BioNutrients-2: Improvements to the BioNutrients-1 Nutrient Production System

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The BioNutrients (BN) project aims to develop an on-demand biological production system capable of rapid and safe delivery of multiple nutrients in single-use production packs for long-duration missions. The general concept of this system is to develop edible engineered microorganisms that produce nutrients for human consumption following long-term storage, revival, and growth. BN-1 is a five-year flight project currently on the International Space Station (ISS). It is testing the long-term storage of various microorganisms for the biomanufacturing of space-relevant compounds and validating the performance of the first generation of production packs. The BN-2 project expands the BN-1 flight project scope by introducing novel products, broadening the range of microbial food sources, and improving upon production pack hardware. We are optimizing the biomanufacturing platform for *in situ* production of yogurt and kefir on the ISS. BN-2 also will test engineered microorganisms, including a probiotic strain, to produce carotenoids or follistatin. To improve the feasibility of this technology for mission use, we transitioned from a hard-shell pack to a second-generation fluorinated ethylene propylene (FEP) bag. The single-use FEP bioreactor facilitates growth of target organisms in a contaminant free environment, while reducing mass and volume compared to the BN-1 hardware. These bioreactors will be employed for production of additional space-relevant products in the BN-2 project. On-orbit testing of FEP production packs will enable optimization of the platform for eventual flight use by the crew.

Nomenclature

ARC = Ames Research Center
ATCC = American Type Culture Collection
BN = BioNutrients
COTS = Commercial off-the-shelf
FEP = fluorinated ethylene propylene
FDA = Food and Drug Administration
GRAS = Generally Regarded as Safe

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GFP = green fluorescent protein
HACCP = Hazard Analysis and Critical Control Point
ISS = International Space Station
MELFI = minus eighty-degree laboratory freezer for the International Space Station
NCYC = National Collection of Yeast Cultures
PWD = Potable Water Dispenser
SABL = Space Automated Bioproduct Laboratory

I. Introduction

THE BioNutrients (BN) project addresses the documented issue of nutrient degradation in the NASA food system that occurs during extended storage by providing on-demand production of high-value nutrients in a self-contained platform technology[1]. The BN project leverages synthetic biology approaches to meet the demands of long-duration missions through the development of biomanufacturing systems capable of operating in a space environment.

The first iteration of the project, BN-1, is currently underway on the International Space Station (ISS)[1]. The overall scope of the BN-1 project is to investigate the use of single-use production packs to grow genetically engineered yeast to produce carotenoids. This investigation will also explore the impact of long-duration storage and the exposure of microbes to the space environment over a five-year period. BN-1 stasis packs store a diverse set of microorganisms with potential for future biomanufacturing applications. Many of the microorganisms included in the stasis packs are “Generally Regarded as Safe” (GRAS) by the Food and Drug Administration and can potentially produce useful products such as vitamins, proteins, pharmaceuticals, biofuels and/or bioplastics. The BN project is testing ambient temperature storage to obviate long-duration cold storage thereby reducing power requirements.

The second iteration of the project, BN-2, expands the breadth of microbially produced food items and is slated for flight by late 2021 or early 2022. In addition to carotenoid production, BN-2 will assess spaceflight production of the fermented milk products, yogurt and kefir. An additional genetically engineered product, follistatin, will be expressed in the yeast *Kluyveromyces lactis*. BN-2 production packs will use a second-generation bioreactor that accommodates each of the microbial food products while reducing the overall volume and mass of the technology. Yeast growth media has also been modified to reduce mass while maximizing product yields. Crew consumption of food products is not planned for BN-2.

Currently the NASA food system does not allow for consumption of microbially enriched foods and no food safety procedure is available. As part of the BN project, we will develop a hazard analysis and critical control points (HACCP) plan to ensure food safety. HACCP plans are a systematic preventative approach to reduce and eliminate any potential hazards associated with food production and use. Development of the HACCP plan is a necessary step to enable future consumption of microbially generated foods and biosynthesized products.

II. BN-2 Bioreactor Design and Optimization

BN-2 bioreactor bags are made from fluorinated ethylene propylene (FEP) that will contain a specific combination of dehydrated edible growth substrate and a GRAS microorganism(s). Each reactor will hold either dehydrated yeast cells, lyophilized commercial yogurt or kefir starter culture, or an Ames Research Center (ARC) developed *Streptococcus thermophilus* lab strain. The FEP bags differ significantly from the production pack developed for the BN-1 on-orbit demonstration (Fig.1). BN-1 production packs are composed of high-density polycarbonate with a perforated screw-top lid that secures a gas permeable membrane. The BN-1 hard-shell polycarbonate is a fixed-volume production pack that occupies unnecessary space during storage; however, the production packs met safety and operation requirements while accommodating a constrained flight build but was not optimized for future missions. To enhance the feasibility of the BN concept for mission use, we have transitioned to a bioreactor that is less mass intensive. The BN-2 bioreactor is primarily composed of flexible FEP plastic membrane that is a familiar form factor to the crew and can be packed in a flattened state to reduce overall volume during storage. A comparison of the BN-1 and BN-2 bioreactor designs can be seen in Table 1.

Production packs require gas exchange due to carbon dioxide off-gassing during yeast fermentation. FEP is gas permeable allowing for gas exchange (Fig. 1B). The flexible plastic walls allow BN-2 bioreactors to be packed flat yet have a larger internal volume than BN-1 production packs. The FEP bioreactor and media are sterilized and assembled with dehydrated microorganisms and heat-sealed on Earth. This produces a contaminant-free bag to store the organism(s) and media. The FEP bag contains a tubing port that is heat sealed directly into the side of the bag. The tubing port is connected to a barbed swabbable injection valve which remains closed until a male luer connector is engaged. This valve will be used with a luer lock syringe filter to inject water to activate the reactor. The BN-2 reactors will use the same type of microbial filter for water injection as the BN-1 production packs.

Oxygen and moisture exposure can affect the viability of the microorganisms by causing oxidative damage to the cells when stored long-term and can also cause degradation to the media. The exterior packaging on the BN-1 production packs is a mylar bag that is vacuumed, nitrogen purged, and heat-sealed to prevent exposure to oxygen and moisture. Each mylar bag also contains a Commercial Off-The-Shelf (COTS) silica-based desiccant bag and oxygen scavenger. BN-2 production packs will also have exterior packaging with an oxygen scavenger and desiccant pack. However, we are flight-testing alternative materials and packaging to improve and iterate on the long-term storage design and enable eventual flight use by the crew.

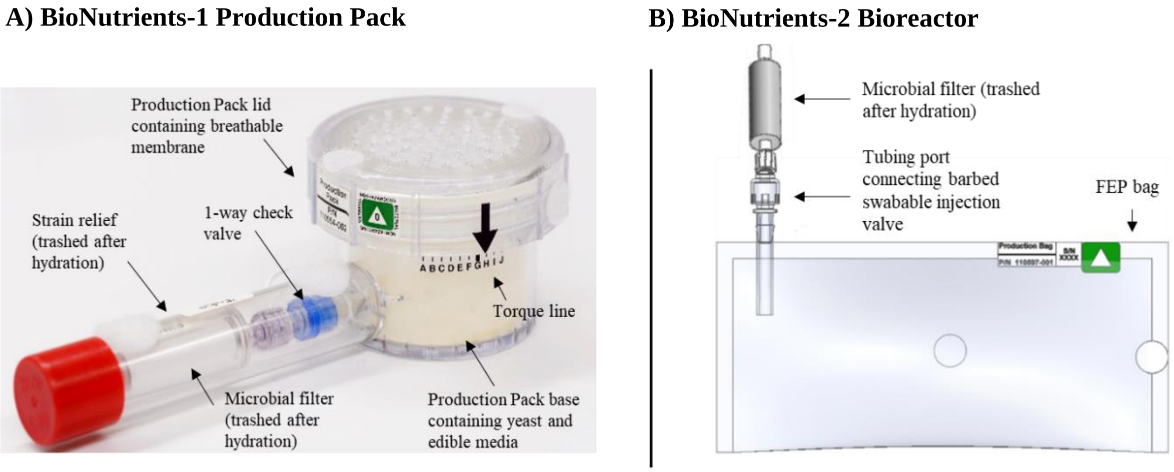

Fig. 1. Visualization of BN-1 and BN-2 Flight Hardware. *The BN-1 production pack consists of a hard-shell pack with screw-top lid. The BN-2 flight hardware has been optimized to reduce mass and volume by using a flexible FEP bag. The bag will still utilize the same microbial filter from the BN-1 design to remove risk of the introduction of contaminants.*

Table 1. Comparison of BN-1 and BN-2 Flight Hardware.

	BioNutrients-1	**BioNutrients-2**
Composition	Polycarbonate	Fluorinated Ethylene Propylene (FEP)
Form Factor	Injection-molded hard-shell container with screw-top lid	Heat-sealed thermoplastic film bag
Gas exchange	Polytetrafluoroethylene membrane	FEP is gas permeable
Internal Volume	115 mL	250 mL
Mass	117 g (Bioreactor only, without water filter/filter cover)	10 g (FEP bag without filter attached)

III. BN-2 Concept of Operations

A. Future on-orbit hydration

The FEP bioreactors will be stored at ambient temperature on the ISS and activated over a six-month period. Two bioreactor activations are planned for the BN-2 project. The first set will be hydrated by a crew member and grown no later than 60 days after launch. The second interval for activation will occur approximately 180 days after launch to understand the shelf-life and performance of the bioreactors over time. Commercial yogurt and kefir starter strains are known to decline in viability in ambient temperature storage, and the second activation will test whether BN-2 packaging maintains viability. Prior to each activation experiment, flown bioreactors will be returned to Earth and parallel bioreactors stored on Earth will serve as ground controls to separate the effects of storage and radiation from growth in the space environment.

Figure 2 illustrates the concept of operations for the BN-2 on-orbit operations. To activate the bioreactors, a crew member will draw an appropriate amount of water into a 1 L bag from the ISS Potable Water Dispenser (PWD) and dispense ambient temperature water into each of the FEP bags. Water is transferred from the 1 L bag by attaching a

50 mL syringe via a luer connector and is added to a 0.2-micron Sterivex filter already connected to the FEP bag (Fig. 1B). The filter will ensure sterile water is injected into the reactor to maintain a contaminant-free environment. Due to the size of the filter, water is trapped in the empty space of the filter and an additional 3 mL of water will be added to each FEP bag to compensate for water loss. Each bioreactor will contain a hydration volume that has been experimentally determined to meet necessary volume and biomass requirements for all downstream analysis once bioreactors are returned to Earth. Specifically, 33 mL of water will be removed from the 1 L water bag and dispensed in the yeast bioreactors for a total volume of 30 mL. The yogurt will contain a total volume of 70 mL and kefir will receive a total volume of 80 mL. Once all bioreactors have been hydrated, filters will be removed from the FEP bag and discarded. The crew will then manually knead the bag and agitate until all contents are completely dissolved. Each experiment will use the same 1 L bag to connect to the PWD for water distribution into the bioreactors and the same 50 mL syringe will be used to hydrate all samples.

The bags containing the yeast, kefir, and yogurt samples will be placed in the Space Automated Bioproduct Laboratory (SABL) incubator at optimal growth temperatures of 30 °C, 22 °C, and 42 °C respectively. The growth duration will be experimentally validated for each set of microorganisms. Growth temperatures and expected incubation times for each set of bioreactors are reported in Table 2. *Saccharomyces cerevisiae*, strain Y55, *S. boulardii*, and *K. lactis* yeast strains will be grown concurrently at 30 °C and will utilize the same SABL incubator.

Although the commercial yogurt (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*) and the engineered laboratory strain of *S. thermophilus* will be grown at 42 °C they may need separate incubators due to differences in growth durations. Optimization of growth duration is being experimentally validated for all fermented milk products. A visual pH indicator will be included within the FEP bag for each of the fermented milk products. The pH indicator, Bromocresol purple, is purple in color at a pH of above 6.8 and will turn yellow below a pH of 5.2. The optimal pH of yogurt and kefir is below 5.2 making bromocresol purple a useful indicator to determine the acidification of the milk. A pH color indicator guide will be provided for the crew member who will assess whether the culture acidified at periodic inspections during the growth cycle. The yogurt and kefir samples will be inspected at four hours and compared to the pH indicator guide, and placed back into the incubator until complete, at which time the temperature will be dropped to 4 °C to stop acidification and growth of the yogurt and kefir producing organisms.

For yeast production, BN-2 bioreactors will be removed from SABL six hours after the hydration activity and agitated to ensure complete dispersion of the yeast pellets and media. After agitation and kneading, they will be placed back in the incubator to complete the remainder of the 48 hour incubation. Once growth is complete all the BN-2 bioreactors will be removed from the SABL incubator and placed in the Minus Eighty-Degree Laboratory Freezer for the International Space Station (MELFI). Samples will be stored at -65 °C or colder and brought back on the earliest returning flight (Fig. 2).

Table 2. Growth, temperature, and volume requirements for each set of the bioreactors.

Organism	Quantity of Samples Per Run	Hydration Volume (mL)	Growth Temp. (°C)	Growth Time (h)	Post-Growth Temp. (°C)	Total Incubation Time (h)	Post-Incubation Cold Stowage Temp. (°C)
S. cerevisiae (Y55)	4	30	30	48	Not applicable	48	≤-65
S. boulardii	4	30	30	48	Not applicable	48	≤-65
K. lactis	4	30	30	48	Not applicable	48	≤-65
Yogurt	4	70	42	6	4	≤24	≤-65
S. thermophilus (GFP)	4	70	42	6	4	≤24	≤-65
Kefir	4	80	22	18	4	≤24	≤-65

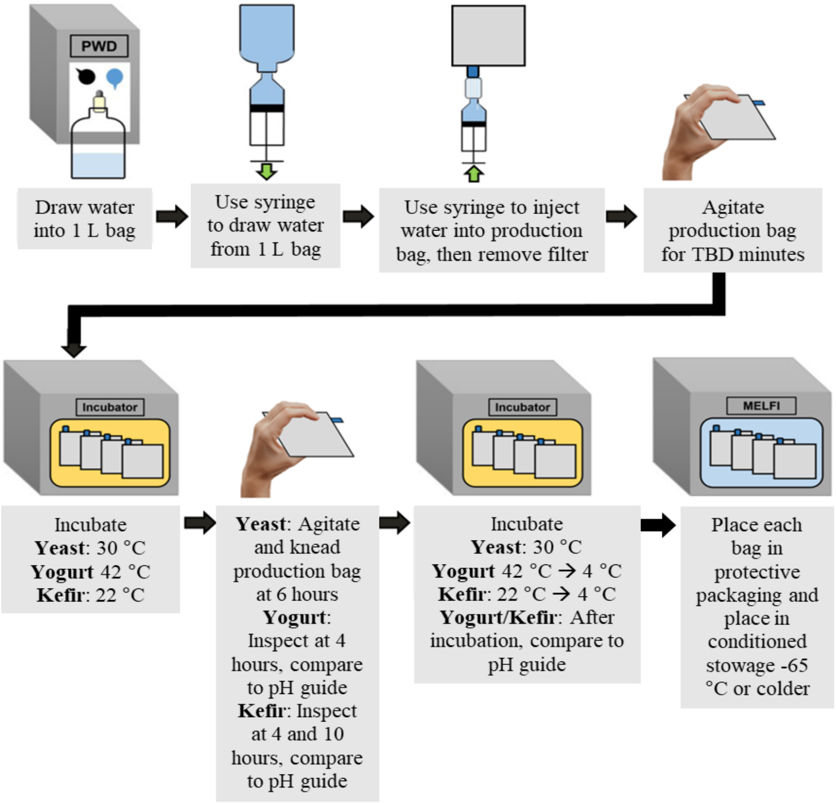

Figure 2. On-orbit operations. *The BN-2 FEP bioreactor bags will be hydrated and grown at their respective temperatures and then frozen in the MELFI until samples can be returned for on Earth analysis.*

B. Yogurt and Kefir Production

Space flight has been shown to alter the composition and physiology of the gut microbiome[2-4]. Reduction in the diversity of organisms present in astronaut microbiomes may make crew members more susceptible to opportunistic pathogens. Probiotics are a potential countermeasure to offset these effects. *In situ* production of fresh yogurt or kefir will facilitate growth of probiotic microbes and could help in maintaining the crew's microbiome health. Furthermore, the addition of fresh food in a crew member's diet can help provide significant psychological benefits during long-duration spaceflights[5].

The BN-2 fermented milk products, yogurt and kefir, will use skim milk powder mixed with dehydrated starter cultures. Commercial yogurt starter cultures include *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*. The commercial kefir starter culture contains a proprietary mix of probiotic bacteria and yeast. Bacteria involved in milk fermentation can also be engineered for production of valuable products. As a proof of concept, the green fluorescent protein (GFP) has been engineered in *S. thermophilus* to demonstrate the feasibility of genetic modification. Genetically engineered yogurt producing bacteria can provide additional vitamins, nutraceuticals, useful enzymes, and medicines within the yogurt.

C. Microbial Production of Space-Relevant Products

Carotenoids are important antioxidants in human diets that are sensitive to degradation in stored foods[6]. Zeaxanthin has been implicated in eye health and vision performance, while β-carotene is a provitamin A and an essential nutrient also aiding in eye health when converted in the body to retinol[7]. The BN-2 project will include two genetically engineered yeast strains from the BN-1 project that produce carotenoids. *S. cerevisiae* Y55 is engineered to produce zeaxanthin and can be stored as spores, while a β-carotene-producing *S. boulardii* probiotic strain will be stored as vegetative cells[1].

A major concern during long-duration space flight is the impact of microgravity on astronaut muscle mass and bone health. Muscle atrophy is a common occurrence among astronauts during exposure to microgravity[8]. Muscle loss is of particular concern for long-duration missions as it will affect the crew members performance and can cause serious health issues. Methods for preventing muscle deterioration have been explored and include muscle-enhancing therapeutics such as follistatin. Overexpression of follistatin can increase muscle mass by actively binding the proteins myostatin and activin. The inhibition of negative regulators by follistatin has been shown to enhance muscle formation[9]. We have genetically engineered *K. lactis* to express follistatin. Furthermore, *K. lactis* can grow on a wider range of substrates and temperatures than that of *S. cerevisiae* and is a useful GRAS organism for bioproduction of additional space-relevant products. The engineered *K. lactis* strain will be grown on the ISS to test for expression of this potential therapeutic as a microgravity countermeasure.

Table 3. Strains and products developed for the BN-2 flight project. *Non-engineered strains were purchased from the American Type Culture Collection (ATCC) and the National Collection of Yeast Cultures (NCYC). The yogurt and kefir strains were purchased from a commercial food supplier.*

Strains for Flight Testing	Product	Origin of Biological Material	Host Strain Designation
Saccharomyces cerevisiae Y55	Zeaxanthin	NASA ARC flown in BN-1	NCYC 3560
Saccharomyces cerevisiae boulardii	β-carotene	NASA ARC flown in BN-1	ATCC MYA-796
Kluyveromyces lactis	Follistatin	NASA ARC developed for BN-2	ATCC 8585
Streptococcus thermophilus/ Lactobacillus bulgaricus	Yogurt	Commercial supplier	YO-MIX 151
Streptococcus thermophilus	GFP	NASA ARC developed for BN-2	ATCC BAA-491
Kefir-producing mixed organism culture	Kefir	Commercial supplier	C-FIR

IV. Future Work

The aim of the BN-2 project is to continue to optimize this platform technology for mission use by iterative testing to increase efficiency and space readiness. In BN-2, we are focusing on optimizing bioreactor design but have also begun to address food safety. Safety protocols must be developed for production of probiotics, microbially-enriched foods and bioproducts, for future consumption or use by crew members. To date, there is no food safety plan for microbially enriched foods in the NASA food system, as NASA does not currently allow astronauts to consume foods with actively growing microorganisms or probiotics. Potential contamination with off-nominal microorganisms can present a dangerous health risk to crew members and could jeopardize mission success. Therefore, as part of the BN-2 project we will integrate a HACCP plan which provides a comprehensive safety protocol developed and tested against standard NASA and FDA safety methods. A HACCP plan is a food safety monitoring system designed to control biological, chemical, as well as physical hazards during food preparation, storage, and transportation. The BN HACCP plan will identify and control potential hazards during all phases of storage and production processes. It will focus on media production, storage, packet materials and assembly, culture purity and contamination testing. It will also include *in situ* procedures such as packet hydration, incubation in space, as well as final product testing. Commercially available systems, such as Petriflms, will be utilized during manufacture of the BN-2 bioreactors to detect food-borne pathogens. These tests will be conducted to ensure only the intended GRAS organisms are present in the bags. In future iterations of the BN projects, grown bioreactors may also be heat processed to meet NASA safety guidelines of no live microorganisms. The HACCP plan will cover both heat killed and live microorganisms. Comprehensive ground safety testing will first be conducted and evaluated, followed by eventual flight testing on ISS. In the development of the HACCP plan, personnel from NASA's Human Research Program (HRP) Food Systems Program will be consulted to ensure that NASA regulations are addressed and adhered to. Another BN priority is to identify technologies for on-demand food safety testing that can potentially be applied, in real time, to ensure the fermented food product is free of contamination.

The planned BN-3 project will further enhance this platform technology by producing additional nutrients per bioreactor. For the current BN-1 and BN-2 projects, a single nutrient is produced per microorganism. Future work will include engineering individual microorganism strains to produce multiple nutrients upon hydration of the production bag. Producing multiple compounds in a single bioreactor will reduce the use of consumables and make the overall technology more efficient for long-duration missions.

V. Conclusion

The BN project seeks to provide advances for in-space biomanufacturing by addressing mission needs for future exploration efforts. Microbially-produced foods and *in situ* production of necessary products offer an alternative to intensive crop-based production methods with substantially less infrastructure and crew member time. The BN-2 flight project will expand the breadth of microbial food sources from the BN-1 project by producing yogurt and kefir on the ISS. This project will also test and analyze an additional space-relevant product follistatin, in a genetically engineered *K. lactis* strain. Furthermore, the GFP producing *S. thermophilus* will determine feasibility of using yogurt as a consumable genetically engineered product. The BN products provide high-value nutrients as supplements for the crew's food supplies, thereby addressing the observed degradation of certain nutrients in the NASA food system and providing fresh and familiar fermented food products.

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