Use of Biodiesel Waste Glycerol in the Bacterial Production of Polyhydroxyalkanoate (PHA) Biodegradable Plastic

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ESF Forest Biorefinery Concept

Autocatalytic water extraction of Hemicellulose

Extracted wood Chips
For pulping/paper production or burning in Cogen Plant

Hardwood Chips

Acetic acid
Valuable side product, used in polymers.

Lignin
Derived into alternative fuels, chemicals, or burned directly to generate steam and electricity.

Oligomer rich solution must be hydrolyzed to simple sugars if fermentation is the goal.
**Hardwood Hemicellulose Structure**

- **Xylan** (type Hemicellulose common in Hardwoods)
  - 7/10 xylose units Acetylated (this is where the acetic acid comes from).
  - Uronic acid residue (every 4-16 xylose units)

- **Cellulose**
  - Glucose unit

**Xylose unit**
Biodiesel is produced on the SUNY ESF campus from waste fryer oil from SU food services, for use in college vehicles and equipment.

- The current US biodiesel **production capacity** is nearing **1 billion gal** per year.
- The co-product stream of waste **glycerol** could surpass **100 million gal** per year in the near future, and many possible uses for this essentially free raw material are under investigation.
### Microbiologically produced poly-3-hydroxyalkanoate (PHA) polymers are a well known biodegradable material with physical characteristics comparable to some commercially available plastics like polypropylene and polyethylene.
Metabolix has teamed up with ADM to manufacture 50,000 tons/yr PHA plastic in the Midwest (Corn Sugar).

Biodegradable thermoplastics produced from biomass
Biodegradability studies have shown that many bacteria and fungi excrete PHA degrading enzymes in soil, sludge, fresh and sea water. These enzymes hydrolyze solid PHA into water-soluble oligomers and monomers, and use the resulting products as nutrients within their cells.

PHA Production Life Cycle

- ESF Biodiesel
- PHA Chemistry
- PHA Life Cycle
- Bacterial PHA
- Results
- Scale Up
- Biotechnology
- Nomura Lab
- Commercial
- Biogas
- Polymer Chem.

Diagram:

- Triacylglycerol
- Transesterification
- Mixed methyl esters (Biodiesel)
- Glycerol
- PHA production via bacteria
- Intracellular PHA inclusion
- Plant crops
- Photosynthesis
- CO₂
- Biodegradation
- BioPlastic (PHA)
- Plant oils
- Fermentor
- Extraction & Purification of PHA
**Burkholderia cepacia** is a bacteria common in soil and water. This organism is being used in research at SUNY ESF, and has been shown to produce PHAs from different food sources.

PHA is the material stored to conserve energy and Carbon for future use.

Benchtop bioreactors can be used to carefully control growth, and to observe product formation and food consumption.
In a typical growth experiment, PHA accumulation in the biomass closely matched the growth rate in the exponential and stationary growth phases.
At the higher BWG levels, bacterial growth rates decreased, suggesting this substrate material contains some inhibitory compounds.
Increased concentration of BWG in the media increased PHA content as a percentage of biomass, despite slower growth rates.
Northeast Biofuels (NEB) facility in Fulton, NY will produce **4M gallons** of Biodiesel per year.

Co-product stream composed of approximately 10% **Biodiesel Waste Glycerol = 400,000 gallons per year.**

\[
\frac{400,000 \text{ gal BWG}}{0.05\% \text{ BWG in Media}} = 8 \times 10^6 \text{ gallons media handled/yr} = 8 \text{M gal/yr.}
\]

\[
\frac{8 \times 10^6 \text{ gal}}{336 \text{ days operation}} \times \frac{3 \text{ days}}{\text{batch}} = 71,500 \frac{\text{gal}}{\text{batch}} \times \frac{1}{0.75 \text{ working volume}}
\]

\[
= 95,000 \text{ gal total bioreactor volume needed}
\]
This could be done with 3 bioreactors of 32,000gal each, with one being harvested every day.

### Seed Train and Bioreactor Cycle Times

<table>
<thead>
<tr>
<th>Vessel, Cycle</th>
<th>Cycle Length, days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seed1-1</td>
<td>3</td>
</tr>
<tr>
<td>Seed2-1</td>
<td>3</td>
</tr>
<tr>
<td>Seed 3-1</td>
<td>3</td>
</tr>
<tr>
<td>Reactor1-1</td>
<td>3</td>
</tr>
<tr>
<td>Reactor2-1</td>
<td>3</td>
</tr>
<tr>
<td>Reactor3-1</td>
<td>3</td>
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</tbody>
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**Diagram:**
- **ESF Biodiesel**
- **PHA Chemistry**
- **PHA Life Cycle**
- **Bacterial PHA**
- **Results**
- **Scale Up**
- **Biotechnology**
- **Nomura Lab**
- **Commercial**
- **Biogas**
- **Polymer Chem.**
PHA Manufacturing Plant
Conceptual Design Based on Scale-up of Laboratory Experiments

Calculated Yields

\[
\frac{1,200 \text{ gal}}{\text{day}} \times \frac{8.33 \text{ lb}}{\text{gal}} \times 0.5 \text{ Biomass Yield} \times \frac{0.71 \text{ g PHB}}{1 \text{ g biomass}} \times 0.80 \text{ Extraction Yield} = \frac{2840 \text{ lb PHA}}{\text{day}} \times 336 \text{ days} = 950,000 \text{ lb/yr}
\]

- **1,200 gal of BWG** used per day in our exercise.
- **Total 500 tons/yr** reflects very low yields and efficiencies.
- Commercial chemical manufacturing facilities are financially viable at 15,000 tons/yr or more.
- Huge jumps in yield will be afforded by:
  - Optimization of reactor conditions
  - Engineered organisms
  - Advances in extraction technology
Conclusions

- Our scale-up to 500 ton/yr is much smaller than 15,000 ton/yr economy of scale.
- Optimization of conditions will increase yields dramatically.
- Extraction technology is advancing quickly, a commercial plant exists!
- Engineered organisms hold great promise.
In vitro evolution of the PHA synthase gene

Gene (DNA) → Enzyme (Protein) → Gene mutation → Mutated Enzyme

Mutated gene
Random mutation may generate highly active mutants

Error prone PCR amplifies the PHA synthase gene under low fidelity conditions.

Random mutant library

Many mutated PHA synthases
Positive mutants are selected by *NileRed* staining

Error prone PCR product (mutated PHA synthase genes)

Introduction into *E. coli* with *phaA* and *phaB* genes

PHA content analysis (HPLC, GC)

Selection of colonies with high P(3HB) accumulation on LB agar plate containing “NileRed” and glucose

Acetyl-CoA $\rightarrow$ Aetoacetyl-CoA $\rightarrow$ 3HB-CoA $\rightarrow$ P(3HB)
Three highly active mutants of PHA synthase were found from Nile Red screening.

Screening of positive clone → PHA content analysis by HPLC → DNA sequence analysis

PHA synthase (559 amino acids)

active center (296)

α/β hydrolase fold region

0 559

Glu130

Glu130 → Aspartic acid
E130D (ED)

Ser325

Ser325 → Threonine
S325T (ST)

Gln481

Gln481 → Lysine
Q481K (QK)
P(3HB) accumulation in *E. coli*

**Diagram:**
- **Promoter**
- **phaC1ps**
- **phaARe**
- **phaBRe**
- **Terminator**

**Gene Expression:**
- *phaA*
- *phaB*
- *PHA synthase*
- *β-ketothiolase reductase*

**Graph:**
- **P(3HB) content (wt%)**
- **Cells were grown on LB-glucose at 30 °C for 96 h.**
- **PHA content was determined by gas chromatography.**
For more information you can email Dr. Nomura ctnomura@esf.edu

Lab webpage: http://www.esf.edu/chemistry/nomura/lab/

- **Current projects**
  - Metabolic and protein engineering for the production of biodegradable plastics
  - Microbial biodiesel production
  - Engineering of cellulase enzymes

- **Looking for graduate student applicants with strong backgrounds in:**
  - Biochemistry
  - Molecular Biology
  - Microbiology
  - Synthetic organic chemistry
Commercial PHA Production Research

Dr. Jim Nakas, Professor
Biology Department
jpnakas@syr.edu

Nakas Research Group:

- Microbial transformation and decomposition processes, nutrient cycling.
- Microbial biomass, solvent production.
- Commercial production of PHAs.
- Microbial ecology.
Dr. Dave Johnson,
Director of the Graduate Program in Environmental Studies (GPES)
dljohnson@esf.edu

Johnson Research Group:

• Biomass energy
• Anaerobic digestion using biodiesel waste
• Design and operation of small-scale anaerobic digestion units.
• Green Entrepreneurship Projects
Mostly Cellulose Research @ ESF...

Dr. Art Stipanovic, Chair
Department of Chemistry
astipano@esf.edu

Stipanovic Research Group:

- Biomass Characterization & Conversion Technology
- Cellulose & Hemicellulose Chemistry and Products
- PHA Physical Characterization/Testing
- PHA Material Development, Blending, Cost Saving Efforts
Thank You

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