New frontiers in synthetic biology for spaceflight

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Picture Credits: NASA
Funding

NASA Space Technology Mission Directorate Center Innovation Fund
NASA Advanced Exploration Systems

Team

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Cameron Sargent  Sarah Rommelfanger
Moving to Deep Space

Challenges...

- Increased radiation exposure risk
- Limited opportunity for crew return (launch window occurs every 25 months)
- Limited opportunity for resupply

Missions to Deep Space are enabled by robust technologies that:

- Make crew more self-sufficient
- Allow utilization of local resources
Potential of Microbial Manufacturing in Space

Scalable

Exponential Growth

1 cell → 2 cells → 4 cells → 8 cells → 16 cells

1 cell

48h

H2O

CO2

NO3-

PO4-

SO4^{2-}

Self-organizing

Programmable

Earth weights

0

0.5

1.0

1.5

0

20

40

Hours
Potential of Microbial Manufacturing in Space

**Biological systems are:**

- Scalable
- Programmable
- Precise (pure isomers)
- The only route of production in some cases (protein therapeutics)
- Low $T^\circ$ and pressure
- Regenerable

Credits: NASA
Barriers to Microbial Manufacturing in Space

Space technologies must be...

• Robust
• Simple to operate
• Stable during storage
• Compatible with available resources and infrastructure

Mount Sharp, Mars. Credits: NASA.

YPD:
0.3% yeast extract
1% peptone
1% glucose
Material conversion

Iowa, Earth

Glucose, Xylose, Lignin

Mount Sharp, Mars

CO₂, NO₃⁻, H₂O, SO₄²⁻, PO₄³⁻
Photoautotrophs

\[6\text{CO}_2 + 6\text{H}_2\text{O} \rightarrow \text{C}_6\text{H}_{12}\text{O}_6 + 6\text{O}_2\]

But...

- Usually have slower growth rates than heterotrophs
- Require photobioreactors

Credits: NASA
MOXIE: Mars Oxygen ISRU Experiment

$2\text{CO}_2 \rightarrow 2\text{CO} + \text{O}_2$

*Clostridium* spp.
# Environmental Control and Life Support System

## U.S. Regenerative Environmental Control and Life Support System (ECLSS)

<table>
<thead>
<tr>
<th>No.</th>
<th>Equipment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Catalytic Reactor</td>
</tr>
<tr>
<td>2</td>
<td>Deionizer Beds</td>
</tr>
<tr>
<td>3</td>
<td>Digital Controller</td>
</tr>
<tr>
<td>4</td>
<td>Distillation Assembly</td>
</tr>
<tr>
<td>5</td>
<td>Electrolysis Cell Stack</td>
</tr>
<tr>
<td>6</td>
<td>Gas Separator</td>
</tr>
<tr>
<td>7</td>
<td>Multifiltration Beds</td>
</tr>
<tr>
<td>8</td>
<td>Particulate Filter</td>
</tr>
<tr>
<td>9</td>
<td>Power Supply</td>
</tr>
<tr>
<td>10</td>
<td>Product Water Tank</td>
</tr>
<tr>
<td>11</td>
<td>Pumps &amp; Valves</td>
</tr>
<tr>
<td>12</td>
<td>Reactor Health Sensor</td>
</tr>
<tr>
<td>13</td>
<td>Storage Tanks</td>
</tr>
<tr>
<td>14</td>
<td>Urine Processor</td>
</tr>
<tr>
<td>15</td>
<td>CO₂ Reduction System (Sabatier)</td>
</tr>
<tr>
<td>16</td>
<td>Water Processor</td>
</tr>
<tr>
<td>17</td>
<td>Delivery Pump</td>
</tr>
<tr>
<td>18</td>
<td>Water Processor Pump &amp; Separator</td>
</tr>
<tr>
<td>19</td>
<td>Water Processor Wastewater Tank</td>
</tr>
</tbody>
</table>

### Oxygen Generation System (OGS) Rack

- 2: Reactor Health Sensor
- 3: Storage Tanks
- 4: Urine Processor
- 5: Pumps

### Water Recovery System Rack 1 (WRS-1)

- 6: Water Processor
- 7: Pump & Separator
- 8: Water Processor
- 9: Wastewater Tank

### Water Recovery System Rack 2 (WRS-2)

- 10: Water Processor
- 11: Pump & Separator
- 12: Water Processor
- 13: Wastewater Tank

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Credits: NASA
“Reference Guide to the International Space Station”
Methane production by Sabatier

- Reacts CO\textsubscript{2} and H\textsubscript{2} to form CH\textsubscript{4} and H\textsubscript{2}O
- H\textsubscript{2}O is recycled
- CH\textsubscript{4} vented to space
- Operational since 2010

“Waste” CH\textsubscript{4} could be fed to methanotrophs in a microbial manufacturing scheme.
Why Not Bacterial Methanotrophs?

M. capsulatus

J. Bacteriol. October 2003 vol. 185 no. 19 5755-5764


• NASA STTR to Mango Materials (Small Technology Transfer Innovation Research)

• “A Novel, Membrane-Based Bioreactor Design to Enable a Closed-Loop System on Earth and Beyond”

• “…a membrane bioreactor system to produce a biopolymer from methane gas…will enable bacterial growth and biopolymer production to occur in microgravity environments…”

Hits in Google Patents

M. capsulatus 356708
P. pastoris 74273
S. cerevisiae 2622
Pichia pastoris (Komagataella phaffii)

Ida J. van der Klei et al. EMBO J. 1998;17:3608-3618

- Diverged from S. cerevisiae ~200 MYA
- Methylotrophic yeast
- Widely used as a protein production host
- Capable of producing reduced and glycosylated proteins
- Grows to very high cell density (optical densities up to 630, Dr. Julia Cino, New Brunswick Scientific)
Why *Pichia pastoris*?

1. Established synthetic biology platform
   Used to produce Trypsin, murine TNFα, and FDA approved drugs Kalbitor (60 amino acid peptide to treat hereditary angioedema) and Jetrea (proteolytic enzyme to treat symptomatic vitreomacular adhesion)

2. Methylotrophic yeast
   Can utilize methanol (CH$_3$OH) as a carbon source
Porting methane metabolism to *P. pastoris*

Methanol (CH$_3$O) is oxidized to formaldehyde (CH$_2$O) by alcohol oxidase (AOD). Formaldehyde can either be oxidized to CO$_2$ through the successive action of formaldehyde dehydrogenase (FLD), S-formylglutathione hydrolase (FGH), and formate dehydrogenase (FDH), or appended to xylulose-5-phosphate and assimilated into biomass through a pathway involving dihydroxyacetone synthase (DAS) and dihydroxyacetone kinase (DAK).
Porting Soluble Methane Monooxygenase to *Pichia*

1. **Hydroxylase: MMOH**
   - alpha
   - beta
   - gamma

   Oxidizes methane hydroxylates methane to methanol

2. **Reductase: MMOR**

   Oxidizes NADH and transfers electrons to MMOH

   1. **Regulatory: MMOB**

   Binds to same site as MMOR. May help drive cycle.

   2. **Assembly chaperone: MMOG**

   Could help in assembly of MMOH

Sirajuddin and Rosenzweig. 2015.
Plasmid I: Single transcript expression of MMOH

```
Plasmid: pPichiaTK052

- Promoter
- Terminus
- Connector
- Zeocin Resistance
- panARS Origin
- ColE1 Origin
- Kanamycin Resistance

Genes:
- MMOH \( \alpha \)
- MMOH \( \beta \)
- MMOH \( \gamma \)

MMOH
```
Plasmid II: Expression of accessory proteins

pPichiaTK050

MMOB
MMOR
MMOG

Promoter
Terminator

ColE1 Origin
Kanamycin Resistance
panARS Origin

Hygromycin Resistance

Kanamycin Resistance

MMOB → MMOR
MMOR → MMOG
M MOG → MMOB
Introducing MMO genes to *Pichia*

**Genome Editing**
- Insert gene into organismal genome
- CRISPR
- Homologous Recombination

**Plasmid Transformation**
- Gene inserted as a small independently replicating circle of DNA
Developing *Pichia* tools

Pichia tools are limited compared to *S. cerevisiae*

Plasmid systems have only recently been developed

John Dueber at Berkeley has developed a “*S. cerevisiae* Toolkit”

This could be adapted for use in *Pichia*

Lee et al. 2015. ACS Synth Biol.
Building a *Pichia* toolkit

Lee et al. 2015. ACS Synth Biol.
Origin of replication: panARS

An autonomously replicating sequence for use in a wide range of budding yeasts

Ivan Liachko & Maitreya J. Dunham

Department of Genome Sciences, University of Washington, Seattle, WA, USA
Adapted plasmid system from John Dueber by incorporating recently identified “panARS” sequence to allow replication of plasmid in *Pichia*.

Most other parts should be re-usable.

Constructed empty vector that should impart resistance to Zeocin.
Plasmid system for *Pichia*
Plasmid demonstration in *P. pastoris*

- **pPichia17** – AOX1p-mTurquoise-ScENO1
- **pPichia18** – AOX1p-Venus-ScENO1
- **pPichia19** – AOX1p-mRuby-ScENO1
Plasmid demonstration in *P. pastoris*

**mTurquoise2**

- Methanol: RFU = 1,200,000
- Glucose: RFU = 200,000

**Venus**

- Methanol: RFU = 250,000
- Glucose: RFU = 100,000

**mRuby2**

- Methanol: RFU = 200,000
- Glucose: RFU = 50,000
Porting Soluble Methane Monooxygenase to *Pichia*

1. Hydroxylase: MMOH
   1. alpha
   2. beta
   3. gamma

Oxidizes methane hydroxylates methane to methanol

2. Reductase: MMOR

Oxidizes NADH and transfers electrons to MMOH

1. Regulatory: MMOB

Binds to same site as MMOR. May help drive cycle.

2. Assembly chaperone: MMOG

Could help in assembly of MMOH

Sirajuddin and Rosenzweig. 2015.
 MMO components are expressed as an operon

- Most studies are sMMO from *Methylococcus capsulatus* (Bath) or *Methylosinus trichosporium* OB3b
- MMO from *M. trichosporium* OB3b has a higher turnover number (3.5 vs. 0.2 – 1.0 s⁻¹)

Sirajuddin and Rosenzweig. 2015.
Proper assembly of MMOH is challenging

Assembled Complex

Pre-assembled Components

Stable. Active complexes are readily purified from *M. capsulatus*.

Unknown stability in *Pichia*. Assumed to be unstable.
Type 2a peptides balance component stoichiometry

VKQLNFCLLKLAGDVESNPGP

Ribosome skips this peptide bond

MMOH
Alpha---2a---Beta---2a---Gamma

Alpha-2a  Beta-2a  Gamma


Stoichiometric production of complex components
Expressing MMOH from single transcript

Single transcript

MMOHα V5 E2A MMOH β V5 P2A MMOHγ V5

3 polypeptides

- MMOHβ
- V5
- P2A

Single hexameric enzyme

MMOH:
- Red: MMOH α
- Blue: MMOH β
- Yellow: MMOH γ

Single plasmid

pPichiaTK052

Promoter
Connector
ColE1 Origin
Kanamycin Resistance
panARS Origin
Terminator
Connector
Zeocin Resistance

Red: MMOH alpha
Blue: MMOH beta
Yellow: MMOH gamma
Western blot to detect MMOH subunits

<table>
<thead>
<tr>
<th>Protein</th>
<th>MW (kDa)</th>
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<tbody>
<tr>
<td>MMOH α</td>
<td>63.5</td>
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<tr>
<td>MMOH β</td>
<td>48.4</td>
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<tr>
<td>MMOH γ</td>
<td>21</td>
</tr>
<tr>
<td>MMOH α + β + γ</td>
<td>132.9</td>
</tr>
<tr>
<td>MMOH α + β</td>
<td>111.9</td>
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<tr>
<td>MMOH β + γ</td>
<td>69.4</td>
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</table>
Porting sMMO to *Pichia*: Current status

\[
\text{CO}_2 \rightarrow \text{H}_2 \rightarrow \text{Sabatier} \rightarrow \text{CH}_4
\]

\[
\text{H}_2 \text{O} \rightarrow \text{Sabatier} \rightarrow \text{Methane}
\]

\[
\text{Methanol} \rightarrow \text{MMO} \rightarrow \text{P. pastoris}
\]

\[
\text{Natural metabolism}
\]

**Status**

<table>
<thead>
<tr>
<th></th>
<th>Designed</th>
<th>Built</th>
<th>Tested</th>
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<tbody>
<tr>
<td>MMOH</td>
<td></td>
<td></td>
<td>n.a.</td>
</tr>
<tr>
<td>MMOB</td>
<td></td>
<td></td>
<td>n.a.</td>
</tr>
<tr>
<td>MMOR</td>
<td></td>
<td></td>
<td>n.a.</td>
</tr>
<tr>
<td>MMOG</td>
<td></td>
<td></td>
<td>n.a.</td>
</tr>
<tr>
<td>MMOH plasmid</td>
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<td></td>
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</tr>
<tr>
<td>MMOR/B/G plasmid</td>
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</tbody>
</table>
Potential of Biotechnology in Space

On ISS, CO\textsubscript{2} is reacted with H\textsubscript{2}. H\textsubscript{2}O is recovered but CH\textsubscript{4} is lost to space.

FIG 1.
Funding
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Samantha Fleury & Lily Neff