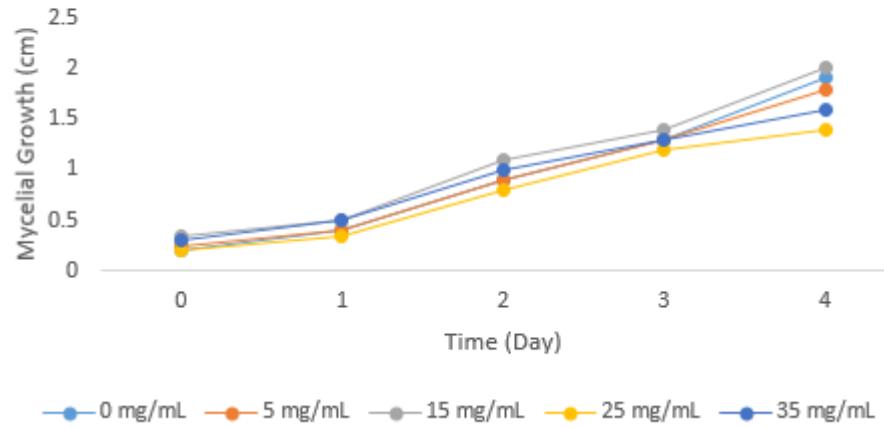
*Sclerotinia sclerotiorum* Mycelial Growth with Pheophorbide a in Light



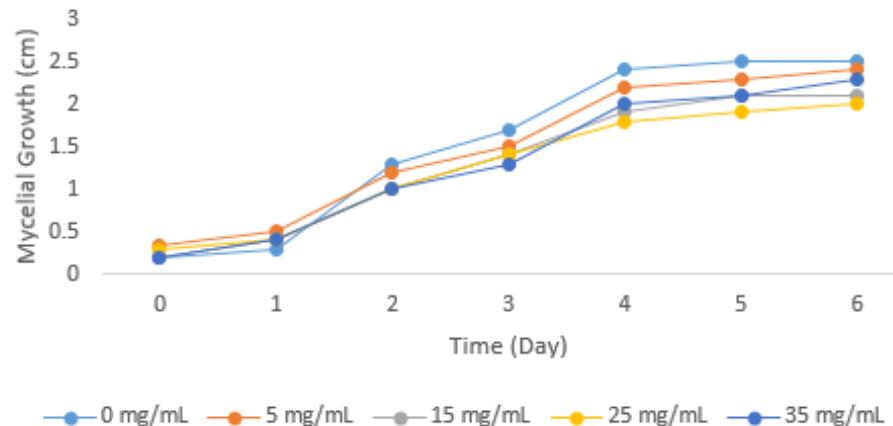
*Sclerotinia sclerotiorum* Mycelial Growth with Pheophorbide a in Light



*Pestalotiopsis microspora* Mycelial Growth with  
Pheophorbide a in Dark



*Pestalotiopsis microspora* Mycelial Growth with  
Pheophorbide a in Light



# Education

&

# Outreach



# In the Community



iGEM Calgary and The Biological Students' Association Present

## THE GOOD, THE BAD, AND THE BIOLOGICAL

Wednesday, September 25, 2019  
5PM – 7PM @ The Hunter Hub in MacHall  
(Food and refreshments will be provided)

A discussion on why we need to innovate with controversial biotechnology and how we can stop it from destroying us.

# Education and Outreach

MDSC 507: Introduction to Synthetic Biology

Lab Skills Workshop (Lethbridge)

Webber Academy (1 and 2)

Canmore Mindfuel Event

CanolaPALOOZA

Faculty Talk

JulyGEM

Telus SPARK

Pacific Northwest Meetup

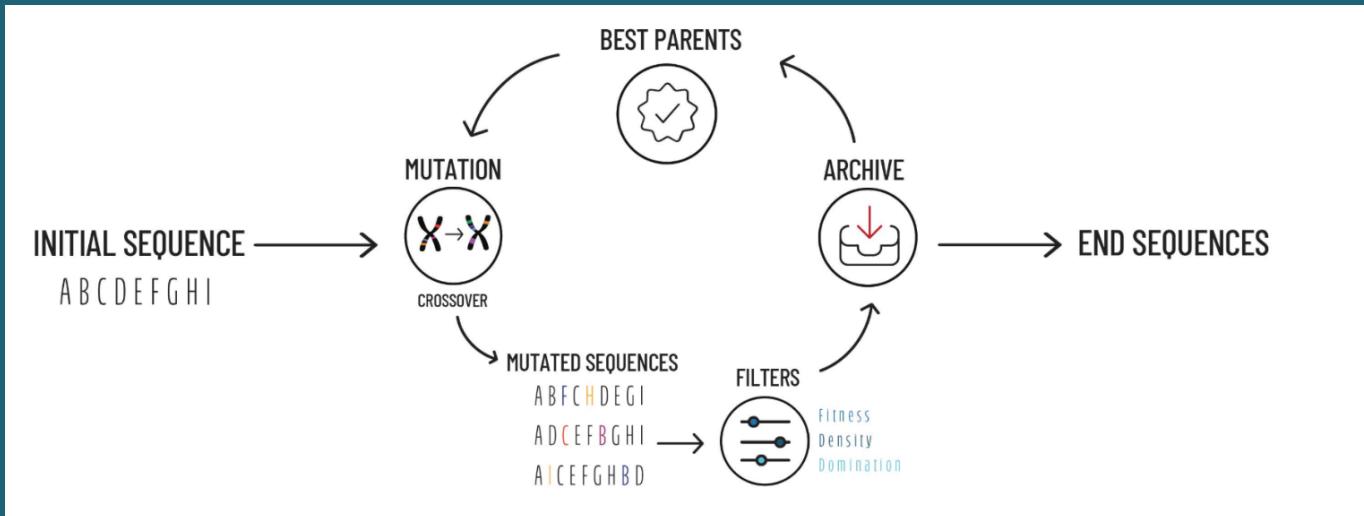
Philosophical Discussion

Bacteria Night

# Functionality

Based on the work of Brian Weitzner/codon-harmony

repeats,  
gc-richness,  
and hairpins.



# Interface

Settings

Txt\*  
 No file selected.

Host  
413997

Host threshold  
0.1

Max generation  
10

Restriction enzymes  
NdeI XbaI HpaI PstI EcoRV NcoI BamHI

Splice sites

Start sites

Gc richness max  
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Gc richness chunk size  
118

Local host profile