Attacking the Plastic Waste Problem: A Two-Pronged Approach

Kevin Chien, Vincent Ling, Sandy Sun, Sam Wu, Lisa Zhang, Peter Zhu
Introduction

- Plastics have become a significant environmental waste problem
- Devised two approaches to tackle this threat
  - Bioplastic synthesis
  - Plastic degradation
Bioplastic Synthesis - Background

- **Polyhydroxyalkanoic acids (PHA)** - natural storage polymers found in bacteria
- **Poly(3-hydroxybutyrate-co-4-hydroxybutyrate), or poly(3HB-co-4HB)** has elastic properties for wide range of applications
- Pathways developed so far in *E. coli* have yielded undesirably low and unpredictable 4HB-to-3HB ratios
To engineer a controlled biopathway for the production of poly(3HB-co-4HB).

To obtain more predictable compositions of the 4HB monomer in poly(3HB-co-4HB).
Bioplastic Synthesis - Methods

- **3HB Genetic Pathway**
  - phaCAB operon genomic DNA from *Cupriavidus necator*
  - Transformed and grown on agar plates with Nile Red

- **4HB Genetic Pathway**
  - Cat2-phaC

- **pASK Constructs**
  - Six different vector constructs in pASK created using PCR-Blunt II-phaCAB and pSOS-cat2 plasmids

- **Polymer extraction/characterization by H1 NMR**

Approach 1 - Background | Goals | Methods | Results | Discussion
# Bioplastic Synthesis - Methods

<table>
<thead>
<tr>
<th>#</th>
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### Approach 1
- **Background**
- **Goals**
- **Methods**
- **Results**
- **Discussion**
Bioplastic Synthesis - Methods

3HB Construct

Approach 1

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Bioplastic Synthesis - Methods

4HB Construct

Approach 1

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Bioplastic Synthesis - Methods

Poly(3HB-co-4HB) Construct

Approach 1

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Bioplastic Synthesis - Results

- Screening using Nile Red indicator
  - Provides a visual indicator of the presence of PHA in *E. coli*
  - Problems: many colonies fluoresce moderately without a strong signal
Sequencing

- Successful: pASKphaCAB-noTag, pASKphaCAB-tag, and pASKphaC were successfully constructed.
- Mutation: pSOSCat-phaC exhibited a frameshift mutation between cat2 and the lacZ promoter.
Bioplastic Synthesis - Results

- NMR of bioplastic
  - Controls:
    - Commercial 3HB
    - Nonpolymer-producing bacteria
  - NMR spectra for the negative control exhibit several peaks not present in commercial and pASKphaCAB polymers
  - 5 ppm and 2.5 ppm area for the commercial and pASKphaCAB-noTag polymers are nearly identical
  - E. coli transformed with pASKphaCAB-noTag did produce poly(3HB) polymer
Bioplastic Synthesis - Results

Approach 1 - Results

pASKphaCAB | Negative Control
Bioplastic Synthesis - Results

- NMR of bioplastic
  - Controls:
    - Commercial 3HB
    - Nonpolymer-producing bacteria
  - NMR spectra for the negative control exhibit several peaks not present in commercial and pASKphaCAB polymers
  - 5 ppm and 2.5 ppm area for the commercial and pASKphaCAB-noTag polymers are nearly identical
  - E. coli transformed with pASKphaCAB-noTag did produce poly(3HB) polymer

Approach 1

| Background | Goals | Methods | Results | Discussion |
Bioplastic Synthesis - Results

NMR for non-polymer producing control

Approach 1

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Bioplastic Synthesis - Results

NMR for commercial 3HB

Approach 1

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- Discussion
Bioplastic Synthesis - Results

NMR for pASKphaCAB-noTag produced polymer
Bioplastic Synthesis - Results

- NMR of bioplastic
  - Controls:
    - Commercial 3HB
    - Nonpolymer-producing bacteria
  - NMR spectra for the negative control exhibit several peaks not present in commercial and pASKphaCAB polymers
  - 5.3 ppm and 2.5 ppm area for the commercial and pASKphaCAB polymers are nearly identical
  - E. coli transformed with pASKphaCAB-noTag did produce poly(3HB) polymer
Bioplastic Synthesis - Results

NMR for commercial 3HB at 2.5 ppm

NMR for pASKphaCAB-noTag at 2.5 ppm

Approach 1

Results

Background

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Bioplastic Synthesis - Results

NMR for commercial 3HB at 5.3 ppm

NMR for pASKphaCAB-noTag at 5.3 ppm
Bioplastic Synthesis - Discussion

- Most of the plasmids have been constructed and verified.
- Need to finish construction of comprehensive plasmid containing the phaCAB operon and the cat2phaC gene.
- Hein et. al. study produced poly(3HB-co-4HB)
  - Used recombinant plasmid consisting of phaC and Cat2
  - Cultivation in the absence of glucose and the presence of 4-hydroxybutyrate
  - 4HB monomers were eventually replaced by increasing amounts of 3HB, producing poly(3HB-co-4HB).
Plastic Degradation

- Biodegradation of polyethylene
- Polyethylene is chemically inert, and accumulating in landfill at a rate of almost 25 million tons/year
Plastic Degradation: LadA

Originally an alkane monooxygenase

Shaped such that catalytic residues are located in deep pocket

When docking alkane substrates, tail of alkanes lie along Insertion Region 4

Requires cofactor FMN, which we can remove to facilitate phage display assay.

Separation of binding and catalytic residues mean that we can engineer binding without interfering with activity

Approach 2

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Plastic Degradation

Goals:
1. Identify the mutable region of LadA
2. Synthesis large library of LadA mutants with mutations in this region
3. Splice LadA mutants into bacteriophages to conduct phage display assay.
Computational Analysis

LadA and highlighted binding pocket

Docking alkanes with 15 carbons to 63 carbons
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Plastic Degradation - Results

Interaction Energies
Native Substrates

- Other 25%
- IS4 44%
- Inner Pocket 31%

Approach 2

Interaction Energies
Non-native substrates

- Other 38%
- IS4 39%
- Inner Pocket 21%
Plastic Degradation - Results

The region outlined in the red box is a subregion of Insertions Region 4 and contains the residues we have identified for mutation.
LadA Sequence + Mutable region

10  20  30  40  50  60
MTKKIHINAF EMNCVGHIAH GLWRHPENQR HRYTDLNYWT ELAQLLEKGK FDALFLADV
70  80  90 100 110 120
GIYDVYRQSR DTAVREAVQI PVNDPLMLIS AMAYVTKHLA FAVTFSTTYE HPYGHARRMS
130 140 150 160 170 180
TLDHTKGR1 AWNVVTSHLP SADKNF1IKK ILEHDERYDL ADEYE1VCYK LWE1GWEDNA
190 200 210 220 230 240
VIRDIENNY TDPSKVHEIN HSGKYVEVPQ PHLCEPSQPR TPVYQAGMS ERGREFAAKH
250 260 270 280 290 300
AECVFLGGKD VETLKFFVDD IRKRAKKYGR NPDHIKMFA1 ICVIVGKTHD EAMEK1NSPQ
310 320 330 340 350 360
KYWSLEGHIA HYGGGTYGDL SKYSSNDYIG S1VGEII1NN MSK1DGKF1K LSVGTPK1VA
370 380 390 400 410 420
DEMQYLVSEA GIDGF1NVQY VSPGETVFDFI ELVVPELQKR GLYRVDYE1G TYREKLF1KG
430 440
NYRLPD1HIA ARYRNISSNV
Plastic Degradation - Discussion

Replaced three residues (Isoleucine 337, Asparagine 340, Tryptophan 348) in PyMOL mutagenesis wizard. The replacements were selected with the considerations of size, shape, and polarity. This mutant showed a six-fold increase in binding affinity to non-native substrates.

Approach 2 - Background | Goals | Methods | Results | Discussion
Experimental

- We have also extracted the genome and designed primers to PCR wild-type LadA for the control run.
- LadA mutant library is synthesized using our mentor’s oligonucleotide synthesizer.
Bioplastic Synthesis - Future Direction

- Optimize the ratio of 4HB to 3HB monomers in poly(3HB-co-4HB).
- Site-directed mutagenesis
- Codon optimization
- Better screening methods for the ratio between 3HB and 4HB in the copolymer
  - Using lipases, which only degrade 4HB monomers, to degrade the plastic and then monitor the lipase activity
Plastic Degradation- Future Direction

- Perform multiple rounds of directed evolution.
- Codon-optimize desired mutants for expression in E.coli.
- Combine LadA with other components of polyethylene-degradation pathway to form a complete system.
Final Remarks

- Increase bioplastic production efficiency
- Obtain more desirable bioplastic elasticity properties
- Biodegrade plastics once thought to be non-biodegradable
Acknowledgements

- iGEM
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