Development of Storage Methods for Saccharomyces Strains to be Utilized for *In situ* Nutrient Production in Long-Duration Space Missions

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From Sea to Space

Nutrient deficiencies occur as a result of limited resupply of fresh foods during long-duration expeditions.
Nutrient Degradation Over Time

Nutritional quality of 109 space food items tested over three years at ambient temperature storage

<table>
<thead>
<tr>
<th>Nutrients below the recommended intake post-processing</th>
<th>Calcium</th>
<th>Potassium</th>
<th>Vitamin K</th>
<th>Vitamin D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamins that may degrade to lower than the recommended daily intake after three years</td>
<td>Vitamin B1</td>
<td>Vitamin C</td>
<td>Vitamin B9*</td>
<td></td>
</tr>
</tbody>
</table>

* Vitamin degradation dependent on food source

Microorganisms for *In situ* Production of Nutrients

In order for *In situ* production of nutrients to occur, microorganisms must maintain high viability during long-duration storage.

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Recommended Dietary Intake (RDI)</th>
<th>Published Nutrient Yields</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin C</td>
<td>75 – 90 mg/day</td>
<td>~100 mg/L³</td>
</tr>
<tr>
<td>Vitamin K</td>
<td>90 – 120 μg/day</td>
<td>85 μg/g wet weight⁴</td>
</tr>
<tr>
<td>Beta-carotene (provitamin A)</td>
<td>6 – 16 mg/day</td>
<td>5.9 mg/g dry cell weight⁵</td>
</tr>
</tbody>
</table>

Citations: ²Code of Federal Regulations, title 21, Sec 101.9, ³Sauer et al., 2004, ⁴Yanagisawa and Sumi, 2005, ⁵Verwaal et al., 2007
**Objective**: To engineer a GRAS (generally regarded as safe) microorganism for the *In situ* production of needed dietary nutrients for long-duration space missions.
Yeast as *In-situ* Production Platform

- **S. cerevisiae**
  - History of Metabolic Engineering
  - Expression Platform Organism
  - Spore Former

- **S. boulardii**
  - Same Engineering Tools can be Applied
  - Probiotic
  - Vegetative Cells

_S. cerevisiae_ is the organism used as the expression platform in-situ production.
Effects of Dehydration on Yeast

- Water evaporation
- Contact with reactive oxygen species (ROS)
- Substitution of water with air
- Dehydrated Cell

- Increase in osmolarity
- Membrane disruption
- Increased intracellular crowding
- Protein aggregation and misfolding
- ROS induced lipid peroxidation and DNA damage

Preservation of Spores and Vegetative Cells

Drying Methods
• Lyophilization (freeze-dry)
• Vacuum (no freezing involved)
• Air-dry

Protectants
• The following protectants are identified as edible and have proven successful:
  – Trehalose, skim milk, monosodium glutamate
  – Proline
  – Sorbitan monostearate
  – Lactose

Storage
• Stored in reduced oxygen environment at room temperature or 4 °C
Methods Flowchart

Vegetative Cells: *Saccharomyces cerevisiae* and *boulardii*

- **Desiccation**
  - Lyophilization
  - Air-drying

- **Storage**
  - Samples stored in an anaerobic chamber in 96 well plates at room temperature

- **Revival**
  - Rehydrated in dilute PBS for 30 minutes, serially diluted, plated, and CFU counted

Spores: *Saccharomyces cerevisiae*

- **Desiccation**
  - Lyophilization
  - Vacuum
  - Air-dry

- **Storage**
  - Sealed in bags without oxygen, and stored at room temperature or 4 °C

- **Revival**
  - Measured by optical density
  - Measured by percent change in biomass
Effect of Drying Methods on Spore Survival

- Protectants did not affect spore survival under vacuum at room temperature
- Protectants increased viability of lyophilized spores
- Lyophilization was overly damaging to spores when compared to vacuum
Optimizing Vegetative Cell Viability

Vegetative cells were allowed to grow in rich media for 3, 5, and 7 days to determine if time spent in stationary phase had an effect on viability after desiccation.

Tested with trehalose as a protectant
Viability of Spores Stored at 4 ºC

- Spores stored at room temperature or at 4 ºC
- No significant difference in viability between spores stored at room temperature vs. 4 ºC after six months
S. cerevisiae Spore Storage

A. Sporulation at room temperature
B. Spores dehydrated in a desiccator
C. Spores dehydrated at 4 °C
D. Spores stored in water
E. Spores dehydrated by vacuum

- No spores survived when stored in water after 6 months
- Minimal decline in viability for spores stored under all parameters
Three-year Spore Storage Study

Spore Viability Measured by Growth Curve

- Initial
- 1 Week
- 1 Month
- 3 Months
- 6 Months
- 1 year

Optical Density 600 nm

Minutes

Spore Viability Measured by Change in Biomass

Percent Change in Biomass

One Week
One Month
Three Months
Six Months
One Year

* Represents 10% less final biomass than samples stored for one week
Conclusions from Storage Study – 1 Year

• Spores have maintained a relatively high viability over time

• After one year there has only been a 10% decline in overall final biomass

• In the event cell viability declines to undesirable levels, a higher starting biomass can be added to the package to offset cell loss over time.
Anhydrobiotic Engineering

Trehalose

- Long-term desiccation leads to loss of molecular chaperone function
- Trehalose may act as a replacement molecular chaperone by inhibiting protein aggregation and misfolding

Traditional Pathway:

\[
\text{Trehalose} \rightarrow \text{Trehalase (NTH1)} \rightarrow \text{Glucose}
\]

Pathway with Engineered NTH1 Knockout:

\[
\text{Trehalose} \rightarrow \text{Trehalase (ΔNTH1) Knockout} \rightarrow \text{Increased Trehalose in Cell}
\]

Engineering Desiccation Tolerance

- After three months the wild type *S. boulardii* strain shows a significant decline in viability compared to the NTH1 deletion strain.
- Longer term data is needed to verify increased desiccation tolerance over time.
Summary

• *S. cerevisiae* spores have maintained high viability over one year

• Lyophilization was dropped as a drying method for spores as the freezing step is likely overly damaging

• Air-drying vegetative cells results in the highest initial viability directly after drying

• Early stationary phase appears to be the optimal time to prepare yeast for desiccation

• NTH1 knockout may increase long-duration survival of *S. boulardii* in a desiccated state although longer term storage data is needed to verify
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References


