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The Integrated Biomass Research Initiative: Pretreatment and Conversion Systems for the Production of Advanced Biofuels and Bioproducts

Presented by Dr. Steven Peretti
Xinglian Geng, Wesley Henderson, Hasan Jameel, Steve Kelley, Sunkyu Park, Richard Phillips, Richard Venditti

20 November, 2012

NCSU Biomass Vision - Lignocellulose

Lignocellulose

Pathway Engineering

Pretreatment

Lignin

Conversion

monomers → chemicals

Depolymerization

Structural modification

High performance material

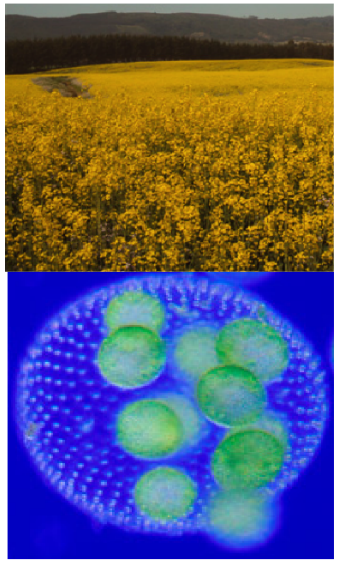
Hydrolysis

Fermentation

Cellulose
Hemi-cellulose

Mixed Sugars → Fuels, Products

Vision for Oil Crops



Oil Crop

Extraction → OIL

OIL → "drop-in fuels" (Deoxygenation and hydrogenolysis)

OIL → biodiesel + glycerol (Transesterification)

glycerol → chemicals (Conversion)

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Value-Added Product from Glycerin Using Lipase Biocatalysis

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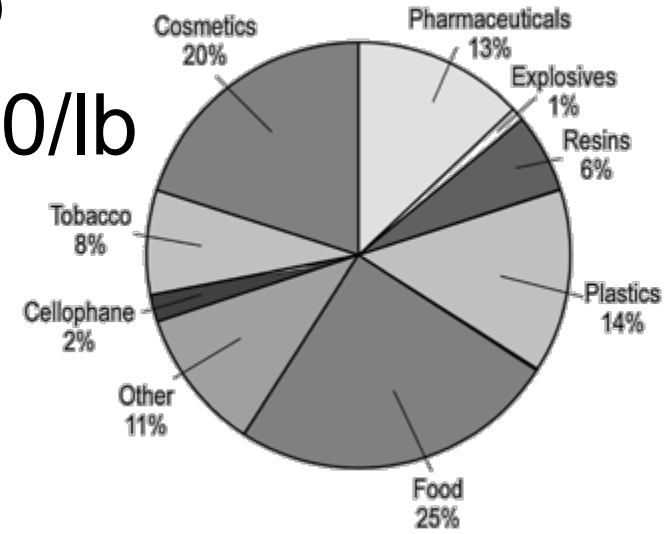
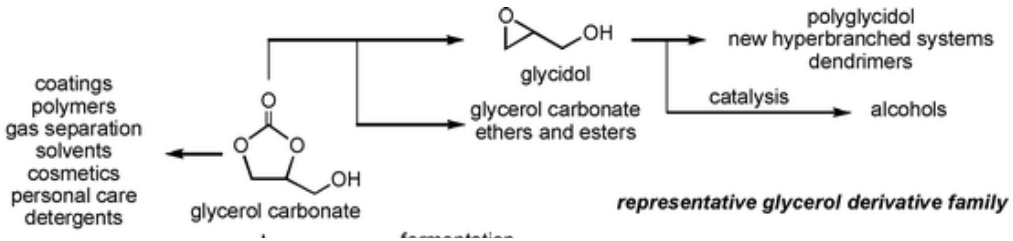
Department of Chemical and Biomolecular Engineering
November 20, 2012

Motivation

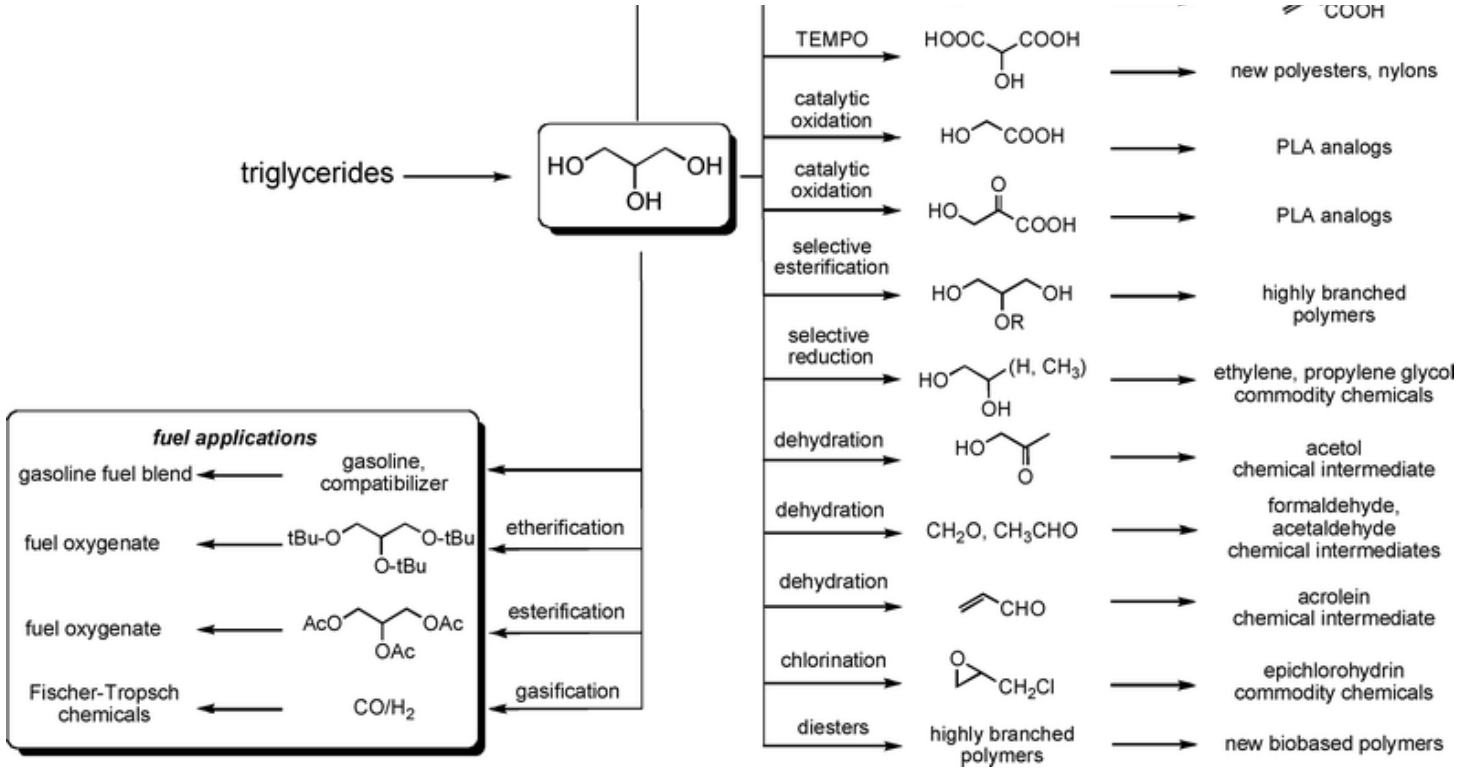
- Global glycerol production: 750,000 tons/ year
  - 90% from non-synthetic sources
  - Supply outpaces demand
  - Projected 6-fold overproduction by 2020
- Crude glycerin: USD $0.09/lb
- Technical glycerin: USD $0.50/lb
- Can be a chemical feedstock



Glycerol Chemistries

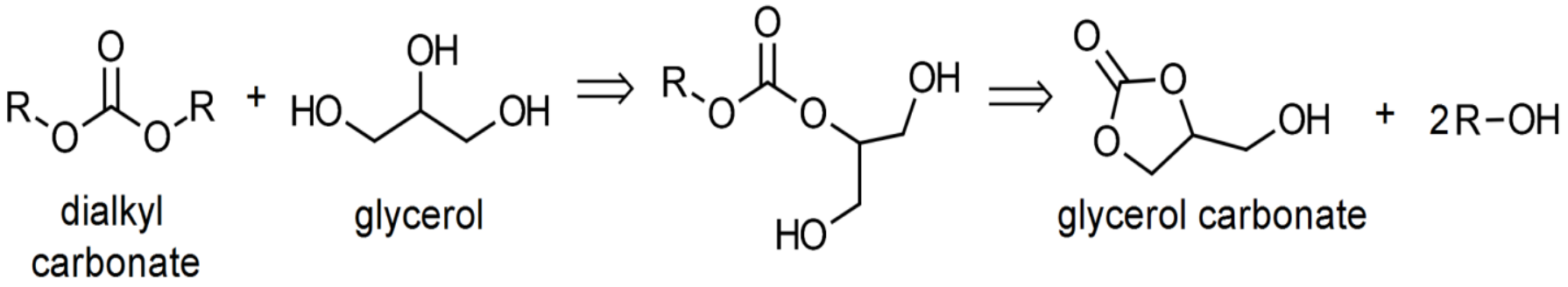


→ **Glycerol carbonate (USD $260/lb)**

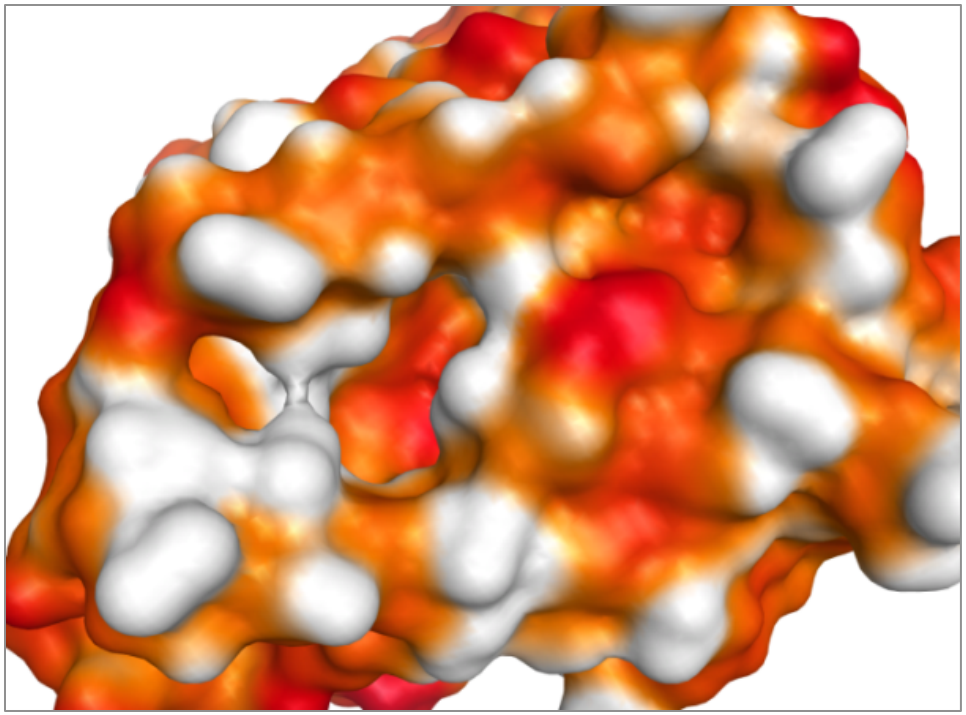


Source: Bozell 2011

Lipase Catalyzed Reaction



- After testing a number of lipases, *Candida antarctica* Lipase B (Novozym 435) was found to most effectively produce glycerol carbonate.



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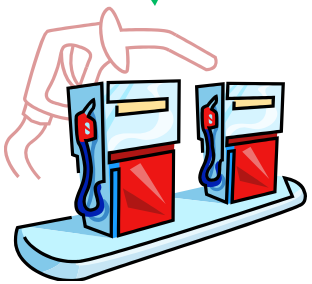
Oils and Fats

Transesterification

Biodiesel Phase

Purification and QC

Clean Biodiesel



Glycerol Phase

Removal of soap, FFA, water, and methanol.

Crude Glycerol

Dimethyl Carbonate and Tert-butanol

Lipase Immobilized Enzyme Reactor

Product Purification

-

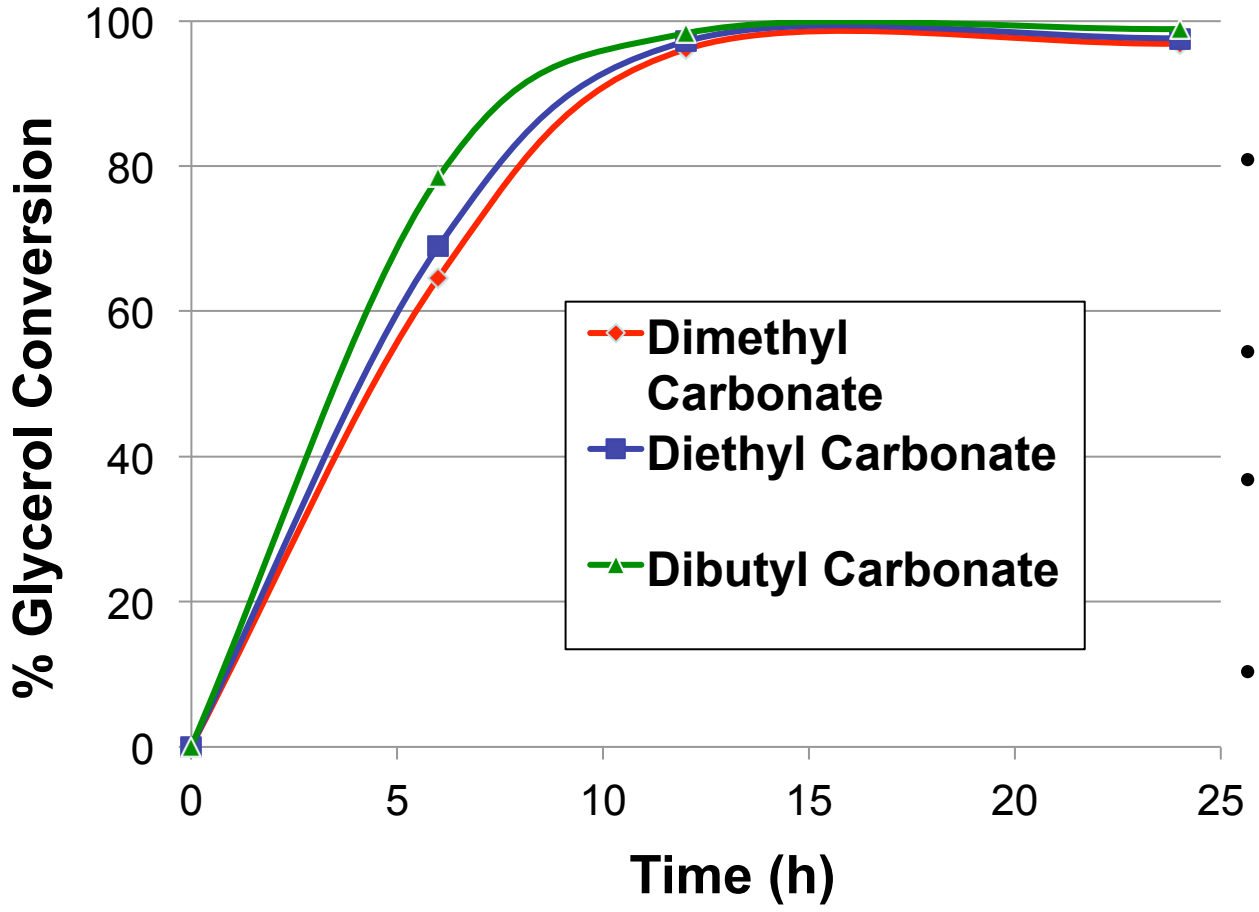
Molecular sieves to remove methanol, recovery of DMC and tert-Butanol

Glycerol Carbonate

Techno-economic hurdles

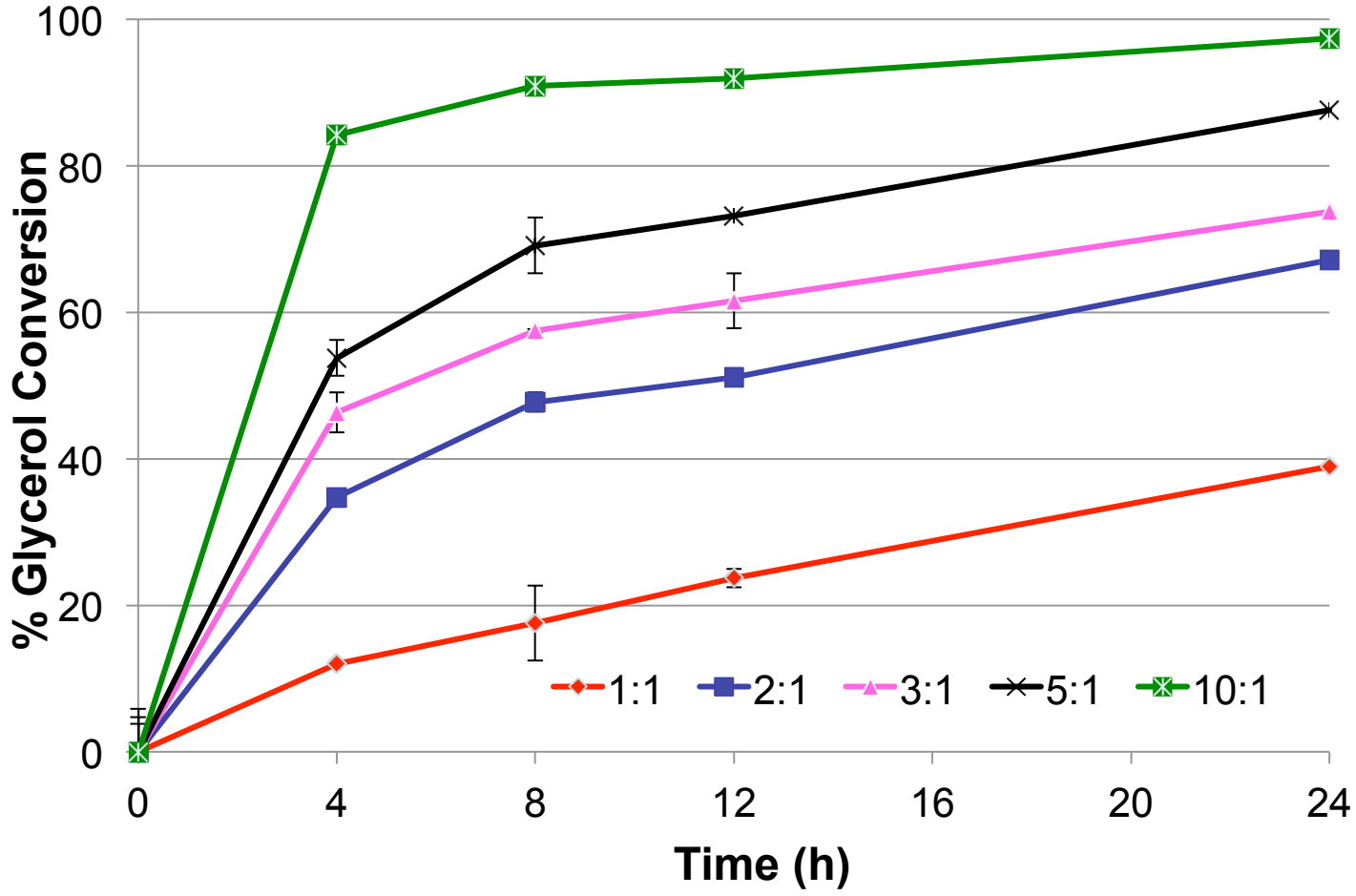
- Selectivity?
- Rapid kinetics?
- Robust catalyst?
- Inexpensive catalyst/process?

Effect of dialkyl/diaryl carbonate choice on glycerol conversion



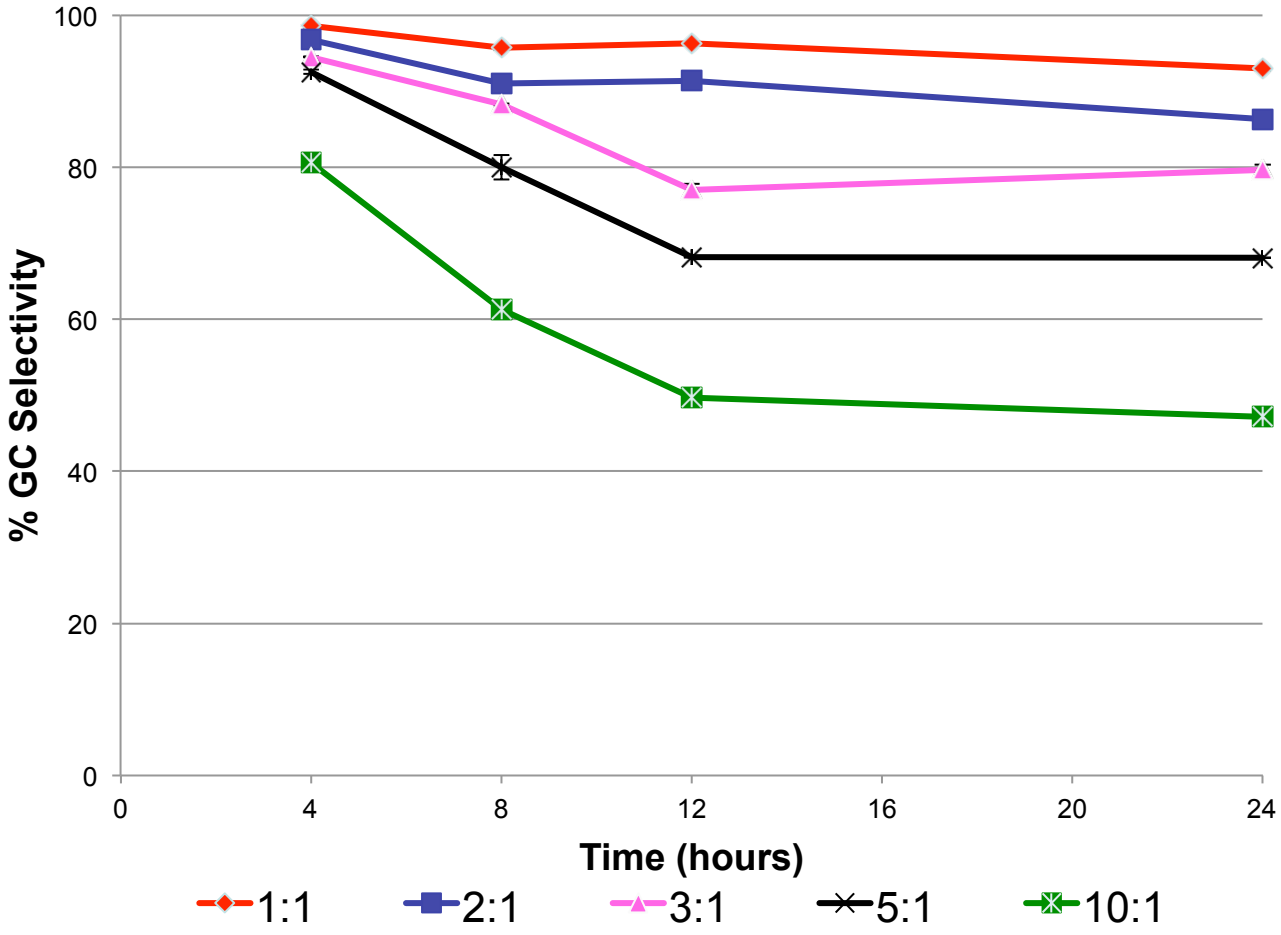
- **20 mmoles glycerol**
- **40 mmoles DAC**
- **5 mL tert-butanol and 0.1 g CalB**
- **Run at 50°C with stir rate of 350 RPM**

Effect of DMC to glycerol molar ratio



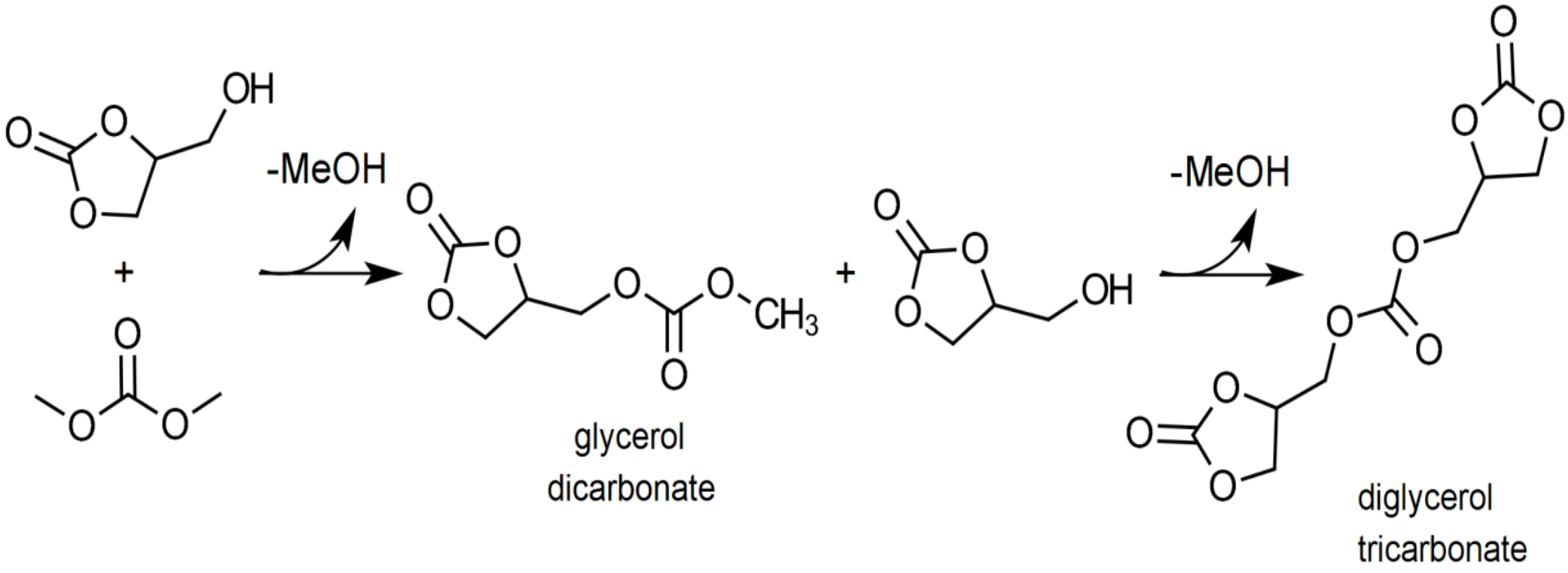
5 mL total reaction volume 0.02 g CalB, 4 mmoles glycerol, varied DMC (4, 8, 12, 20, 40 mmoles), 60°C, shaken at 250 RPM

Effect of DMC to glycerol molar ratio on glycerol carbonate selectivity

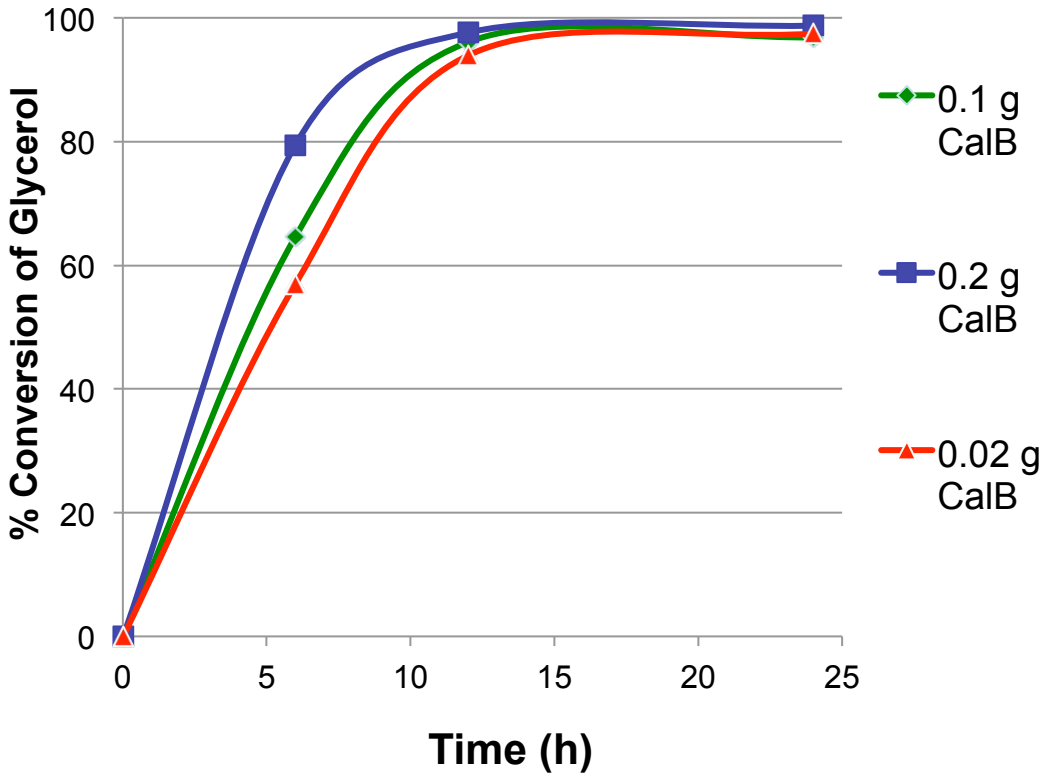


- **5 mL total reaction volume**
- **0.02 g CalB, 4 mmoles glycerol, varied DMC (4, 8, 12, 20, 40 mmoles),**
- **60°C, shaken at 250 RPM**

Where is the glycerol carbonate?



Effect of lipase loading on glycerol conversion



- **20 mmoles glycerol, 40 mmoles DMC, 5 mL tert-butanol,**
- **50°C, 350 RPM stir rate.**
- **Lipase loading varied from 1% to 10% glycerol weight (0.02 and 0. 2 g, respectively)**

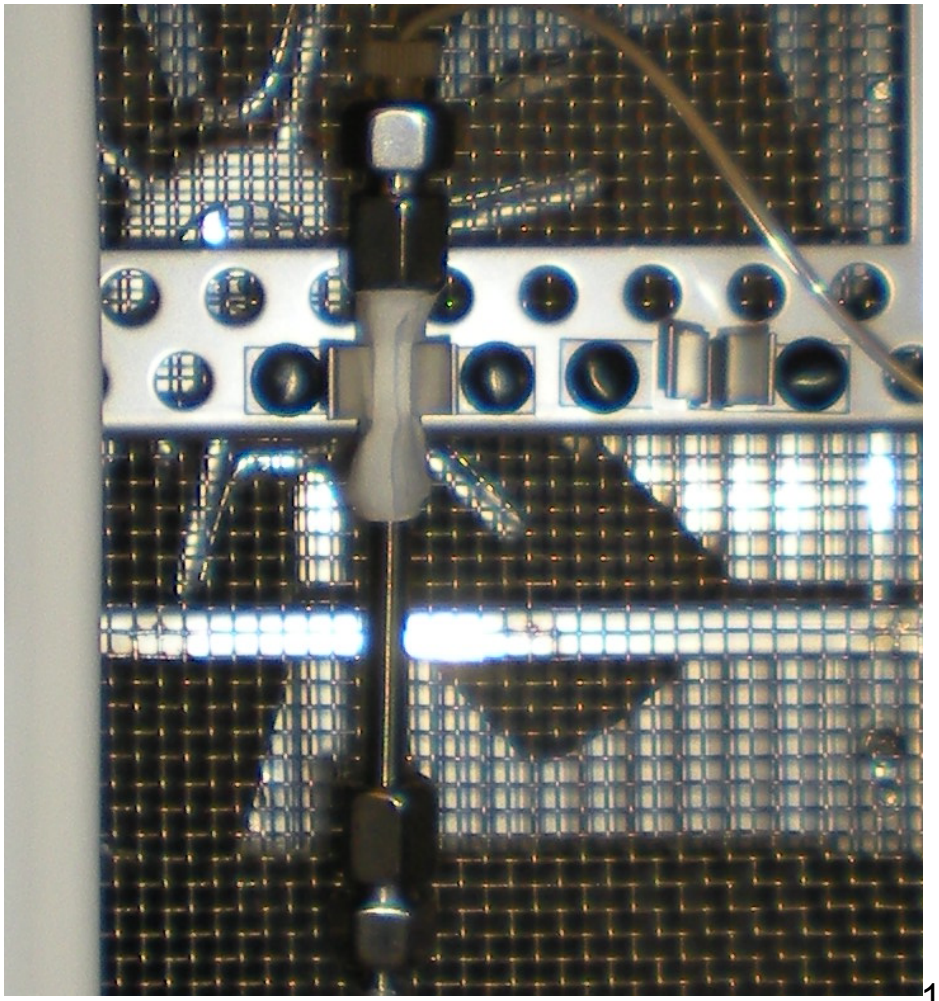
Comparison of enzymatic routes to glycerol carbonate synthesis

Process	% Glycerol Conversion	% Product Selectivity	Conditions
This study	99	>95	50°C, 12 hours, 2:1 DMC to glycerol, tert-butanol, 5% CalB loading (w/w glycerol)
Kim	94	94	60°C, 30 hours, 1:1 DMC to glycerol, THF, 55% CalB loading (w/w glycerol)
Lee	90	>90	70°C, 48hours, 10:1 DMC to glycerol, glycerol coated on silica gel; 5-20% CalB loading (w/w glycerol)
Tudorache	74	80.3	60°C, 4 hours, 10:1 DMC to glycerol, 12% Asp. niger lipase (w/w glycerol)
Tudorache	48.6	85	60°C, 6 hours, 10:1 DMC to glycerol, 2-8% Asp. niger lipase (w/w glycerol) immobilized on magnetic particles

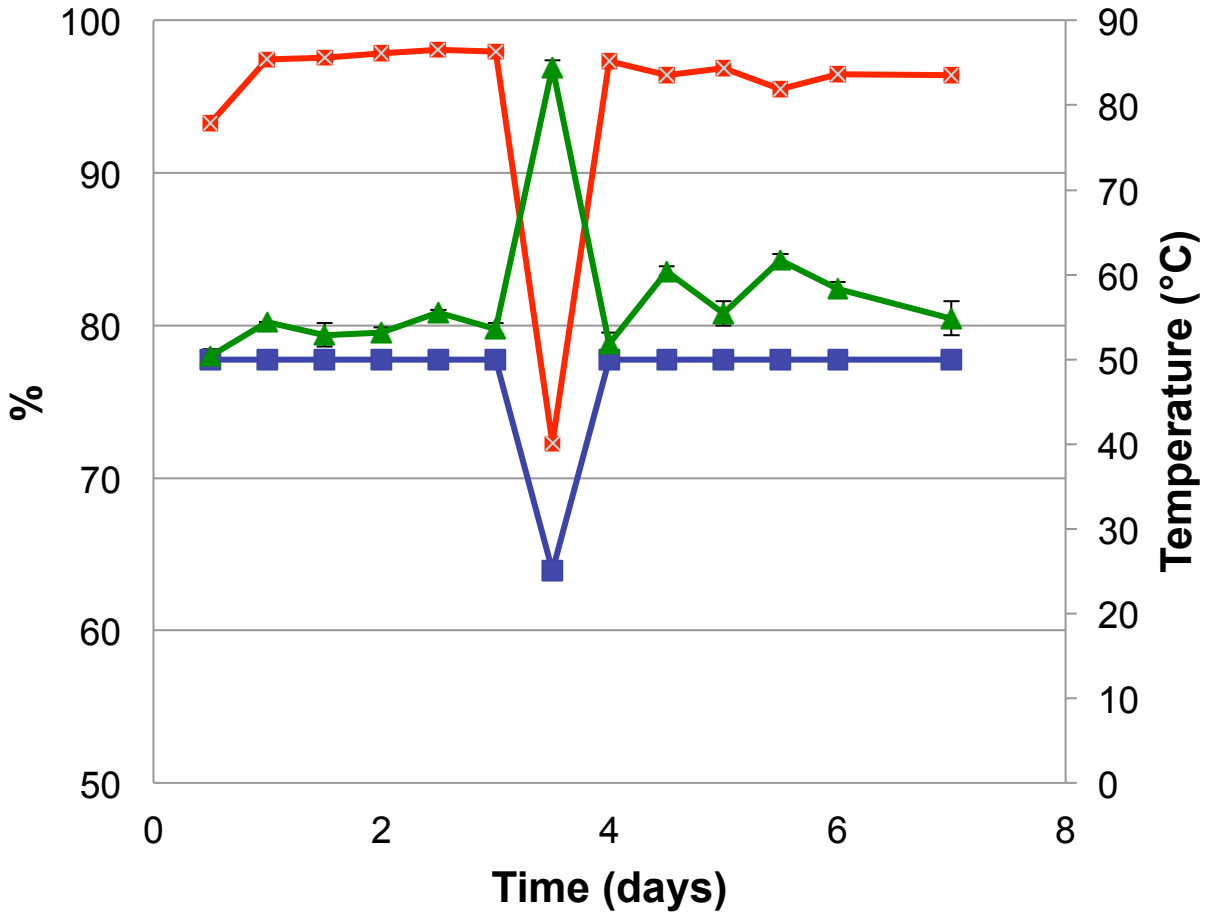
Techno-economic scorecard

- Selectivity: >95% √
- Rapid kinetics: 12 hours = not yet; try packed bed (engineering approach)
- Robust catalyst?
- Inexpensive catalyst/process?

Packed Bed reaction system

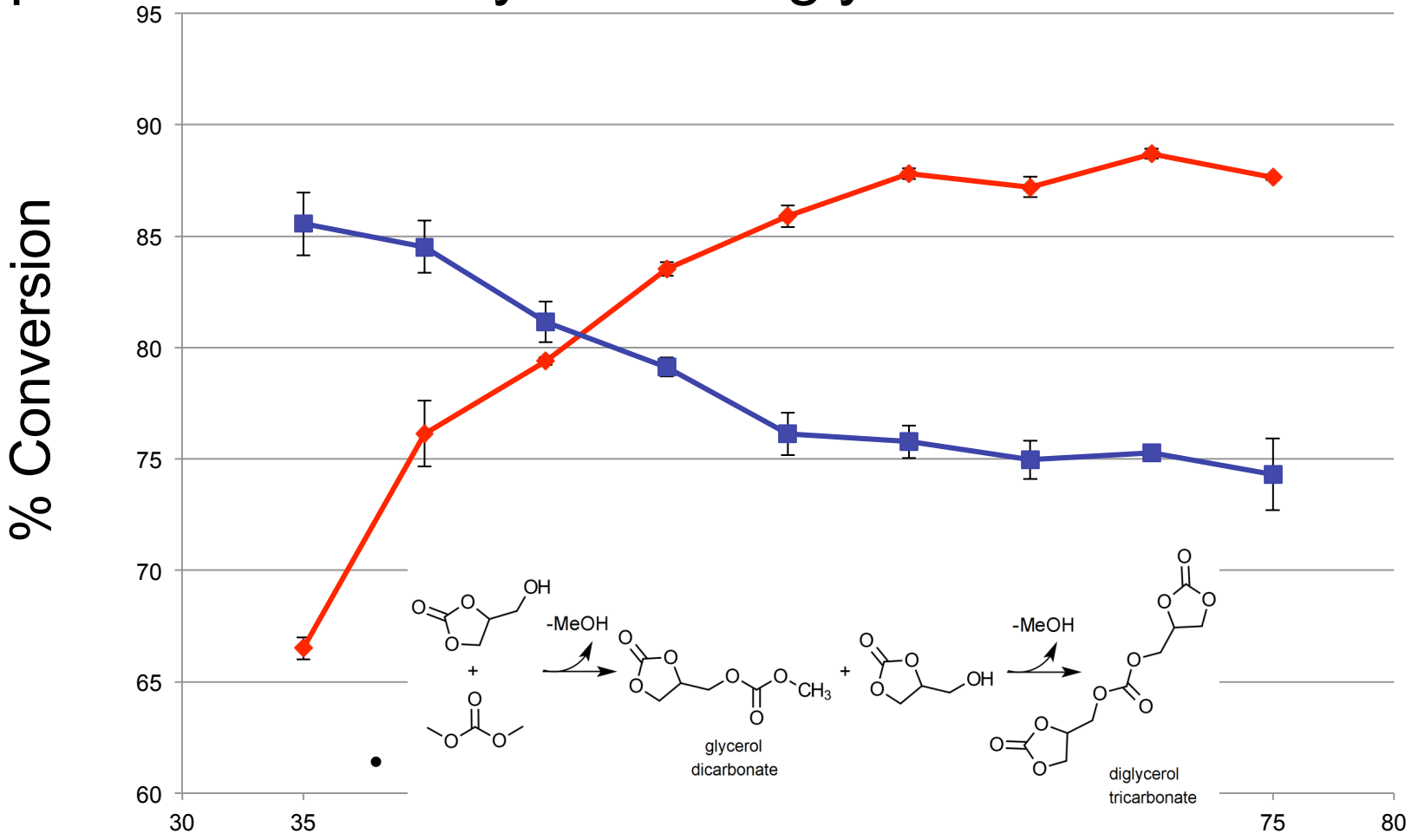


Packed Bed Reactor

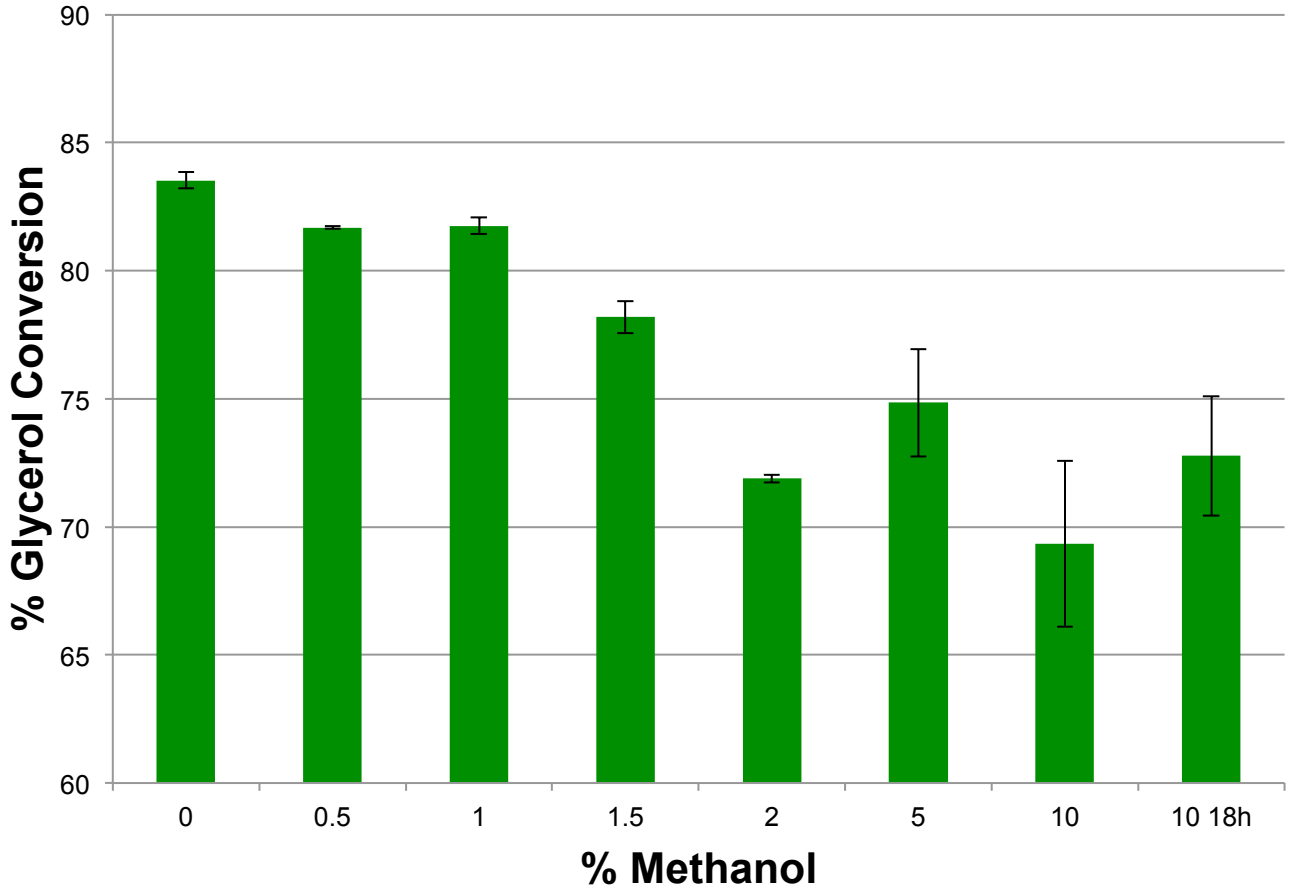


- **5:1 DMC to glycerol ratio**
- **0.55g CalB**
- **flow rate 4.8 mL/h**
- **residence time 4 m**

Effect of temperature on glycerol conversion and product selectivity towards glycerol carbonate



Effect of increased methanol (v/v glycerol) in glycerol feedstock

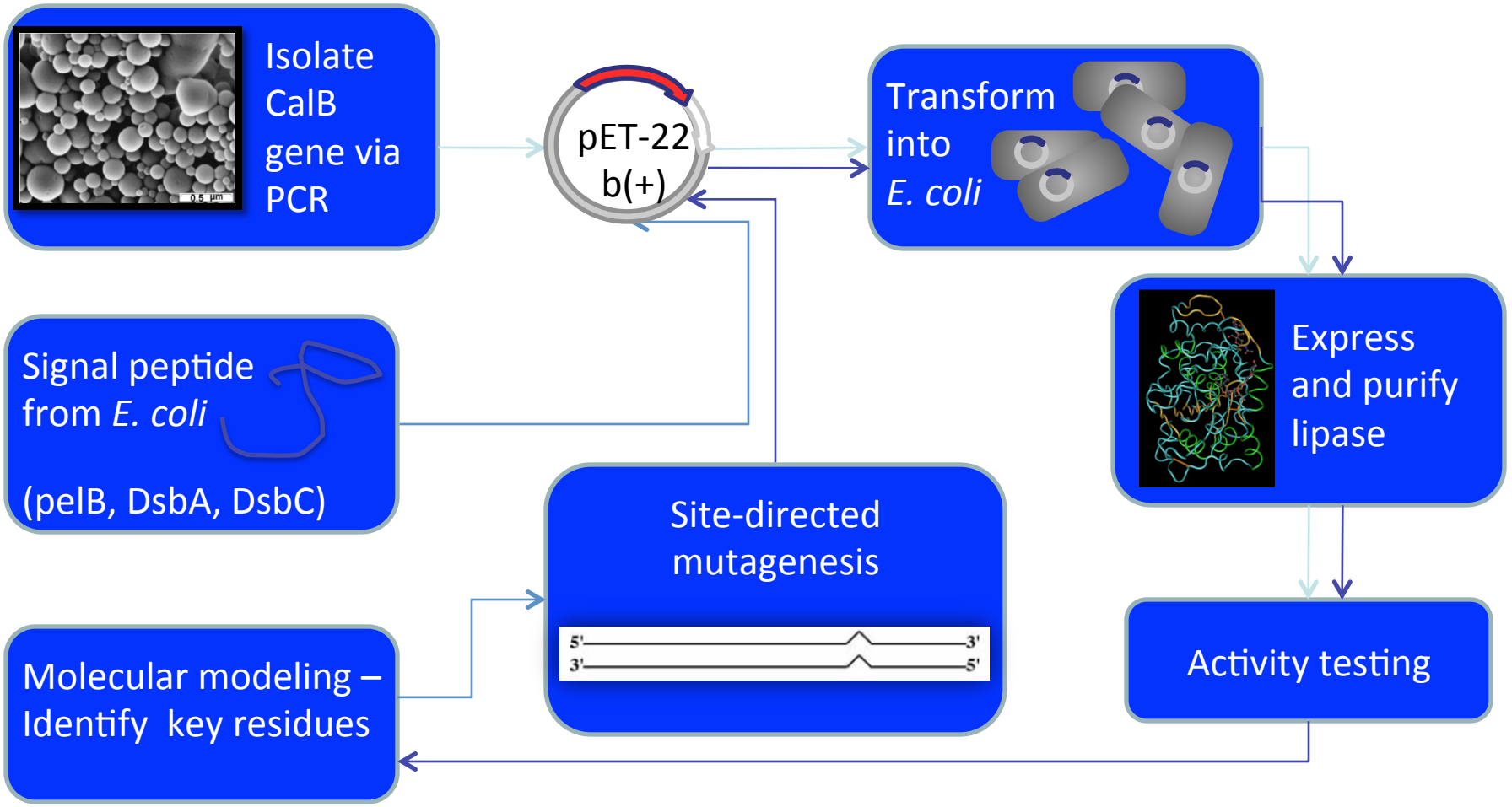


- **6 hour at each methanol level**

Techno-economic scorecard

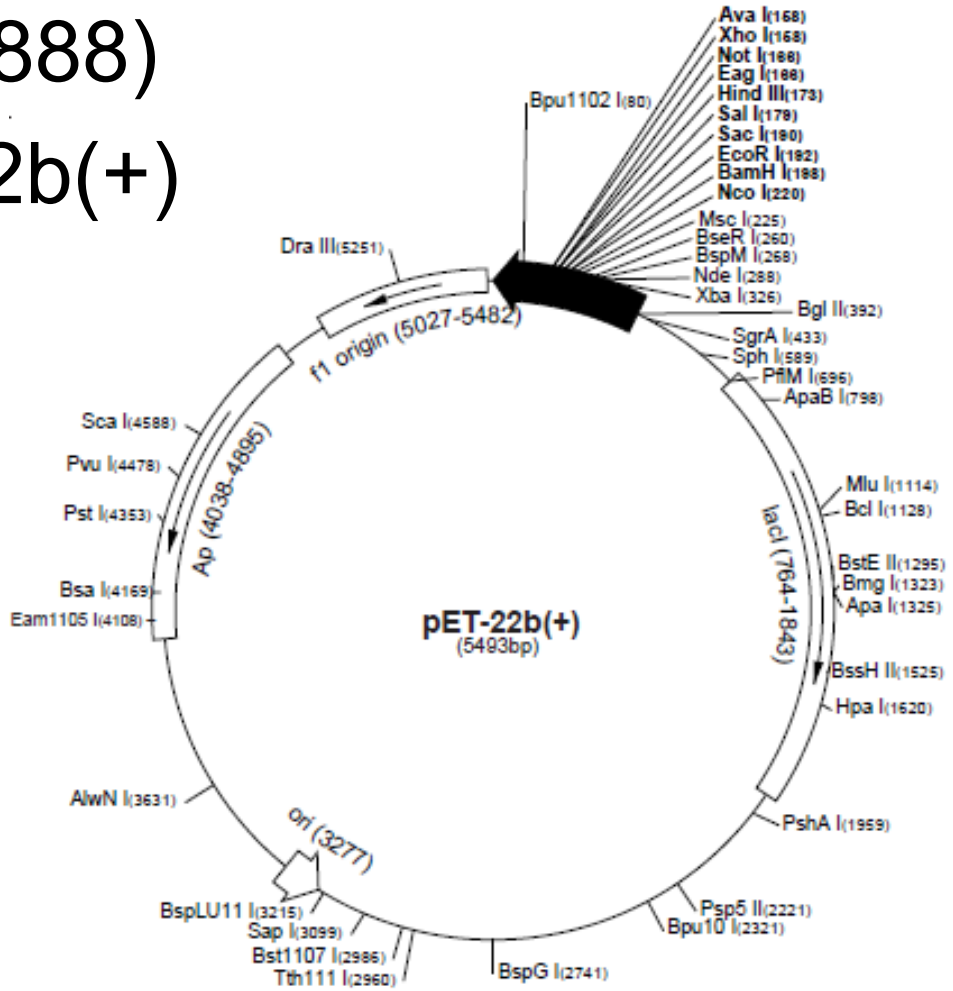
- Selectivity: >95% √
- Rapid kinetics: 4 min, > 85%, continuous √
- Robust catalyst: methanol sensitive – try mutagenesis or new enzyme
- Inexpensive: unexplored – consider methods to minimize enzyme purification

Mutagenesis to improve enzyme performance



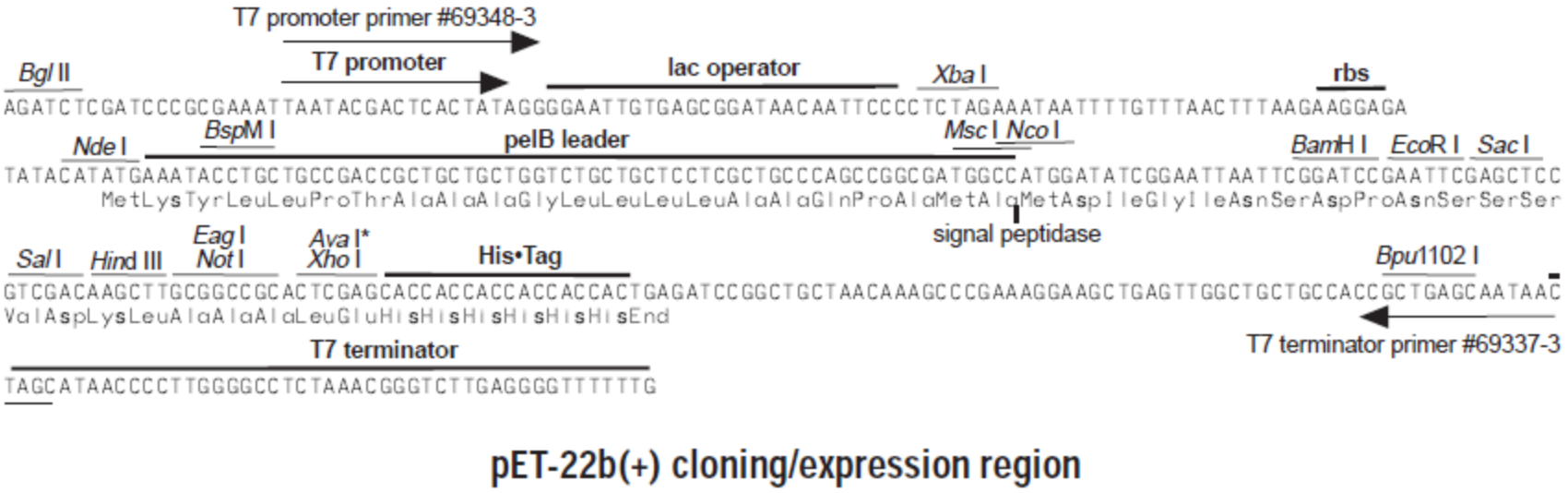
Functional Expression of CalB in *E. coli*

- Isolated CalB gene from wild type (ATCC 34888)
- Inserted into pET-22b(+) vector



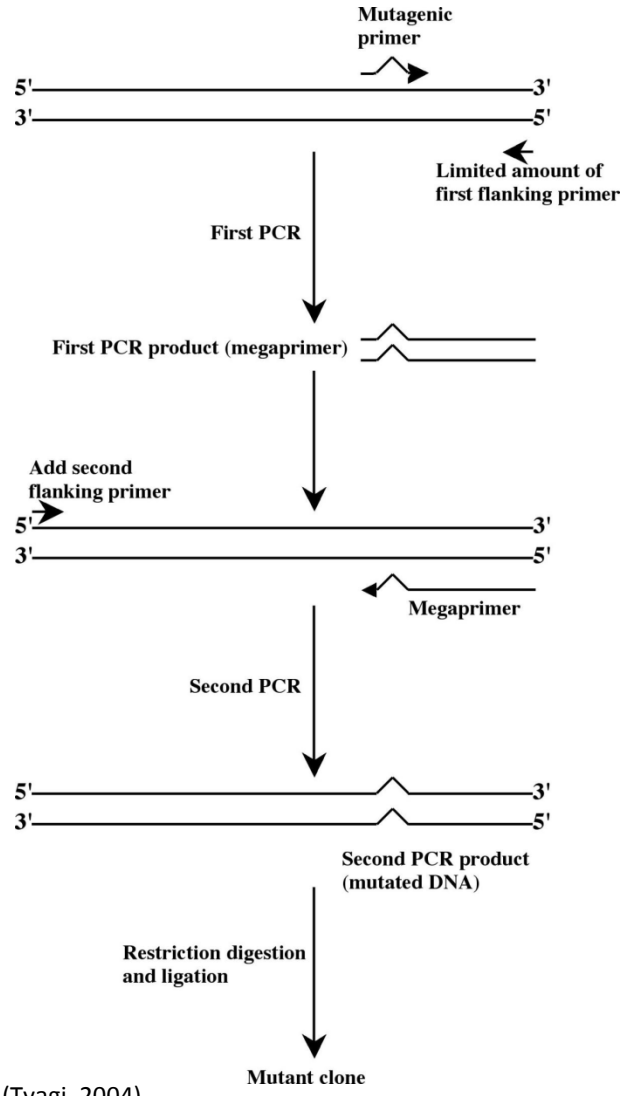
Signal Sequences

- Three signal sequence genes were separately co-inserted with the lipase gene – pelB, DsbA, and DsbC



pET-22b(+) cloning/expression region

Site Directed Mutagenesis



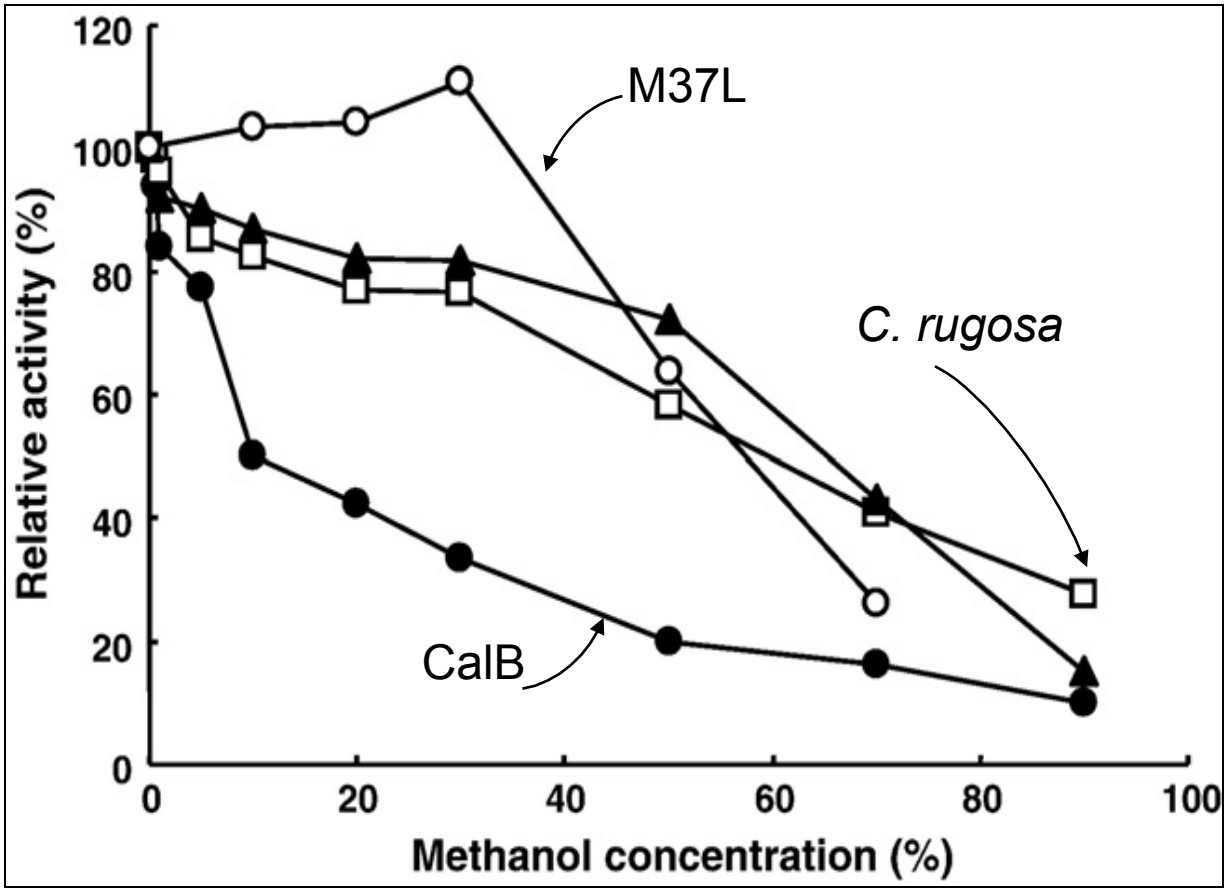
(Tyagi, 2004)

Mutations:

- **Manipulation of active site size**:
  - Leu278 →Ala, Val, Gly, or Trp
  - Trp104 →Phe or Leu
- **Entrance to pocket**:
  - Asp223 →Asn
  - Glu188 →Gln
  - Ile189 →Ala
- **Charge surrounding active site**:
  - Thr103 →GlyD223NF

A better catalyst?

- Methanol effects

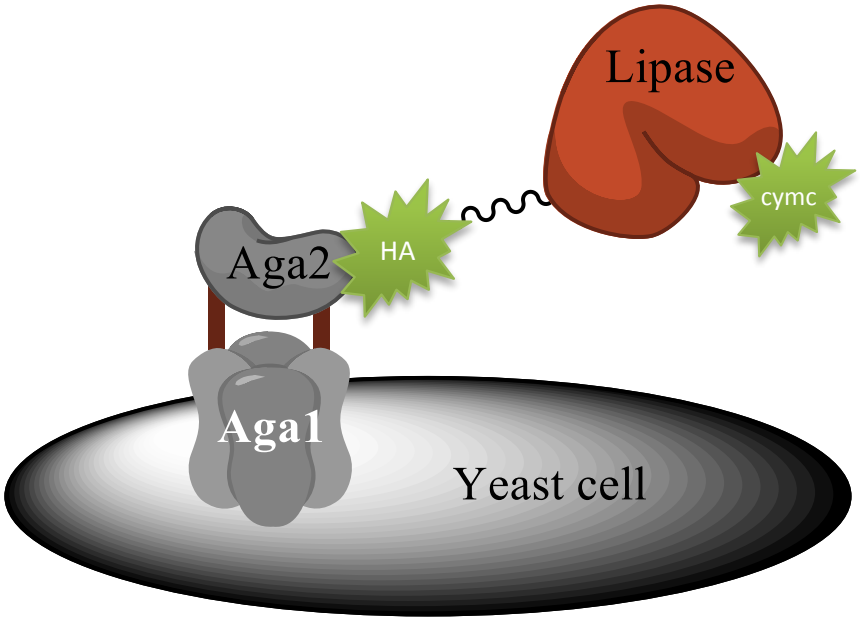


Yang, K. S. *et al*. *J Biosci Bioeng* **2009**, *107*, 599-604.

Inexpensive enzymes?

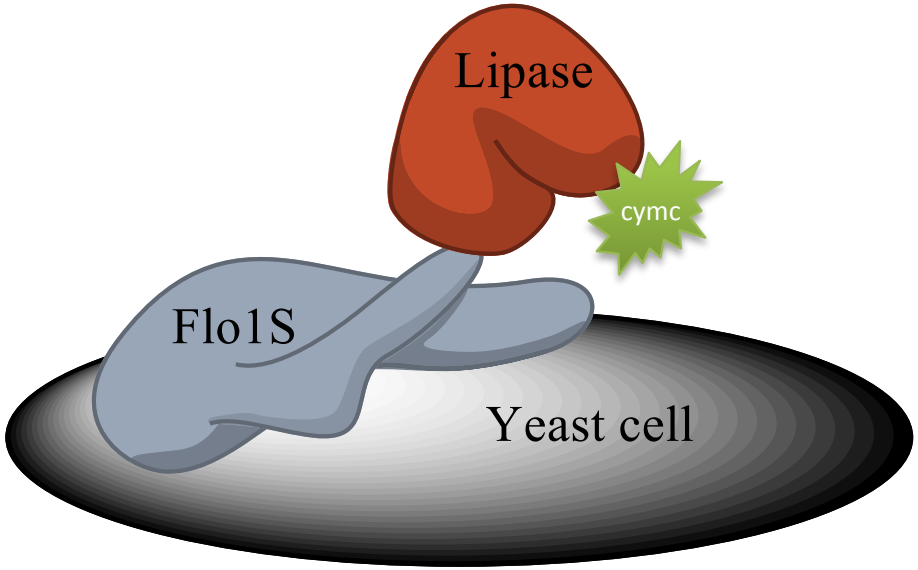
- Enzyme utilization options
  - Cell-free (*in vitro*)
    - Protein solution
    - Immobilized
  - Cellular systems (*in vivo*)
    - Excretion/metabolism (fermentation)
    - Cellular display (surface display, SD)
- Surface display advantages:
  - No protein purification required
  - Higher surface:volume ratio than macroscopic supports
  - Tunable surface coverage

Surface Display



α-Agglutinin (Aga)

- Dimeric mating protein
- Covalently attached

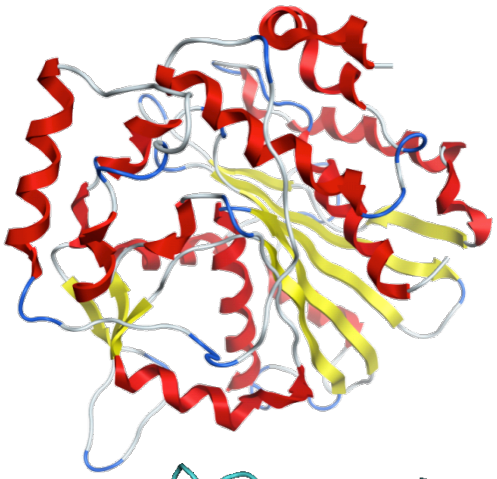


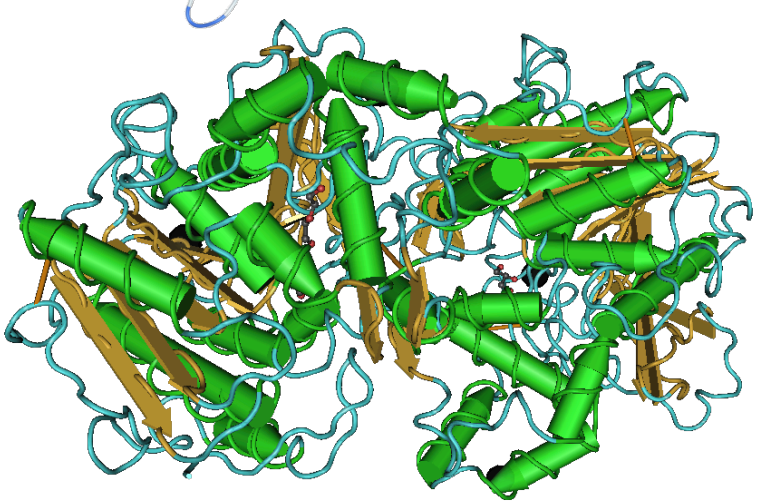
Flo1

- Endogenous flocculation protein
- Displays proteins from N-terminus
- Non-covalent attachment

Surface Display

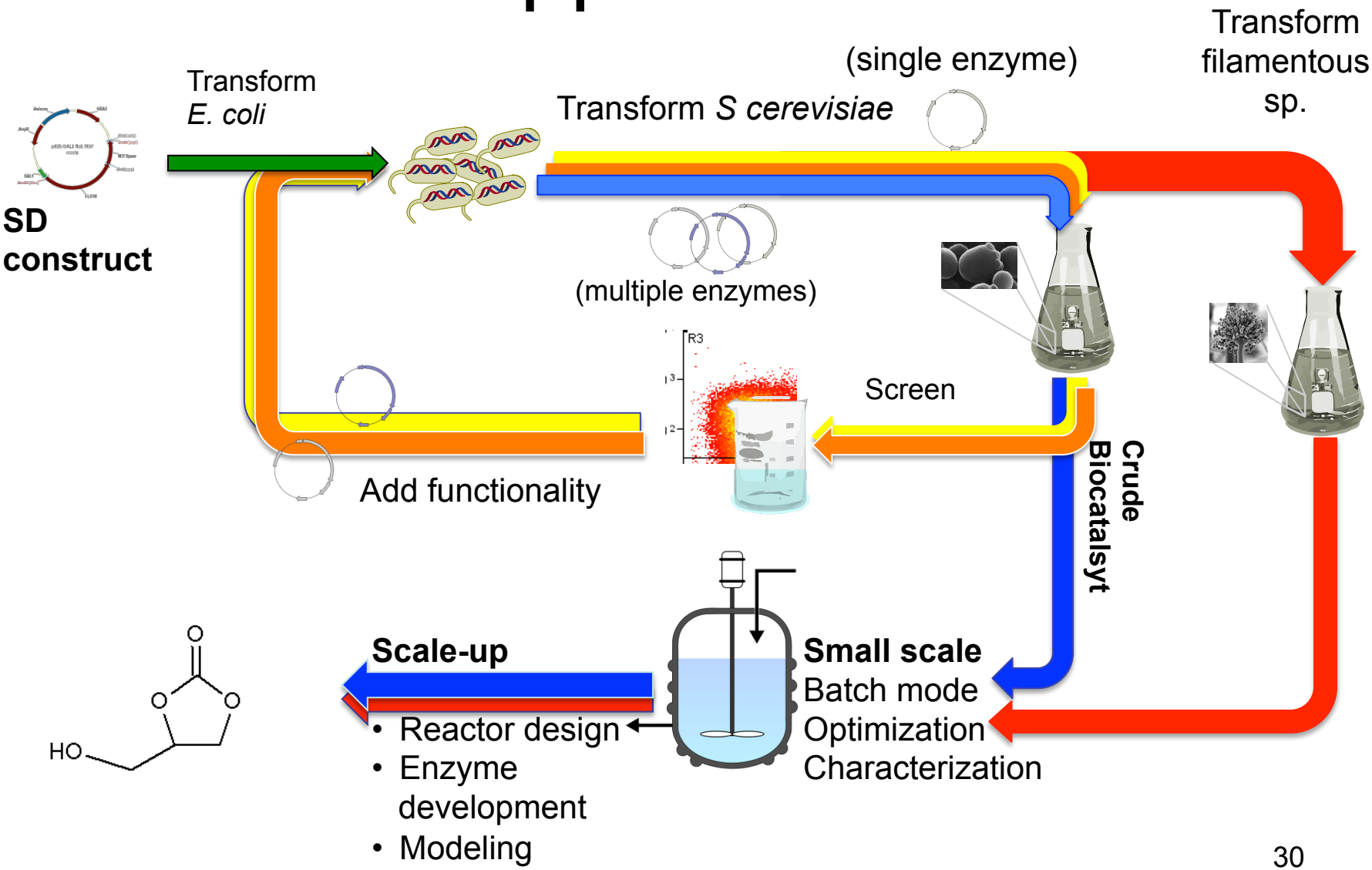
- Proteins for display:
  - M37L
    - Methanol resistant
    - Low active temperature
    - Codon-optimized for yeast
  - CalB
    - Well-characterized
    - Proven effective





Uppenberg, J. *et al. Biochemistry (N. Y.)* **1995**, *34*, 16838-16851.

Approach


Transform
E. coli
SD
construct
Transform S cerevisiae
(single enzyme)
(multiple enzymes)
Transform
filamentous
sp.
Screen
Add functionality
Crude
Biocatalsyt
Scale-up
Small scale
Batch mode
Optimization
Characterization
• Reactor design
• Enzyme development
• Modeling
HO

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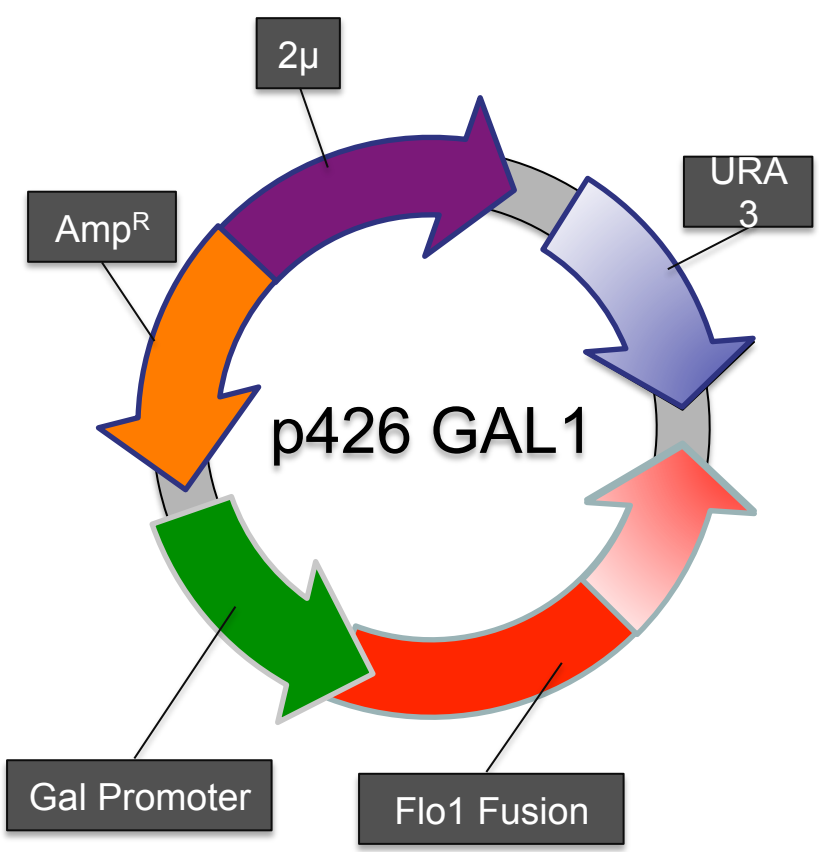
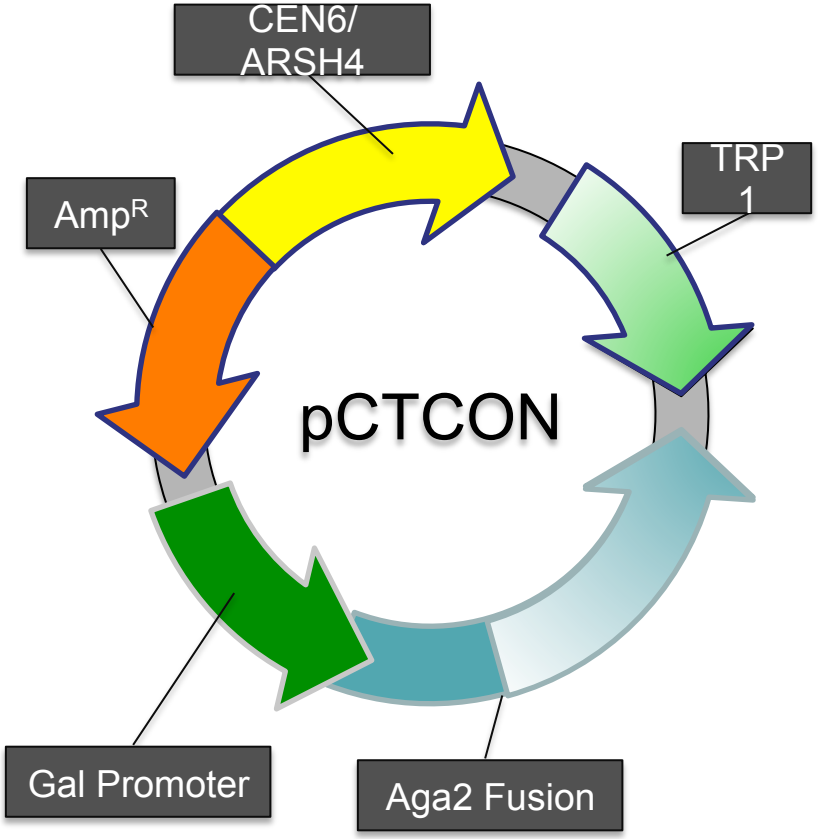
Plasmid Construction

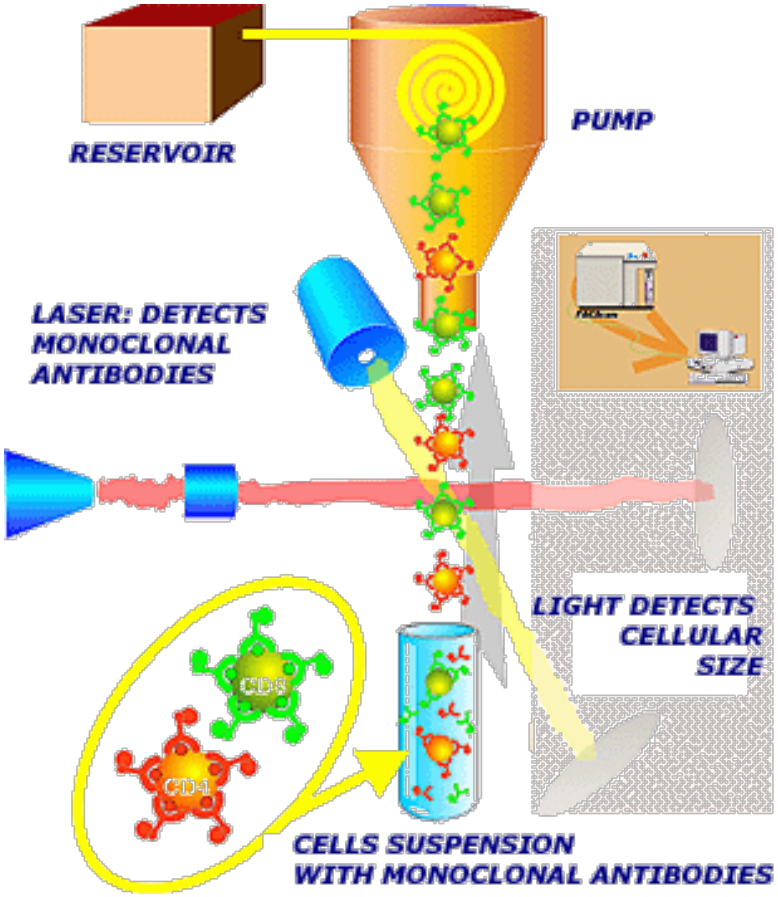


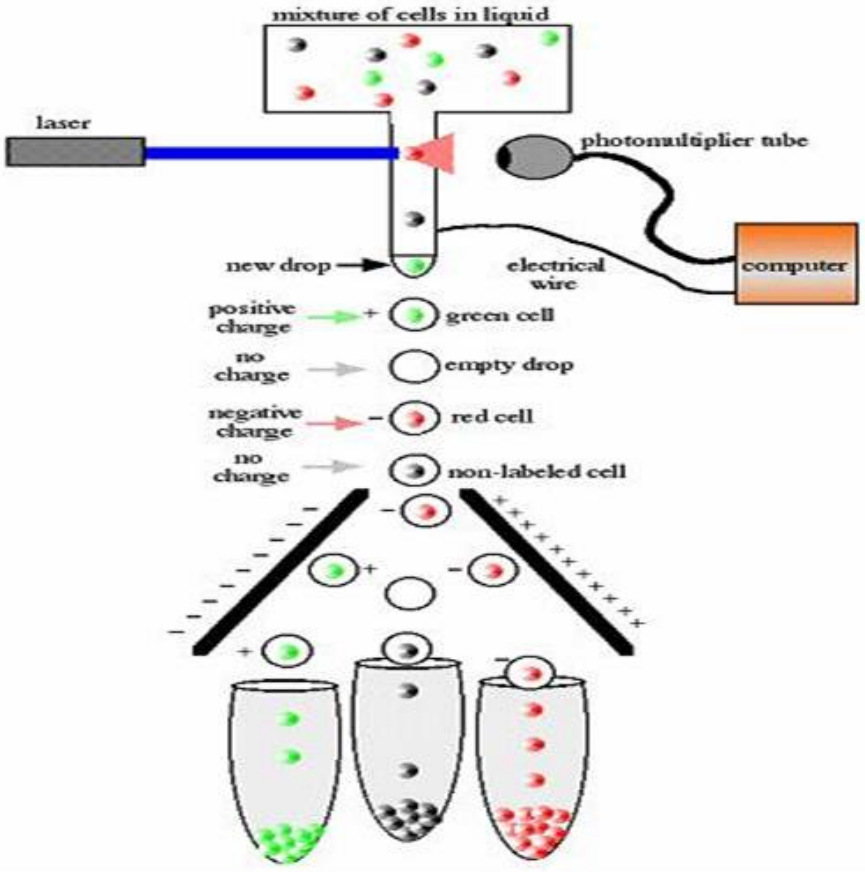


Methods

- Transform *E. coli* DH5α, *S. cerevisiae* MT8-1
  - Bacteria used to produce, store plasmid
  - Yeast used for protein expression
- Induce enzyme production with galactose
- Yeast can be used directly or **lyophilized**
- Assay for lipase activity, measure expression
  - p-Nitrophenol assay in aqueous buffer
  - Epitope tags with immuno-fluorescent labels
- Select productive cells

Selection: Flow Cytometry



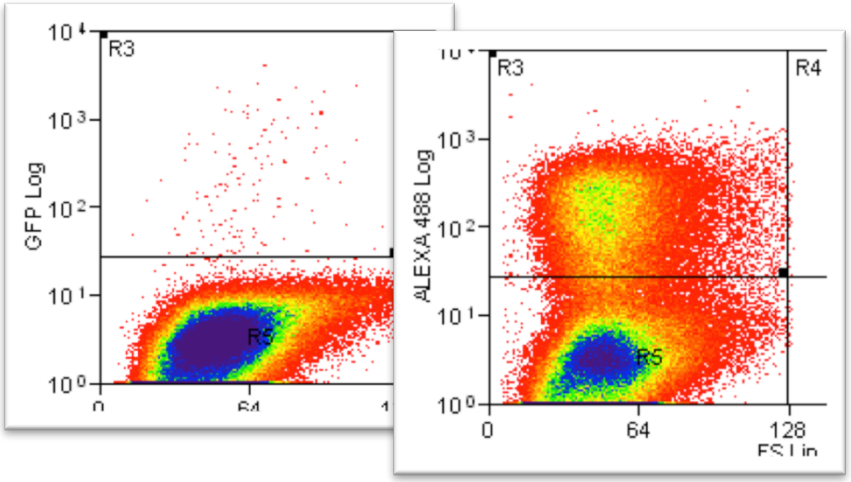


http://www.sanidadanimal.info/cursos/inmun/images/3citomet2.gif

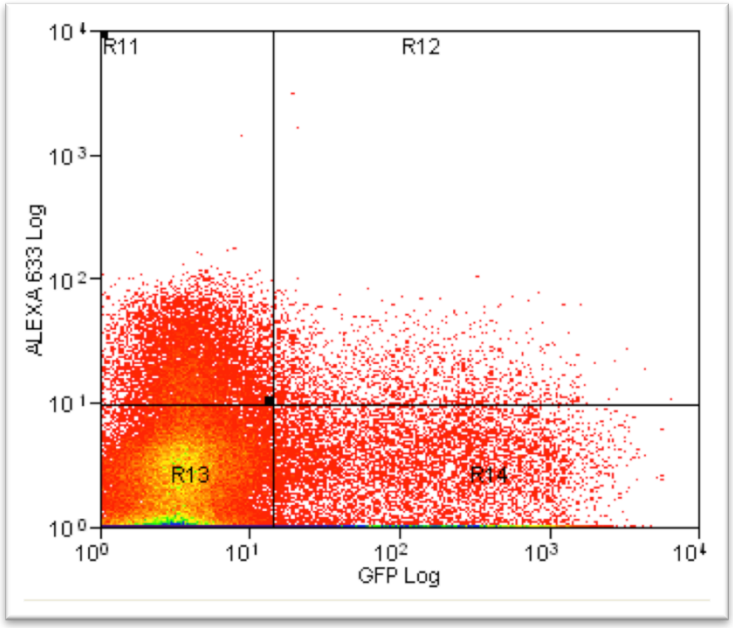
http://bitc2-preview.sr.unh.edu/TRC/INSTRUMENTATION/FCsummary.html

Initial Results

Single enzyme vs Enzyme/fluorophore



- Expressed in 50-60% of pop.
- Codon optimization improved M37L



- Lower-than-expected expression
- Single epitope tag used

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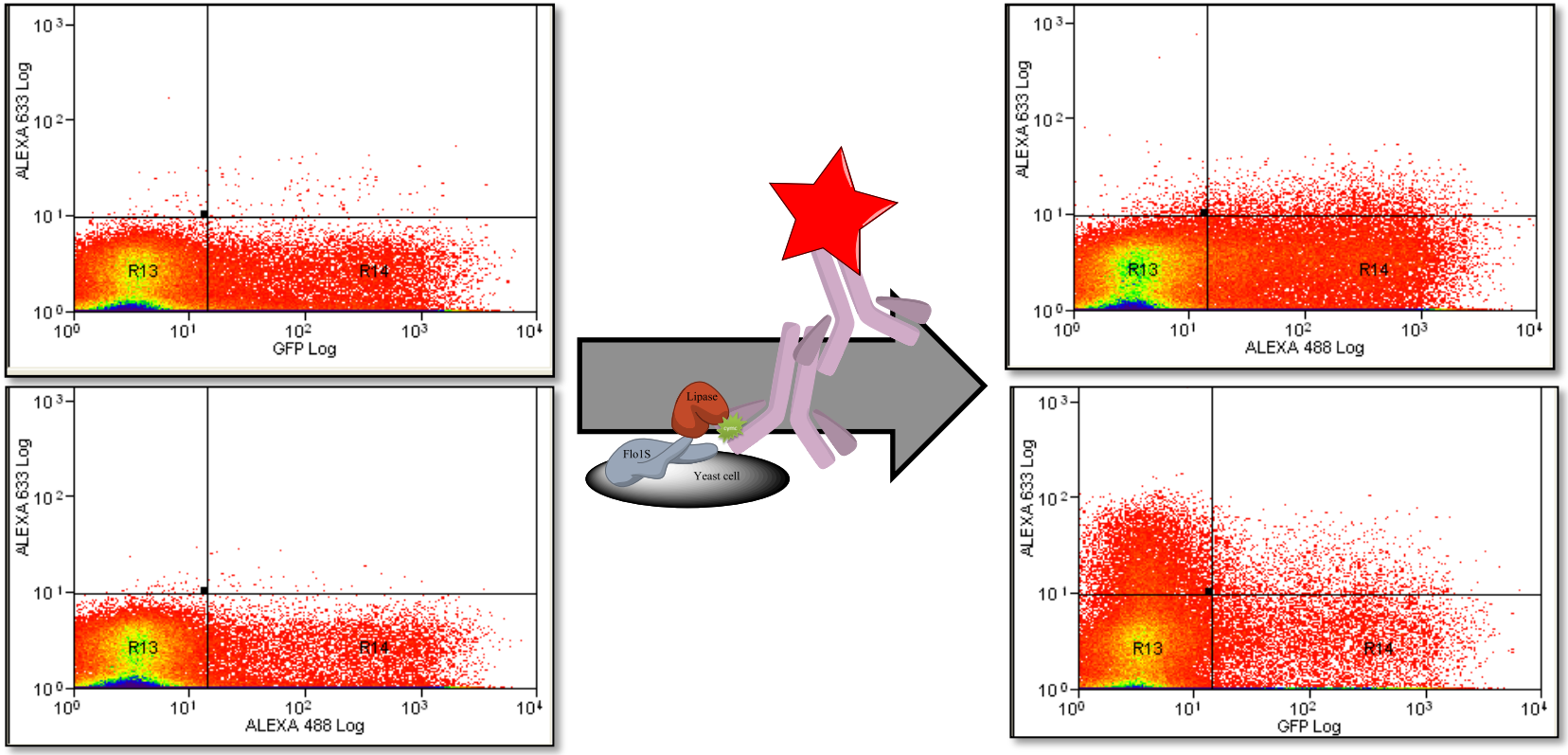
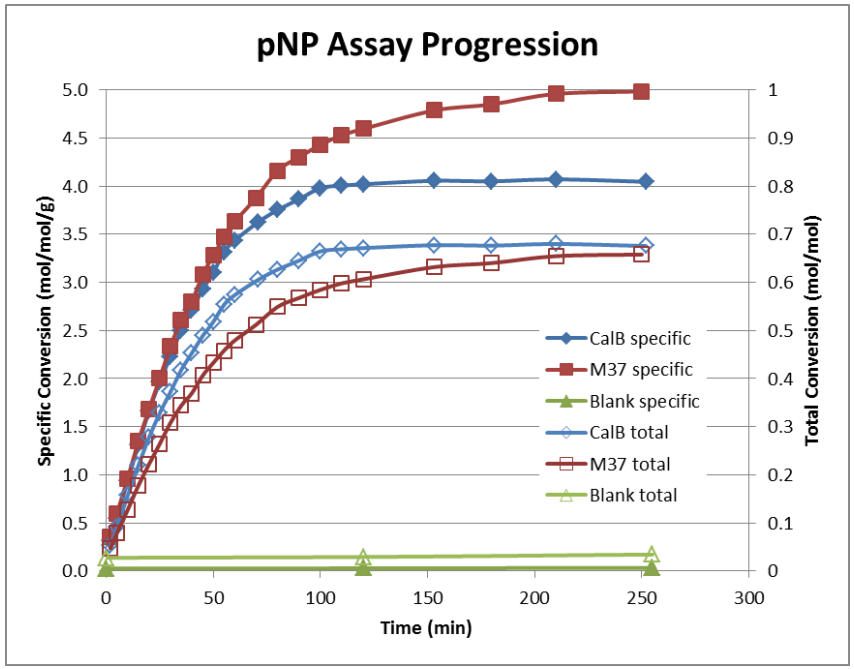
Initial Results

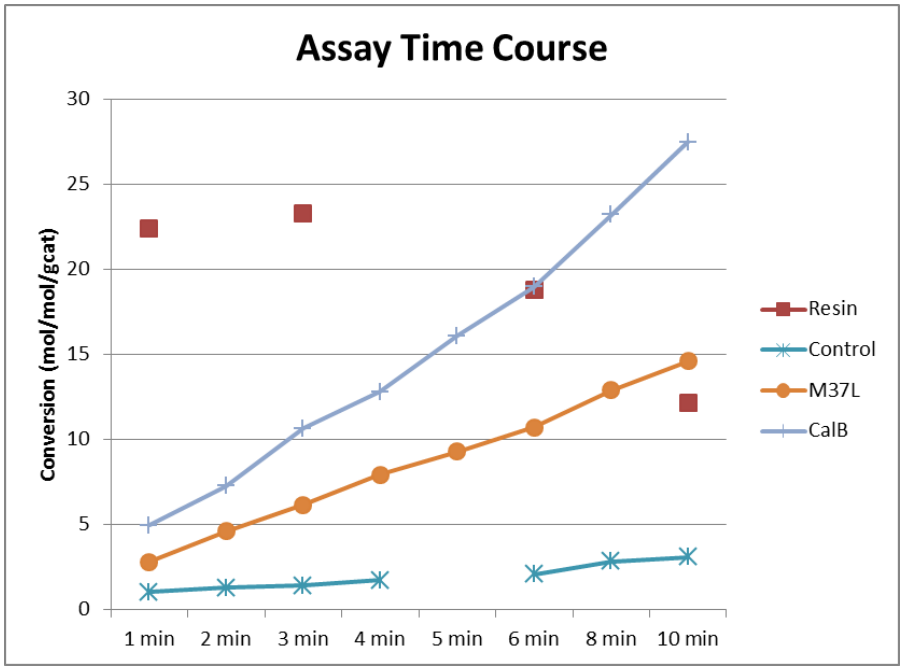
Sorting



Initial Results

- Esterase assays - lyophilized cells





Techno-economic scorecard

- Selectivity: >95% √
- Rapid kinetics: 4 min, > 85%, continuous √
- Robust catalyst: mutagenesis results in process, evaluating M37L √-
- Inexpensive catalyst/process: surface display is promising = ?
- **Glycerol carbonate is possibly an economically viable product !**

Oils and Fats

Transesterification

Biodiesel Phase

Purification and QC

Clean Biodiesel

Glycerol Phase

Removal of soap, FFA, water, and methanol.

Crude Glycerol

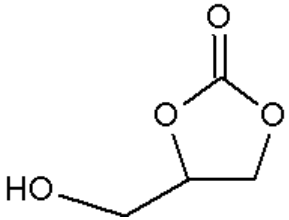
Dimethyl Carbonate and Tert-butanol

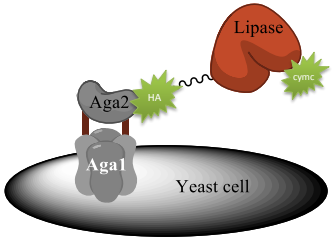
Lipase Immobilized Enzyme Reactor

Product Purification -

Molecular sieves to remove methanol, recovery of DMC and tert-Butanol

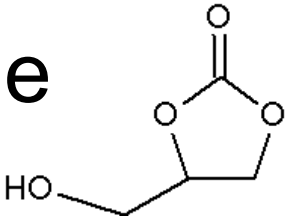
Glycerol Carbonate





Take home concepts!

- Biomaterials are complex – even the simple ones



- Complexity requires multiple approaches
- In addition to macro-scale approaches (reactor design, etc), molecular level tools will often be appropriate, including molecular biology! **Learn many techniques!**
- Is biomaterial the primary product?

Muchas gracias!