Microorganism Utilization for Synthetic Milk Production

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History

• Astronauts spend extended periods of time in space aboard the ISS
• Experience the effects of microgravity
• Dietary requirements require a certain amount of plant and animal material not readily available
• Grow vegetable crops as lettuce, radish, and tomato aboard the ISS, creating a large amount of plant waste
History

- Use of biological agents may serve as a synthetic food processor
  - Cellulolytic microbes
    - Converts the waste into multiple, high value, synthetic ‘animal’ products
  - Milk

- Bovine milk components include protein, sugar, lipid

- Multi-stage bioprocessing required to create the fuel for each successive stage.
History

- Concept of converting crop residue to a useable product
- Could be adapted for other space mission solid waste
- Provides the benefit of reducing solid waste in space.
- TA06-Human health, life support, and habitation systems> ECLSS and Habitation Systems>Waste Management
Explore the use of biological agents- bacteria, fungi, and yeast- as synthetic food processors.
Introduction

— Desired architecture for long duration spaceflight,
  — International Space Station (ISS)
  — Future missions to Mars
— Provide a supply of fresh food crops
  — Crops create a high proportion of inedible plant waste.
Background

- Fungi, bacteria, and yeasts possess metabolic pathways fueled by cellulosic biomass
  - Fungi can produce glucose from cellulose
  - Oleaginous yeasts accumulate upwards of 80% of their dry weight as lipids.
  - Microbes produce proteins
Background

• Fungi produce a number of polysaccharide hydrolyzing enzymes and enzyme complexes.
  - Monosaccharide glucose useful as fuel for other organisms

• Yeast can be fueled by glucose to produce lipids.
  - Milk lipids are the fatty portion of milk and make up 33 g total lipid/L in bovine milk
Goals

• Produce the components of milk from inedible plant waste by utilizing microorganisms
  - Sugar - glucose
  - Lipid - milk fats
  - Protein – casein

• Utilize fungi that possess cellulolytic metabolic pathways
  - produce glucose from cellulose to fuel future reactor stages
  - substitute glucose for lactose component of milk

• Production of milk lipids through oleaginous yeasts.
Materials and Methods: Environmental Conditions

Environmental conditions optimized:

- **TIME COURSE BATCH STUDIES**
- pH
- Temperature
- Carbon source
- Aeration
- Choice microorganisms
## Materials and Methods - Conditions

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Experimental Conditions</th>
<th>Duration(d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FB 1*</td>
<td>2% or 8% Avicel feed; no additional feed, no pH control</td>
<td>25</td>
</tr>
<tr>
<td>FB 2</td>
<td>fed 8% Avicel as replacement volume</td>
<td>23</td>
</tr>
<tr>
<td>FB 3</td>
<td>Replaced sampled volume every 7 days with 8% Avicel</td>
<td>21</td>
</tr>
<tr>
<td>FB 4</td>
<td>No additional feed, pH controlled D1-7</td>
<td>14</td>
</tr>
<tr>
<td>FB 4b</td>
<td>8% Avicel added to FB 4 supernatant</td>
<td>7</td>
</tr>
<tr>
<td>FB 5</td>
<td>2% Avicel daily feed; pH controlled D1-7</td>
<td>14</td>
</tr>
<tr>
<td>FB 5b</td>
<td>8% Avicel added to FB 5 supernatant</td>
<td>7</td>
</tr>
<tr>
<td>FB 6</td>
<td>No daily feed; pH controlled D1-7</td>
<td>7</td>
</tr>
<tr>
<td>FB 6b</td>
<td>8% Avicel added to FB 6 supernatant</td>
<td>7</td>
</tr>
<tr>
<td>FB 7</td>
<td>2% daily feed; pH controlled D1-7</td>
<td>7</td>
</tr>
<tr>
<td>FB 7b</td>
<td>8% Avicel added to FB 7 supernatant</td>
<td>7</td>
</tr>
<tr>
<td>FB 8</td>
<td>2% Avicel daily feed; no pH control</td>
<td>7</td>
</tr>
<tr>
<td>FB 8b</td>
<td>8% Avicel added to FB 8 supernatant</td>
<td>7</td>
</tr>
<tr>
<td>FB 9</td>
<td>0% Avicel starting concentration, no pH control</td>
<td>14</td>
</tr>
<tr>
<td>FB 10</td>
<td>Temperature changed to 26 °C at D3, no feed, no pH control</td>
<td>14</td>
</tr>
</tbody>
</table>
Materials and Methods: Microorganisms

- *Trichoderma reesei*, cellulolytic fungus, BSL I
  - QM 9414 (ATCC 26921), Rut C30, (ATCC 56765)
  - Drive the production of glucose
  - Known for its ability to convert native and derived cellulose
  - Produces a number of polysaccharide hydrolyzing enzymes

- *Rhodosporidium toruloides*, yeast, BSL I
  - Strain Y-1091 (ATCC 10788), USDA ARC
  - Lipid production found in milk fats
  - Can accumulate up to 76% dry weight as lipid
Materials and Methods: Sugar Production

• Time course batch study of *T. reesei* cultured in Mandel’s media
  - supplemented with 0 – 8 % Avicel
  - Incubated at 30°C, static or shaken at 125 rpm
  - Monitored environmental conditions

• Glucose and cellobiose detected over time
  - Agilent 1100 HPLC with an Aminex HPX-87H ion exclusion column
Results: Environmental Conditions - Glucose

Glucose Production Rate & Avicel

[Graph showing production rate of glucose in shaken and static conditions with percentage of Avicel on the x-axis and production rate on the y-axis.]
Results: Environmental Conditions, pH

Glucose Production

Static       Shaken
Results: Environmental Conditions & Sugar Production

![Graph showing sugar concentration over time with temperature change from 30°C to 50°C.](image-url)
Results: Overall Glucose Production

- glucose production below what was expected
Results: Glucose Averages
Results: Enzymatic Hydrolysis

Cellobiose

Glucose

\[ y = 0.7446x + 0.8393 \]
\[ R^2 = 0.992 \]
Materials and Methods: Lipid production

- *Rhodosporidium toruloides*
  - Cultured in 1 L minimal media supplemented with glucose
  - 30 °C with a rotary rate of 200 rpm
  - Optical density (600 nm) used to determine concentrations
  - Environmental conditions monitored & controlled
  - Glucose & lipid levels monitored over time as previously described
### Materials and Methods - Conditions

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<thead>
<tr>
<th>Experiment</th>
<th>Experimental Conditions</th>
<th>Duration (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RB 1</td>
<td>90 g/L glucose in minimal media; no pH control</td>
<td>14</td>
</tr>
<tr>
<td>RB 2</td>
<td>60 g/L glucose in modified minimal media; pH control D7-14</td>
<td>21</td>
</tr>
<tr>
<td>RB 3</td>
<td>60 g/L glucose in minimal media; pH control D1-7</td>
<td>14</td>
</tr>
<tr>
<td>RB 4</td>
<td>60 g/L glucose in modified minimal media; pH control D1-7</td>
<td>14</td>
</tr>
<tr>
<td>RB 5</td>
<td>60 g/L glucose in minimal media; no pH control</td>
<td>14</td>
</tr>
<tr>
<td>RB 6</td>
<td>60 g/L glucose in modified minimal media; no pH control</td>
<td>14</td>
</tr>
</tbody>
</table>
Materials and Methods: Lipid detection

- Lipid composition occurred in three stages
  - freeze drying
  - lipid extraction
  - trans-esterification/methylation

Step 1:
>30ml x 3

Yeasts Culture
- OD600nm measured
Materials and Methods: Lipid Analysis

- Fatty acids analyzed with a Trace Ultra gas chromatograph (GC)
  - fitted with a 30m x 0.25mm x 0.25μm DB-225ms column
- Determined % total lipid recovery of dry cell weight
- Quantified 6 fatty acids of interest
  
  | oleic acid   | palmitic acid   |
  | stearic acid | myristic acid   |
  | linoleic acid| linolenic acid  |
Results: Growth & Environmental Conditions - Lipids
Results: Environmental Factors & Lipid Production

- pH level decreased to about 3.0 or below
- Glucose decreased indicating culture growth
Results: Lipid Production & Fatty Acid Analysis

- Total lipid content below what expected
- Individual analysis of six fatty acids revealed the percentage of each fatty acid was lower than naturally produced bovine milk.

- RB1 - minimal media with 90g/L glucose.
- RB5 minimal media with 60g/L glucose.
- RB6 modified minimal media with 60g/L glucose.
# Results: Lipid Production – Average Fatty Acid Content

<table>
<thead>
<tr>
<th>Day of Experiment</th>
<th>Methyl Linolenate</th>
<th>Methyl Linoleate</th>
<th>Methyl Oleate</th>
<th>Methyl Palmitate</th>
<th>Methyl Myristate</th>
<th>Methyl Stearate</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>8.54</td>
<td>44.45</td>
<td>2.05</td>
<td>21.32</td>
<td>19.78</td>
<td>17.76</td>
</tr>
<tr>
<td>5</td>
<td>8.81</td>
<td>49.03</td>
<td>1.32</td>
<td>17.76</td>
<td>20.98</td>
<td>16.03</td>
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<tr>
<td>8</td>
<td>12.08</td>
<td>46.11</td>
<td>2.42</td>
<td>16.03</td>
<td>22.28</td>
<td>18.03</td>
</tr>
<tr>
<td>9</td>
<td>13.67</td>
<td>44.25</td>
<td>1.11</td>
<td>18.03</td>
<td>20.65</td>
<td>18.34</td>
</tr>
<tr>
<td>12</td>
<td>11.23</td>
<td>46.47</td>
<td>1.05</td>
<td>17.84</td>
<td>21.58</td>
<td>17.84</td>
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<tr>
<td>14</td>
<td>10.53</td>
<td>45.26</td>
<td>1.21</td>
<td>19.25</td>
<td>22.16</td>
<td>19.25</td>
</tr>
</tbody>
</table>
# Results: Lipid Production & Bovine Comparison

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Linolenic</th>
<th>Linoleic</th>
<th>Myristic</th>
<th>Stearic</th>
<th>Oleic</th>
<th>Palmitic</th>
</tr>
</thead>
<tbody>
<tr>
<td>RB1</td>
<td>0.27</td>
<td>1.45</td>
<td>0.16</td>
<td>2.37</td>
<td>6.09</td>
<td>2.79</td>
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<tr>
<td>RB2</td>
<td>0.12</td>
<td>0.45</td>
<td>0.08</td>
<td>3.63</td>
<td>3.25</td>
<td>1.38</td>
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<tr>
<td>RB3</td>
<td>0.24</td>
<td>0.93</td>
<td>0.13</td>
<td>1.37</td>
<td>3.20</td>
<td>1.47</td>
</tr>
<tr>
<td>RB4</td>
<td>0.38</td>
<td>1.19</td>
<td>0.19</td>
<td>2.43</td>
<td>6.62</td>
<td>2.69</td>
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<tr>
<td>RB5</td>
<td>0.12</td>
<td>0.58</td>
<td>0.05</td>
<td>0.80</td>
<td>21.45</td>
<td>1.07</td>
</tr>
<tr>
<td>RB6</td>
<td>0.19</td>
<td>0.73</td>
<td>0.06</td>
<td>1.11</td>
<td>3.15</td>
<td>1.41</td>
</tr>
<tr>
<td>Bovine Milk</td>
<td>2.30</td>
<td>3.60</td>
<td>9.00</td>
<td>11.00</td>
<td>24.00</td>
<td>24.00</td>
</tr>
</tbody>
</table>
Discussion and Conclusions

- Glucose and fatty acid production were lower than expected

- Sugar produced by breakdown of cellulose could serve as a resource for other microorganisms to produce lipids

- Of 6 fatty acids investigated, all were present but in lower percentage than bovine milk
Discussion and Conclusions

- Environmental parameters were crucial to continued performance of cultures
  - Fed-batch culture with a biphasic pH and temperature regime required.

- Cultures need to increase biomass at optimal pH before recovery of glucose

- Microorganisms could be utilized to breakdown inedible solid waste to produce glucose
Further Studies:

- Continue with fed-batch experimentation, glucose extraction and reactor scale studies
- Proceed to fed-batch & reactor scale studies, as well as lipid extraction techniques for use in simulant milk.
- Investigate additional organisms for increased lipid production
- Remaining third component, casein or milk protein became the missing piece of the puzzle.
  - Required a GMO, beyond the scope of this project
Research Support Acknowledgements

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Questions?