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

Natalie Ball[1] and Hiromi Kagawa[2]
KBR Wyle[1], SETI Institute[2], NASA Ames Research Center, Moffett Field, CA 94035

Aditya Hindupur[3]
KBR Wyle, NASA Ames Research Center, Moffett Field, CA 94035

and

John A. Hogan[4]
NASA Ames Research Center, Moffett Field, CA 94035

Long-duration space missions will benefit from closed-loop life support technologies that minimize mass, volume, and power as well as decrease reliance on Earth-based resupply. A system for *In situ* production of essential vitamins and nutrients can address the documented problem of degradation of stored food and supplements. Research has shown that the edible yeast *Saccharomyces cerevisiae* can be used as an on-demand system for the production of various compounds that are beneficial to human health. A critical objective in the development of this approach for long-duration space missions is the effective storage of the selected microorganisms. This research investigates the effects of different storage methods on survival rates of the non-sporulating probiotic *S. boulardii*, and *S. cerevisiae* spores and vegetative cells. Dehydration has been shown to increase long-term yeast viability, which also allows increased shelf-life and reduction in mass and volume. The process of dehydration causes detrimental effects on vegetative cells, including oxidative damage and membrane disruption. To maximize cell viability, various dehydration methods are tested here, including lyophilization (freeze-drying), air drying, and dehydration by vacuum. As a potential solution to damage caused by lyophilization, the efficacy of various cryoprotectants was tested. Furthermore, in an attempt to maintain higher survival rates, the effect of temperature during long-term storage was investigated. Data show spores of the wild-type strain to be more resilient to dehydration-related stressors than vegetative cells of either strain, and maintain high viability rates even after one year at room temperature. In the event that engineering the organism to produce targeted nutrient compounds interferes with effective sporulation of *S. cerevisiae*, a more robust method for improving vegetative cell storage is being sought. Therefore, anhydrobiotic engineering of *S. cerevisiae* and *S. boulardii* is being conducted.

Nomenclature

CFU	=	colony forming unit
FDA	=	food and drug association
ISS	=	International Space Station
MSG	=	monosodium glutamate
OD	=	optical density
ROS	=	reactive oxygen species
RPM	=	rotations per minute
YPD	=	yeast extract, peptone, dextrose (a growth media for yeast)

[1] Junior Scientist, Bioengineering Branch, M/S 239-15, Moffett Field, CA 94035
[2] Research Scientist, Bioengineering Branch, M/S 239-15, Moffett Field, CA 94035
[3] Research Scientist, Bioengineering Branch, M/S 239-15, Moffett Field, CA 94035
[4] Physical Scientist, Bioengineering Branch, M/S 239-15, Moffett Field, CA 94035

I. Introduction

Throughout long-duration space missions essential amino acids and nutrients must be available in adequate quantities to maintain crew health and safety. Deficiency in any essential nutrient can have a detrimental effect on human health and therefore mission success. Shelf-life studies have indicated that some nutrients, such as vitamins C and K, folate, and thiamin to name a few, are susceptible to substantial degradation over time.[1] Additionally, not all required nutrients have been evaluated. Evidence indicates current storage and food processing techniques are not sufficient for the preservation of certain nutrients during extensive long-duration missions without intermittent resupply.[2] Efforts to remediate these problems have included the use of vitamin supplements, the development of enhanced food storage techniques with temperature control, and the implementation of bioregenerative food systems including crop growth.[1,3] To add to this ongoing research, we have begun developing and testing a low mass production system for on-demand synthesis of nutrients using the edible yeast *Saccharomyces cerevisiae*. *In situ* production of nutrients in space could serve to ameliorate the health risks associated with the decrease in bioavailability of certain nutrients in foods stored for an extended duration.[1]

In situ production of essential nutrients by *S. cerevisiae* and *S. cerevisiae* var. *boulardii* is dependent on the survival of the organisms. For this production system to be successful, yeast cells must maintain their viability through multiple years of storage for rapid, on-demand revival and synthesis of the compound of interest. The efforts presented in this paper involve developing methods to enable the long-term viability of yeast spores and vegetative cells. Multiple mechanisms were employed and tested to increase longevity including dehydration, oxygen exclusion, selective metabolic engineering, and the use of protectants. Testing of various treatments via revival in an edible media is ongoing, and is anticipated to continue for a period of three years. The results of these tests are leading to knowledge that informs additional long-duration storage testing. This work is intended to culminate in a flight experiment where treatments are stored for up to three years on the International Space Station (ISS), with intermittent revival testing and subsequent ground characterization of growth and expression of target nutrients.

II. Background

S. cerevisiae has an extensive history of metabolic engineering making it an ideal organism for production of compounds with dietary, pharmaceutical, or therapeutic applications.[4] *S. cerevisiae* has already been used for the production of important compounds such as insulin, beta-carotene, and artemisinic acid (an anti-malarial drug precursor) among others.[5-7] The same tools for genetic engineering of *S. cerevisiae* can also be applied to *S. boulardii*. Although both *S. cerevisiae* and *S. boulardii* have been identified as the same species, both of which are edible and have been granted "Generally Regarded as Safe" status by the Food and Drug Administration (FDA), they also exhibit key genetic differences.[8,9] *S. cerevisiae* for example, is capable of undergoing sporulation allowing it to drastically reduce its metabolic activity and survive in extreme environments. *S. boulardii* is unable to produce spores, presenting a potential disadvantage for long-term storage. However, *S. boulardii* has been characterized as a probiotic. These characteristics make both strains desirable for development and testing of *in situ* nutrient production.

In addition to the benefits of the nutrients being produced by the yeast, probiotic organisms are favorable because they are a prophylactic. *S. boulardii* has been implicated in the treatment of various gastrointestinal disorders such as antibiotic-associated diarrhea infections possibly by aiding in the re-establishment of normal gut microbiota.[10-12] *S. boulardii* can also inhibit the growth and adherence of opportunistic pathogenic bacteria and yeast either directly or by excretion of inhibitory compounds.[13,14] Space flight has been shown to alter the physiology and composition of the gut microbiome.[15-17] Reduction in the diversity of the normal microbiota can potentially lead to infection by opportunistic pathogens. Furthermore, it has been shown that previous NASA Space Transportation System (STS) missions as well as simulated microgravity experiments increase virulence factors of some pathogenic organisms.[18-20] Pre- and probiotics can potentially offset the effects of an altered microbiome. To utilize probiotic *S. boulardii* vegetative cells, experiments must be conducted to optimize survival during long-duration storage, as vegetative cells typically have a reduced shelf-life compared to spores.

S. cerevisiae and *S. boulardii* are anhydrobiotes that are capable of surviving in the dehydrated state as vegetative cells.[21] The desiccation process involves the removal of intracellular water allowing the yeast to undergo a reversible delay in their metabolism. Metabolic reduction combined with the yeast's innate ability to withstand desiccation ensures a relatively high survival directly after dehydration as well as during long-term storage.[22] Dehydration still imposes major stresses on the cell causing a global change in its metabolism and structure. The removal of water can cause ruptures in the plasma membrane, increased intracellular crowding, and damage to the cell wall.[23,24] Furthermore, as water molecules are replaced by air, oxidative damage occurs causing a build-up of reactive oxygen

species (ROS) within the cell.[25,26] ROS can damage proteins, DNA, and cause lipid peroxidation in cell membranes.[25] Modifications of vital structures that occur during desiccation can cause irreparable damage resulting in cell death.

In order to mitigate some of the damage caused by freezing and desiccation, we have evaluated the use of protectants. Sugars and sugar alcohols such as lactose, sucrose, trehalose, and xylitol have often been used as protective agents during dehydration and freezing.[27,28] Trehalose, one of the most commonly used protectants for dehydration, is a non-reducing disaccharide found to naturally occur in various organisms including yeast, nematodes, and plants.[29] Trehalose has been cited as providing exogenous protective effects during freezing and dehydration, as well as endogenous effects on yeast survival during long-term storage in a desiccated state.[30,31] Two significant models have been proposed to explain the sugar's protective mechanism of action. The water displacement model suggests sugars form hydrogen bonds with soluble proteins, stabilizing their three-dimensional structure as water diminishes.[32] The vitrification model suggests that at low water levels, sugars form a glassy matrix, acting as a shell around proteins and thus protecting the cell from desiccation stress.[33] These two models are unlikely to be mutually exclusive.

Trehalose has been shown to increase cell viability when used for lyophilization, a process of freeze-drying by vacuum.[34] Commercial industries have long used lyophilization for long-duration storage of microbes.[35,36] Studies have shown lyophilized vegetative *S. cerevisiae* stored under vacuum at 5 °C can last up to 20 years with no major decline in viability after dehydration.[22] Alternate methods of drying include spray drying, air-drying, and vacuum drying at ambient temperatures. The major stresses affecting the viability of stored yeast cells in the dehydrated state are temperature, residual water content, and exposure to oxygen. Anhydrobiotic organisms have an optimal water activity between 5-8% for long-duration storage.[20] These factors have been considered when designing experiments to optimize dehydration and storage conditions for long-duration storage of *S. cerevisiae* spores and vegetative cells, as well as *S. boulardii* vegetative cells. Although it is ideal to store *S. cerevisiae* in its spore state, genetic engineering of the yeast to produce desired nutrients may interfere with its ability to form spores. Therefore, *S. cerevisiae* vegetative cells will be tested for long-term viability.

Anhydrobiotic engineering of *S. boulardii* is being explored as a more robust method to maximize long-term survival in the vegetative state. The gene NTH1 encodes for trehalase which is localized in the cytosol and has an optimal activity at neutral pH. NTH1 is the major enzyme involved in the hydroylization of the non-reducing disaccharide trehalose into two glucose molecules.[37] Trehalose is a storage carbohydrate in yeast and has been shown to accumulate in cells with nutrient-poor conditions[38,39] It has also been implicated in desiccation tolerance during long-term storage.[31] As water is removed from the cell, intermolecular non-polar interactions occur that normally cause protein aggregation. Tapia et al.[31] have shown that long-term desiccation leads to the loss of molecular chaperone function that normally inhibits protein aggregation (eg. HSP proteins). Intracellular trehalose may act as a replacement molecular chaperone, inhibiting such protein aggregation and misfolding. The NTH1 deletion resulted in higher trehalose stores and, as a result, an increased viability during long-term storage of *S. cerevisiae* cells.[28,31]

In order for *In situ* production of nutrients in space to occur, the yeast must produce a desired amount of the compound of interest within a reasonable time. The successful revival of the yeast is dependent on the cells' ability to maintain a high viability during long-duration storage. This paper will outline experiments aimed at finding optimal conditions for the long-term preservation and storage of both spores and vegetative cells.

III. Materials and Methods

The methods in which *S. cerevisiae* spores and *S. cerevisiae* and *S. boulardii* vegetative cells were dehydrated, stored, and revived are outlined in Figure 1.

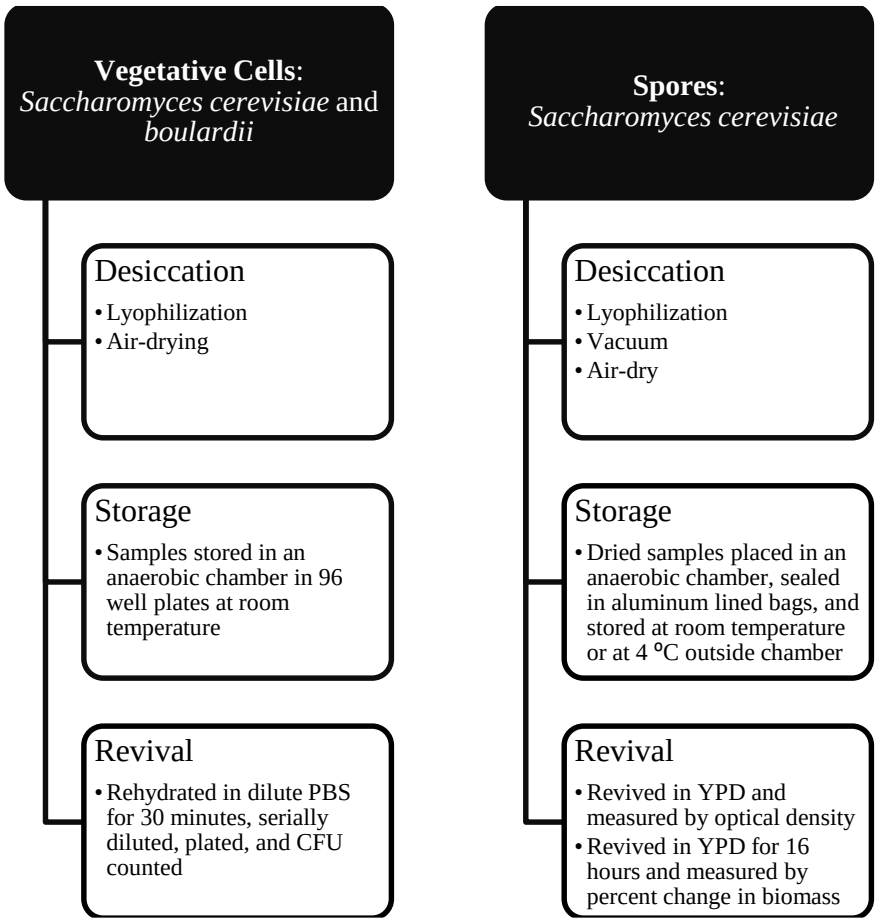

Figure 1. Flow chart of methods for long-duration storage studies of *S. cerevisiae* spores and *S. cerevisiae* and *S. boulardii* vegetative cells.

A. Yeast Strains and Media

The yeast strains used in this study are *Saccharomyces cerevisiae* Y55-3560 and *Saccharomyces boulardii* MYA-796 (ATCC). Yeast cells were revived in 10 g/L yeast extract, 20 g/L soy peptone, and 100 g/L glucose (YP5D), or 20 g/L glucose (YPD), or an edible version of YP5D composed of edible yeast extract made by Ohly®, PeptoPro® casein based protein powder, and dextrose. Cells were grown at 30 °C in a shaker incubator at 200 rpm or on YPD plates at 30 °C.

1. *Sporulation:* An overnight culture of Y55 was grown in YPD from frozen stock. Cells were transferred to pre-sporulation media; 10 g/L yeast extract, 20 g/L peptone, and 10 g/L potassium acetate (YPA), and allowed to grow for 18 – 24 hours. Cells were then transferred to sporulation media; 10 g/L potassium acetate supplemented with all necessary amino acids and allowed to sporulate for 72 hours. Hemocytometer counts were used to determine sporulation efficiency.
2. *Vegetative cells*: An overnight culture of Y55 or MYA-796 was grown from frozen stock. Cells were transferred to a baffled Erlenmeyer flask containing YPD at an optical density (OD) of 0.02 and allowed to grow to stationary phase at a temperature of 30 °C in a shaking incubator (Innova 4300, New Brunswick Scientific) at 200 rotations per minute (rpm).

B. Dehydration

1. *Spores* – Y55 spores were washed 2 times with water and concentrated to a weight between 0.06 to 0.1 grams. Spores were dehydrated by vacuum at an ambient temperature, air-drying, or lyophilization.
2. *Vegetative cells* – Stationary phase vegetative cells were collected from batch cultures and washed 2 times with water.
    - **i.** Lyophilization – Spores and vegetative cells were suspended in water or an edible cryoprotectant, frozen at -80 °C, and dehydrated on a manifold FreeZone console freeze drying system (Labconco), overnight.
    - **ii.** Air Drying- Vegetative cells were dehydrated in a biological hood or in an anaerobic chamber at room temperature. 100 µL aliquots were placed in 96 well plates, centrifuged, and 30 µL of water was removed. Dehydration time varied by experiment and typically required up to three days.
    - **iii.** Vacuum – Spores and vegetative cells were placed in a Thermo Scientific Savant ISS110 Speed Vacuum concentrator until dried.

C. Storage

1. Once dehydrated, vegetative cells or spores were purged with nitrogen, and either stored in an anaerobic chamber, or sealed in nitrogen purged foil lined bags. Spores were either stored in contact with the edible media itself or separate inside Eppendorf tubes. The foil lined bags and edible media were sterilized by electron beam processing (44 kGy) prior to the addition of test organisms. Cells were stored at room temperature unless listed otherwise.

D. Revival

1. *Spore Growth Curves* – Spores were rehydrated in YP5D or edible media and measured by OD. For OD measurements spores were suspended in YP5D and sonicated on ice for two minutes to disperse spore clumps**.** Once dispersed, spores were placed in flasks containing YP5D at a starting OD of 0.5 and measurements were taken every hour for seven hours omitting the four hours for germination. A twenty-four hour time point was also included to determine OD at the end of fermentative growth.
2. *Spore Biomass* – Spores were rehydrated in YP5D and allowed to growth over 16 hours. Biomass was measured by the percent change in biomass starting with initial and ending mass. After 16 hours spores were washed 3 times with water, lyophilized, and weighed as dry mass. Accurate plate counts of viable spores were unobtainable due to spore clumping and were rejected as a test method.
3. *Vegetative Cell Viability* – Vegetative cells were rehydrated in dilute phosphate buffer (1:8), serially diluted, and plated on YPD. The relative viability was determined by colony forming units per milliliter (cfu/mL) as compared to the average cfu/mL of the control plates prior to dehydration (done in biological triplicates). Each experiment was run in triplicates.

IV. Results

A. Protectants

A variety of protectants have been utilized to increase freezing and desiccation tolerance for long-duration storage.[40] However, all protectants utilized in this application should be FDA approved and safe for crew consumption. The National Collection of Yeast Cultures has successfully used an edible mixture of skim milk, trehalose, and monosodium glutamate (MSG) as cryoprotectants with lyophilization. We have tested this mixture for the lyophilization of *S. cerevisiae* spores. Results indicate lyophilized spore viability is higher when using cryoprotectants, as opposed to only water (Fig. 2). Proline has also been found to be an effective cryoprotectant.[41] In this instance, the substitution of proline for skim milk was also successful (Fig. 2). Using protectants during ambient temperature vacuum drying had a negligible effect on viability. As such, they will not be pursued further for vacuum drying testing. All tests of lyophilized spores had lower viability than their vacuum-dried counterparts, regardless of use of cryoprotectants, suggesting the freezing step during lyophilization is overly damaging to the yeast spores. Lyophilization was therefore dropped as a method of dehydration of spores for remaining experiments.

Trehalose was also used as an exogenous protectant in experiments to determine the viability of *S. boulardii* and *S. cerevisiae* vegetative cells in YPD media for up to seven days directly after air-drying. Both strains were dehydrated in a 10% trehalose mixture or water. The survival of yeast cells, after 3, 5, and 7 days of growth, were tested to determine if time spent in stationary phase had any effect on long-term survival of vegetative cells. *S. boulardii* was grown in YPD for at least 72 hours to ensure the transition from fermentation to respiration. The transition to respiration is a key factor in increasing desiccation tolerance in yeast.[42] When *S. cerevisiae* begins exhausting a necessary nutrient such as glucose, it transitions into a quiescent state, which is characterized by an arrested cell cycle and the accumulation and conservation of storage carbohydrates such as trehalose and glycogen.[43] Quiescent cells form during stationary phase, which typically occurs in rich (YPD) liquid media after three to five days of growth, depending on the cell line's doubling time.[39] Stationary phase cells tend to have higher survival after desiccation and during long-term storage.[38,42]

Trehalose had a negligible effect on the survival of *S. boulardii* and *S. cerevisiae* vegetative cells once they reached stationary phase and were dehydrated by air-drying. The viability of *S. boulardii* tends to decrease as more time is spent in stationary phase, which is consistent with data by Calahan et al.[42] of *S. cerevisiae* (Fig. 3A). However, our Y55 *S. cerevisiae* strain did not show a significant reduction in viability over time when compared to *S. boulardii*. (Fig. 3B). Longer-duration storage testing is needed to determine if increased time spent in stationary phase will affect long-term viability.

B. Dehydration and Storage

Three different methods of dehydration were employed to determine maximum initial viability directly after desiccation for both yeast spores and vegetative cells. Vacuum dehydration resulted in a higher viability when compared to lyophilization for *S. cerevisiae* spores (Fig. 2) and was used as an internal parameter to test viability during air-drying. Multiple parameters were tested to determine the effects of temperature and water on yeast viability during desiccation and long-term storage. An average of 0.06 grams of spores were air-dried on Parafilm® situated over 24 well plates and covered to reduce potential for contamination (Fig. 4C). Spores were air-dried as flat discs to maximize their surface area, thereby reducing drying time and moisture content. All spores were sealed in aluminum lined bags in an anaerobic chamber to remove oxygen, and subsequently stored at room temperature. Spores were rehydrated in YP5D and grown for 16 hours before final biomass was determined. The first experiment was to determine effects of temperature on sporulation rates. Normally *S. cerevisiae* cells are placed in sporulation media (1% potassium acetate) at 30 °C.[44] In our experiments with *S. cerevisiae*, Y55 has a sporulation efficiency of approximately 85% (spores to vegetative cells). By reducing the temperature during the last phase of sporulation we hoped to increase sporulation efficiency and reduce vegetative cell

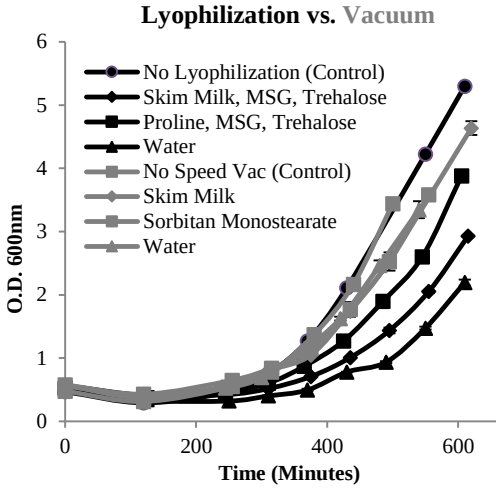

Figure 2. Lyophilization vs. vacuum. *S. cerevisiae spores were suspended in an edible protectant and dehydrated by either lyophilization or vacuum. Prior to dehydration untreated spores were revived as a baseline to measure later viability experiments.*

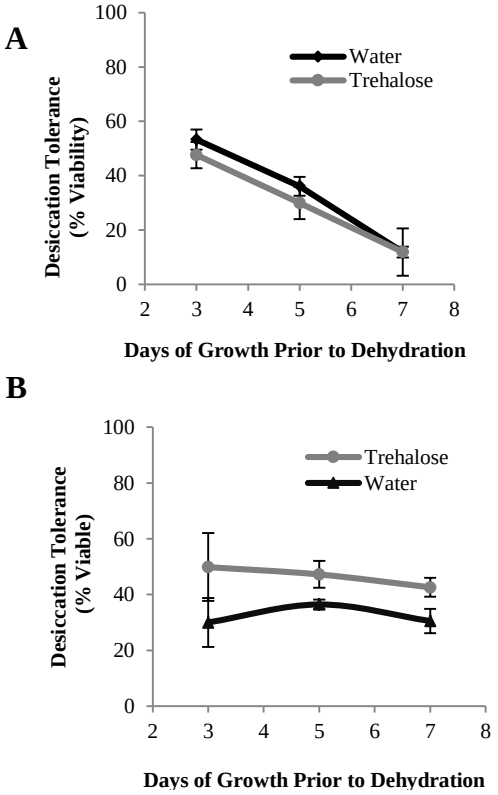

Figure 3. Desiccation tolerance of vegetative cells by air-drying with 10% trehalose as a protectant. *(A) S. boulardii was grown in YPD for 3, 5, and 7 days and assayed for desiccation tolerance (see Materials and Methods: vegetative cells). (B) S. cerevisiae viability after desiccation at 3, 5, and 7 days of growth in YPD.*

contamination. However, room temperature sporulation did not significantly affect sporulation for the Y55 strain or the viability for long-term storage. Spores were also air-dried within a desiccator to determine the effects of moisture on spores during storage. Water was shown to negatively affect spore viability as seen in Figure 4A. Spores stored in water after six months had very few surviving cells after 16 hours of growth in YP5D. Spores were also dehydrated at 5 °C to determine the effects of temperature on spores during dehydration. After one year of storage there is no significant difference between any of the parameters that were dried by air-drying. Spores dehydrated by vacuum are showing a decrease in viability after one year.

Storage temperatures were also tested to determine if temperature would have any effect on long-term storage of spores (Fig. 4B). Spores were stored in the same way as the previous experiment, except storage occurred at both room temperature and at 4 °C. After six months of storage, we have yet to observe any significant decline in viability between room temperature and 4 °C. These two experiments will extend for the duration of two years.

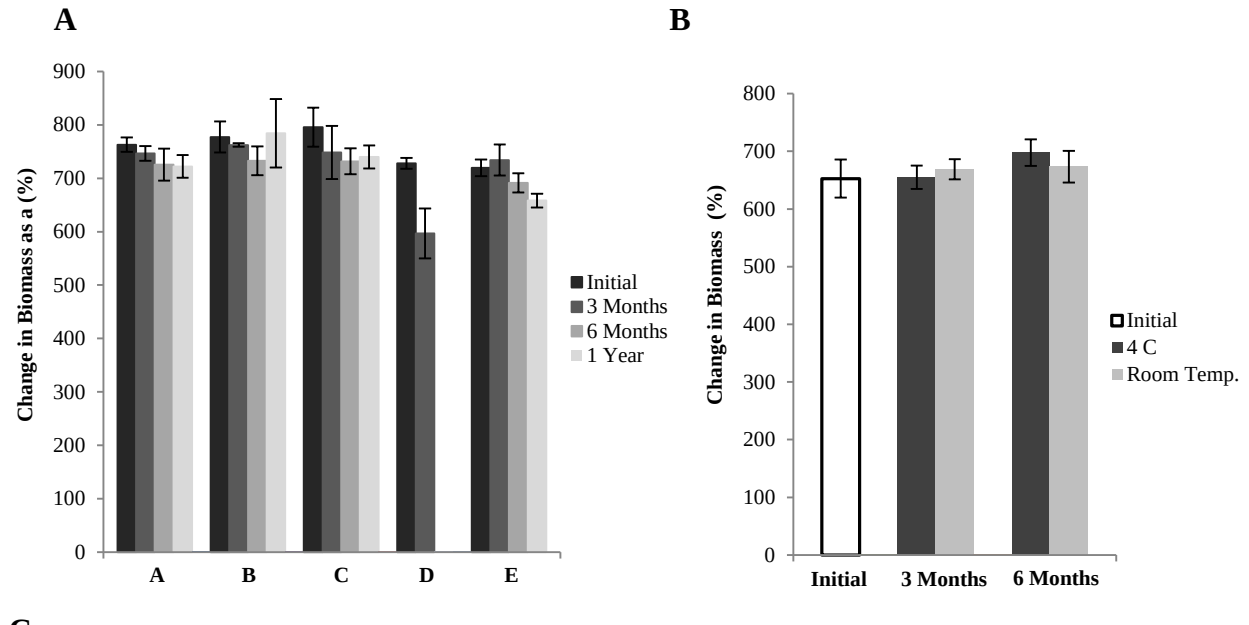

Figure 4. *S. cerevisiae* spore storage. *Spores were stored anaerobically in aluminum lined bags and revived in YP5D for 16 hours. Germinated cells were washed 3 times, frozen, lyophilized, and weighed for final biomass. (A) A. Spores grown at room temperature in sporulation media B. Spores dehydrated in a chemical desiccator C. Spores air-dried at 5 °C D. Spores stored in water E. Spores dehydrated by vacuum (B) spores stored at room temperature or at 4 °C. (C) Image of S. cerevisiae spores air-drying in 24 well plate of parafilm with lid slightly ajar to allow for dehydration.*

A three-year storage study is currently underway for *S. cerevisiae* spores (Fig. 5). Spores were dehydrated by vacuum and stored in the same way as experiment 4A. Spores were revived in YP5D at a starting OD of 0.5 or grown in YP5D for 16 hours to determine final biomass. Growth curve and biomass data were collected to determine loss in viability over time. The initial drop in viability shown in Figure 5A is likely a result of vegetative cell death. Approximately 15% of the total culture after sporulation is comprised of vegetative cells, due to incomplete sporulation. Samples stored for one year showed on average 10% less biomass than samples stored for 1 week, a statistically significant difference (Fig. 5B). These results are promising in that the loss in viability is relatively minimal for spores stored up to one year at room temperature.

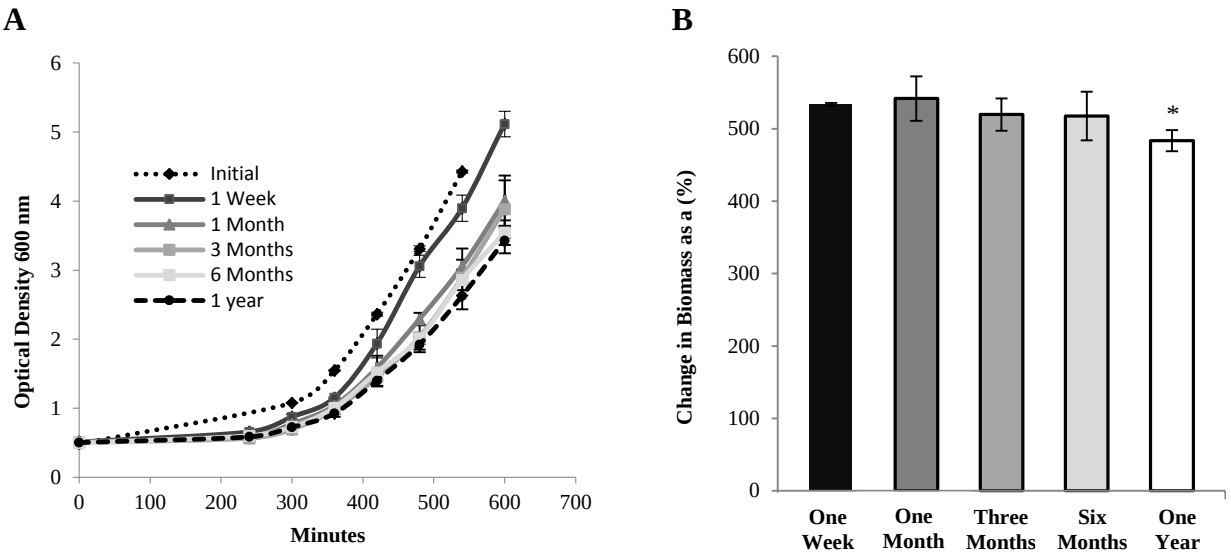

Figure 5. *S. cerevisiae* spore revival in YPD 5x dextrose*: Experiment (A) and (B) were dehydrated by vacuum. (A) Prior to vacuum, an untreated initial sample of spores was stored at 4 °C for 24 hours and revived as a baseline to measure later viability experiments. Growth was measured over 10 hours allowing for a four hour gap required for germination. (B) Percent change in biomass is measured by the starting dry mass of the dehydrated spores compared to the final biomass. Spores were allowed to germinate and grow for 16 hours in YP5D, washed 3 times with water, frozen, lyophilized, and weighed for final biomass. Biomass loss from one week compared to one year represents a statistically significant difference. Asterisk represents the statistical significance ($p \leq 0.005$, t test)*

C. Anhydrobiotic Engineering

To enhance survival during long-duration storage of *S. boulardii*, alternative methods to increase viability are being sought. We have created a knock-out of the neutral trehalase gene NTH1 using the Clustered Regularly Interspaced Short Palindromic Repeats-Cas9 based genome editing tool. The gene NTH1 is involved in the hydrolyzation of intracellular trehalose. As discussed, trehalose has been implicated in the prevention of cytoplasmic and membrane protein aggregation, which occurs during desiccation. It has been shown that increasing endogenous trehalose stores by reducing the yeasts' ability to breakdown intracellular trehalose will increase its ability to survive in the desiccated state longer.[28,31] The NTH1 knockout has already been tested in *S. cerevisiae*, but never in *S. boulardii*. Early data shows a significant difference in viability between the wild type and the NTH1 deletion strain (Fig. 6). However, longer-duration testing will be needed to determine if a NTH1 deletion will follow this pattern of increased viability. It is important to note that after three months of storage, *S. boulardii* NTH1 knockout has not had a significant decline in viability directly after desiccation. Further testing is required to conclude the rate of decline in viability over time.

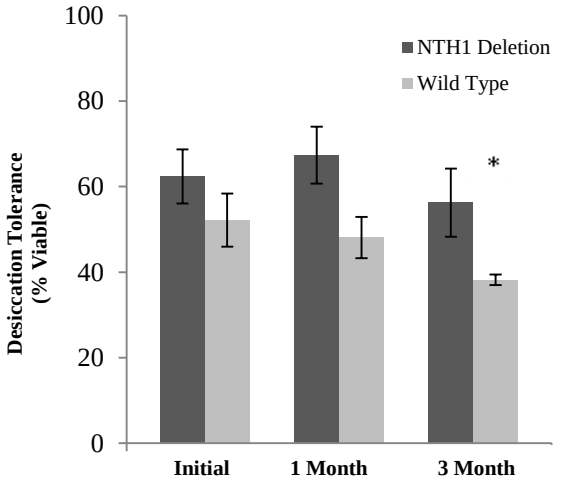

Figure 6. Desiccation tolerance of wild type and NTH1 mutant *S. boulardii*. *After one month of dehydration the wild type strain shows a significant decline in viability as compared to the NTH1 mutant. Asterisk represents the statistical significance ($p \leq 0.005$, t test)*

V. Conclusion

As evidenced by the above results, stored *S.* cerevisiae spores have maintained high viability over the course of one year. This indicates that the *In situ* production of nutrients by *S. cerevisiae* represents a promising approach to problems of nutrient degradation in long-duration space missions. Continued testing is needed to verify that this approach works over multiple years. In the event cell viability continues to decline in subsequent years, a higher starting biomass can be added to the package to offset cell loss over time.

These experiments have contributed to our understanding of optimal conditions for long-duration storage of spores and vegetative cells. We have found that vacuum drying and air-drying spores result in a similar survival directly after dehydration. Lyophilization resulted in a lower initial viability and continued to decline over time. Continued testing is needed to determine the effects of temperature, desiccant, air-drying and vacuum on long-term survival of spores. For vegetative cells, we have found that air-drying provides the highest initial viability directly after desiccation. Once *S. boulardii* and *S. cerevisiae* reach stationary phase, trehalose has minimal to no effect on the organisms' ability to survive desiccation. Early stationary phase appears to be the optimal time for desiccation to occur as cell viability continues to decline over time for *S. boulardii.* However, longer-term data is needed to determine the effects of time spent in stationary phase on long-term survival on both strains. The anhydrobiotic engineering of *S. boulardii* will require longer-duration testing to determine if the NTH1 deletion follows a pattern of increased viability over time. We have found that optimal storage conditions are those in which the yeast is stored with minimal residual water, and without oxygen. Storage temperature may also affect the long-term viability of spores however, further testing is required. Our data shows that after six months there has been no significant difference between storage at room temperature versus 4 °C.

References

[1]Zwart, S., Kloeris, V., Perchonok, M., Braby, L. and Smith, S., "Assessment of Nutrient Stability in Foods from the Space Food System after Long-Duration Spaceflight on the ISS," *Journal of Food Science*, Vol. 74, No. 7, 2009, pp. H209-H217.

[2]Cooper, M., Douglas, G. and Perchonok, M., "Developing the NASA Food System for Long-Duration Missions," *Journal of Food Science*, Vol. 76, No. 2, 2011, pp. R40-R48.

[3]Perchonok, M., Cooper, M., Catauro, P., "Mission to Mars: Food Production and Processing for the Final Frontier," *Annual Review of Food Science Technology*, Vol. 3, 2012, pp. 311-330.

[4]Nevoigt, E., "Progress in Metabolic Engineering of *Saccharomyces cerevisiae*," *Microbiology and Molecular Biology Reviews*, Vol. 72, No. 3, 2008, pp. 379-412.

[5]Ro, D.-K., et al., "Production of the Antimalarial Drug Precursor Artemisinic Acid in Engineered Yeast," *Nature*, Vol. 440, No. 7086, 2006, pp. 940-943.

[6]Thim, L., Hansen, M. T. and Sørensen, A. R., "Secretion of Human Insulin by a Transformed Yeast Cell," *FEBS Letters*, Vol. 212, No. 2, 1987, pp. 307-312.

[7]Verwaal, R., et al., "High-Level Production of Beta-carotene in Saccharomyces Cerevisiae by Successive Transformation with Carotenogenic Genes from *Xanthophyllomyces dendrorhous*," *Applied and Environmental Microbiology*, Vol. 73, No. 13, 2007, pp. 4342-4350.

[8]Edwards-Ingram, L., et al., "Genotypic and Physiological Characterization of *Saccharomyces boulardii*, the Probiotic Strain of *Saccharomyces cerevisiae*," *Applied and Environmental Microbiology*, Vol. 73, No. 8, 2007, pp. 2458-2467.

[9]MacKenzie, D. A., et al., "Relatedness of Medically Important Strains of *Saccharomyces cerevisiae* as Revealed by Phylogenetics and Metabolomics," *Yeast*, Vol. 25, No. 7, 2008, pp. 501-512.

[10]Kotowska, M., Albrecht, P. and Szajewska, H., "*Saccharomyces boulardii* in the Prevention of Antibiotic Associated Diarrhoea in Children: A Randomized Double-Blind Placebo Controlled Trial," *Alimentary Pharmacology & Therapeutics*, Vol. 21, No. 5, 2005, pp. 583-590.

[11]McFarland, L. V., "Systematic Review and Meta-Analysis of *Saccharomyces boulardii* in Adult Patients," *World J Gastroenterol*, Vol. 16, No. 18, 2010, pp. 2202-2222.

[12]Moré, M. I. and Swidsinski, A., "*Saccharomyces boulardii* Cncm I-745 Supports Regeneration of the Intestinal Microbiota after Diarrheic Dysbiosis–a Review," *Clinical and Experimental Gastroenterology*, Vol. 8, No. 2015, pp. 237.

[13]Kelesidis, T. and Pothoulakis, C., "Efficacy and Safety of the Probiotic *Saccharomyces boulardii* for the Prevention and Therapy of Gastrointestinal Disorders," *Therapeutic Advances in Gastroenterology*, Vol. 5, No. 2, 2012, pp. 111-125.

[14]Murzyn, A., et al., "The Effect of *Saccharomyces boulardii* on *Candida albicans*-Infected Human Intestinal Cell Lines Caco-2 and Intestin 407," *FEMS Microbiology Letters*, Vol. 310, No. 1, 2010, pp. 17-23.

[15]Taylor, G. R., "Space Microbiology," *Annual Reviews in Microbiology*, Vol. 28, No. 1, 1974, pp. 121-137.

[16]Taylor, P. W., "Impact of Space Flight on Bacterial Virulence and Antibiotic Susceptibility," *Infection and Drug Resistance*, Vol. 8, No. 2015, pp. 249.

[17]Ilyin, V., "Microbiological Status of Cosmonauts During Orbital Spaceflights on Salyut and Mir Orbital Stations," *Acta Astronautica*, Vol. 56, No. 9, 2005, pp. 839-850.

[18]Chopra, V., et al. "Alterations in the Virulence Potential of Enteric Pathogens and Bacterial–Host Cell Interactions under Simulated Microgravity Conditions," *Journal of Toxicology and Environmental Health, Part A*, Vol. 69, No. 14, 2006, pp. 1345-1370.

[19]Searles, S. C., Woolley, C. M., Petersen, R. A., Hyman, L. E. and Nielsen-Preiss, S. M., "Modeled Microgravity Increases Filamentation, Biofilm Formation, Phenotypic Switching, and Antimicrobial Resistance in *Candida albicans*," *Astrobiology*, Vol. 11, No. 8, 2011, pp. 825-836.

[20]Wilson, J., et al., "Space Flight Alters Bacterial Gene Expression and Virulence and Reveals a Role for Global Regulator Hfq," *Proceedings of the National Academy of Sciences*, Vol. 104, No. 41, 2007, pp. 16299-16304.

[21]Dupont, S., Rapoport, A., Gervais, P. and Beney, L., "Survival Kit of *Saccharomyces cerevisiae* for Anhydrobiosis," *Applied Microbiology and Biotechnology*, Vol. 98, No. 21, 2014, pp. 8821-8834.

[22]Miyamoto-Shinohara, Y., et al., "Survival Rate of Microbes after Freeze-Drying and Long-Term Storage," *Cryobiology*, Vol. 41, No. 3, 2000, pp. 251-255.

[23]Borovikova, D., Teparić, R., Mrša, V. and Rapoport, A., "Anhydrobiosis in Yeast: Cell Wall Mannoproteins are Important for Yeast *Saccharomyces cerevisiae* Resistance to Dehydration," *Yeast*, Vol. 33, No. 8, 2016, pp. 347-353.

[24]Dupont, S., Beney, L., Ritt, J.-F., Lherminier, J. and Gervais, P., "Lateral Reorganization of Plasma Membrane is Involved in the Yeast Resistance to Severe Dehydration," *Biochimica et Biophysica Acta (BBA)-Biomembranes*, Vol. 1798, No. 5, 2010, pp. 975-985.

[25]de Jesus Pereira, E., Panek, A. D. and Eleutherio, E. C. A., "Protection against Oxidation During Dehydration of Yeast," *Cell Stress & Chaperones*, Vol. 8, No. 2, 2003, pp. 120-124.

[26]França, M., Panek, A. and Eleutherio, E., "Oxidative Stress and its Effects During Dehydration," *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, Vol. 146, No. 4, 2007, pp. 621-631.

[27]Rozenfelde, L. and Rapoport, A., "Anhydrobiosis in Yeast: is It Possible to Reach Anhydrobiosis for Yeast Grown in Conditions with Severe Oxygen Limitation?" *Antonie van Leeuwenhoek*, Vol. 106, No. 2, 2014, pp. 211-217.

[28]Tapia, H., Young, L., Fox, D., Bertozzi, C. R. and Koshland, D., "Increasing Intracellular Trehalose is Sufficient to Confer Desiccation Tolerance to *Saccharomyces cerevisiae*," *Proceedings of the National Academy of Sciences*, Vol. 112, No. 19, 2015, pp. 6122-6127.

[29]Goddijn, O. J. and van Dun, K., "Trehalose Metabolism in Plants," *Trends in Plant Science*, Vol. 4, No. 8, 1999, pp. 315-319.

[30]Elbein, A. D., Pan, Y., Pastuszak, I. and Carroll, D., "New Insights on Trehalose: A Multifunctional Molecule," *Glycobiology*, Vol. 13, No. 4, 2003, pp. 17R-27R.

[31]Tapia, H. and Koshland, D. E., "Trehalose is a Versatile and Long-Lived Chaperone for Desiccation Tolerance," *Current Biology*, Vol. 24, No. 23, 2014, pp. 2758-2766.

[32]Golovina, E. A., Golovin, A. V., Hoekstra, F. A. and Faller, R., "Water Replacement Hypothesis in Atomic Detail—Factors Determining the Structure of Dehydrated Bilayer Stacks," *Biophysical Journal*, Vol. 97, No. 2, 2009, pp. 490-499.

[33]Jain, N. K. and Roy, I., "Effect of Trehalose on Protein Structure," *Protein Science*, Vol. 18, No. 1, 2009, pp. 24-36.

[34]Berny, J.-F. and Hennebert, G., "Viability and Stability of Yeast Cells and Filamentous Fungus Spores During Freeze-Drying: Effects of Protectants and Cooling Rates," *Mycologia*, Vol. No. 1991, pp. 805-815.

[35]Połomska, X., Wojtatowicz, M., Zarowska, B., Szołtysik, M. and Chrzanowska, J., "Freeze-Drying Preservation of Yeast Adjunct Cultures for Cheese Production," *Polish Journal of Food and Nutrition Sciences*, Vol. 62, No. 3, 2012, pp. 143-150.

[36]Florastor, "Frequently Asked Questions," https://florastor.com/faqs/ Accessed Feb 28,2017

[37]Nwaka, S. and Holzer, H., "Molecular Biology of Trehalose and the Trehalases in the Yeast *Saccharomyces cerevisiae*," *Progress in Nucleic Acid Research and Molecular Biology*, Vol. 58, No. 1997, pp. 197-237.

[38]Gadd, G., Chalmers, K. and Reed, R., "The Role of Trehalose in Dehydration Resistance of *Saccharomyces cerevisiae*," *FEMS Microbiology Letters*, Vol. 48, No. 1-2, 1987, pp. 249-254.

[39]Li, L., et al., "Key Events During the Transition from Rapid Growth to Quiescence in Budding Yeast Require Posttranscriptional Regulators," *Molecular Biology of the Cell*, Vol. 24, No. 23, 2013, pp. 3697-3709.

[40]Hubalek, Z., "Protectants Used in the Cryopreservation of Microorganisms," *Cryobiology*, Vol. 46, No. 3, 2003, pp. 205-229.

[41]Rudolph, A. S. and Crowe, J. H., "Membrane Stabilization During Freezing: The Role of Two Natural Cryoprotectants, Trehalose and Proline," *Cryobiology*, Vol. 22, No. 4, 1985, pp. 367-377.

[42]Calahan, D., Dunham, M., DeSevo, C. and Koshland, D. E., "Genetic Analysis of Desiccation Tolerance in Saccharomyces Cerevisiae," *Genetics*, Vol. 189, No. 2, 2011, pp. 507-519.

[43]Gray, J. V., et al., "Sleeping Beauty": Quiescence in *Saccharomyces cerevisiae*," *Microbiology and Molecular Biology Reviews*, Vol. 68, No. 2, 2004, pp. 187-206.

[44]Elrod, S. L., Chen, S. M., Schwartz, K. and Shuster, E. O., "Optimizing Sporulation Conditions for Different *Saccharomyces cerevisiae* Strain Backgrounds," *Meiosis: Volume 1, Molecular and Genetic Methods*, Vol. No. 2009, pp. 21-26.