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Astropharmacy: in-space pharmaceutical manufacturing for deep space missions

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Abstract

During long-duration spaceflight beyond Low Earth Orbit (LEO), astronauts will be exposed to increased health risks that will require medical countermeasures. Biologics (e.g., peptide-based drugs) are particularly well-suited to treat many of the spaceflight-induced medical conditions. However, supplying these pharmaceuticals will be challenging due to reduced drug stability in space, limited shelf-life (6 months for biologics) even when refrigerated, and mass/volume constraints. To address this challenge, we are developing a platform for on-demand production of small doses of pharmaceuticals in deep space. This platform uses engineered *Bacillus subtilis* bacteria, which can be transported and stored in space as durable dried spores. When an astronaut gets sick, these spores will be activated inside a custom hardware system to initiate production of the required drug. Seven small peptide drugs with relevant indications (e.g., bone loss and post-radiation exposure treatments) have been successfully expressed in *B. subtilis* to date. Several prototypes have been developed to translate laboratory culture and purification procedures into a hardware system to enable start-to-finish production in space, with minimal crew involvement. For each prototype, we are deriving the Figures of Merit (mass, power, volume, crew time), which are being compared to traditional laboratory protocols. The implementation of the "Astropharmacy" during long-duration missions beyond LEO will be discussed using a test scenario where an astronaut develops neutropenia (low white blood cells) following a solar particle event. Overall, the ability to produce small quantities of pharmaceuticals on-site, on-demand, with minimal power, mass and crew time requirements will address a major barrier to keeping astronauts healthy during long-duration spaceflight beyond LEO. On Earth, this platform could be beneficial in field situations with similar logistical constraints, such as in war zones, extended submarine deployments, or remote communities. This approach could enable personalized treatments in space and on Earth and make orphan drugs more affordable, while making the development of new drugs more rapid.

Keywords: in-space drug manufacturing; on-demand drug manufacturing; space medicine; long-duration human spaceflight; engineered microbes; peptide medications

Acronyms/Abbreviations

National Aeronautics and Space Administration (NASA), granulocyte colony-stimulating factor (G-CSF), figures of merit (FOM), Tobacco Etch Virus (TEV), granulocyte-macrophage colony-stimulating factor (GM-CSF), Luria Broth (LB), room temperature (RT), optical density (OD), relative luminescence units (RLU), fluorinated ethylene propylene (FEP), critical quality attributes (CQA), stereolithography (SLA), Bicinchoninic Acid (BCA), to be determined (TBD), not applicable (NA), package (pkg).

1. Introduction

As we prepare to enter the new era of human space exploration with plans for long-duration missions to the Moon and Mars, astronauts will increasingly rely on medications to mitigate the health risks due to prolonged periods of reduced gravity, increased radiation exposure and prolonged isolation in a high-stress environment [1, 2]. However, supplying required medications from Earth will not be feasible due to limited shelf-life of many medications, reduced drug stability in space, upmass and volume constraints, and limited resupply opportunities [1, 3–6].

Peptide and protein-based pharmaceuticals can be particularly well-suited to treat medical conditions

during long-duration spaceflight [7]. The hazards of long-duration spaceflight, especially prolonged reduced gravity and radiation exposure, can cause degenerative conditions such as bone and muscle loss, cytopenias, and dysregulation of the immune system [8–10]. On Earth, peptide-based pharmaceuticals are increasingly being investigated and approved to treat similar metabolic, cardiovascular, endocrine, and radiation-induced conditions [11–13]. Despite their promising efficacy in treating spaceflight-induced medical conditions, biologics are not currently used in medical kits onboard the International Space Station (ISS) as they often require refrigeration for storage and their shelf-life is even more limited to as little as ~6 months [14].

The challenge of supplying astronauts with required medications in deep space and the "risk of ineffective or toxic medications during long-duration exploration spaceflight" that was identified by the Human Research Program at the National Aeronautics and Space Administration (NASA) could be addressed by producing required pharmaceuticals *on site, on demand*. These capabilities would also open the door for potential use of peptide and protein-based pharmaceuticals to treat spaceflight-induced medical conditions.

To enable on-site production of pharmaceuticals in deep space, we are developing a compact platform called "Astropharmacy" that uses engineered *Bacillus subtilis* bacteria to produce small doses of peptide-based pharmaceuticals on demand. We have demonstrated production of granulocyte colony-stimulating factor (G-CSF) for treatment of radiation-induced neutropenia and teriparatide for treatment of osteoporosis by engineered *B. subtilis* in a laboratory setting [15].

However, there remains a need to translate these laboratory procedures to spaceflight suitable hardware. Earth-based culture systems for recombinant protein production are unsuitable for space exploration as they are often optimized for large-scale production, require equipment that is heavy and complex, and use large amounts of consumables [16]. Technologies for space are commonly developed with the goal of minimizing the system's launch cost by minimizing its mass, power, volume, and crew time requirements [17]. These parameters are commonly referred to as the Figures of Merit (FOM). Additional factors to consider include variable gravity (e.g., microgravity during transit to Mars versus. ~⅓ of Earth's gravity on the Martian surface), storage and stability of raw materials under variable temperatures and space radiation, and utility of reusable versus disposable components.

In this study, we report on the ongoing work related to the development and testing of hardware prototypes for operation of the Astropharmacy in deep space and determination of the FOM for traditional laboratory methods and the developed prototypes.

2. Material and methods

2.1 Engineered microbial strains used in this study

Two genetic constructs encoding for (1) eforRED chromogenic protein fused with Strep-Tag® II and (2) *ydjM* secretion peptide + Strep-Tag® II + HiBiT + GS linker + Tobacco Etch Virus (TEV) cleavage site + molgramostim pharmaceutical peptide were genome-integrated into protease-deficient RIK1285 strain of *Bacillus subtilis* (TaKaRa Bio, San Jose, USA) as described in [15]. The resulting engineered strains of *B. subtilis* expressing eforRED and molgramostim (a.k.a., granulocyte-macrophage colony-stimulating factor, GM-CSF) were denoted as strains AK45 and AK29, respectively.

2.2 Culture conditions

B. subtilis strains AK45 and AK29 were streaked from glycerol stocks onto Luria Broth (LB) plates containing 1.5% agar and 100 µg/mL spectinomycin antibiotic. Plates were incubated overnight at 37°C. Then 5 mL of liquid LB containing 100 µg/mL spectinomycin were inoculated from solid plates and incubated overnight at 37°C. These overnight cultures were used to inoculate larger cultures for prototype testing or for production of eforRED or GM-CSF for FOM analysis. Unless otherwise specified, cultures were incubated at 37°C, 200 rpm.

2.3 Quantification of culture growth

Culture growth was quantified based on optical density at 600 nm (OD600). OD600 was measured in 200 µL samples in 96-well, transparent, flat bottom plates (Thermo Fisher Scientific, Waltham, USA) using SpectraMax® 384 spectrophotometer (Molecular Devices, San Jose, USA). LB media was used for a blank measurement, which was subtracted from sample measurements.

2.4 Quantification of peptide expression

Expression of GM-CSF in strain AK29 was quantified based on expression of the HiBiT tag that produces a luminescent signal when bound to its complimentary LgBiT peptide. HiBiT expression was quantified using commercial Nano-Glo® HiBiT Extracellular Detection System (Promega, Madison, USA), following manufacturer's instructions. Luminescence was read using SpectraMax® M5 microplate reader (Molecular Devices) at 500 ms integration time. LB media with HiBiT detection reagents was used for a blank measurement, which was

subtracted from sample measurements. Measurements were reported in relative luminescence units (RLU).

2.5 Spaceflight hardware prototype development and testing

A 3D-printed expression chamber (Fig. 1A) and commercial fluorinated ethylene propylene (FEP) PermaLife™ culture bags (OriGen Biomedical, Austin, USA) (Fig. 1C) were investigated for culturing of engineered *B. subtilis* and expression of target pharmaceutical peptide, as alternatives to traditional laboratory flasks (Fig. 1B). The 3D-printed expression chamber was designed in the computer aided modeling software Fusion 360 (Autodesk, San Francisco, USA), built using a stereolithography (SLA) Form3+ printer (FormLabs, Somerville, USA) from a translucent, durable photocurable resin (FormLabs), with 25 mL or 50 mL culture volume capacity. It was designed to be compatible with a plate reader for potential monitoring of culture growth and a plate centrifuge. It also contains a port in the middle that would allow the culture to be drawn via a syringe into a lysis chamber. The FEP culture bag also has two ports that can be connected to a syringe or tubing to draw the culture out and could be advantageous due to lower mass/volume, spaceflight heritage [18], and a gas-permeable material.

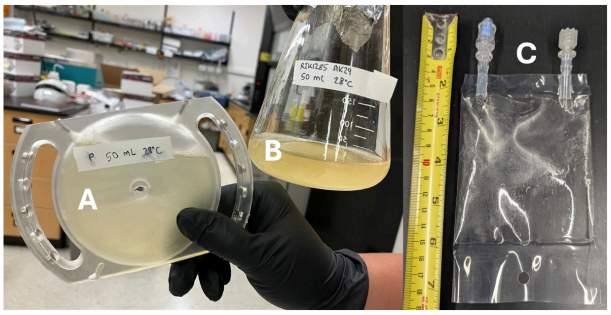

Fig. 1. Culture vessels used in the study. A) 3D-printed growth/expression prototype. B) Traditional 250 mL flask. C) Commercial FEP culture bag.

For cell lysis, we tested a commercially available OmniLyse device (Claremont BioSolutions, Upland, USA) that was previously used onboard the International Space Station as part of the WetLab-2 experiment (Fig. 2) [19]. Traditional laboratory lysis protocol was used as a positive control. In short, cell pellets were resuspended in Milli-Q® water containing 5 mg/mL lysozyme, incubated on ice with gentle shaking for 1 hour, and centrifuged at 16,000 rcf for 10 min to obtain the clarified lysate. As an alternative to lysis, we are also investigating the feasibility of engineering *B. subtilis* to secrete produced pharmaceutical peptides.

For purification, we have set-up a system that uses a small peristaltic pump controlled by Arduino Mega microcontroller and tubing to pass lysate, wash, elution, and regeneration buffers through a spin tube containing resin that selectively binds proteins containing the Strep-Tag® II. This system would allow to automate and achieve continuous purification, thus minimizing the need for astronaut time, compared to traditional spin column or magnetic bead batch purifications. This would also eliminate the need for a centrifuge or a magnetic rack for the purification step. We tested this purification prototype with eforRED protein using Strep-Tactin®XT 4Flow® resin (IBA Lifesciences, Gottingen, Germany) with pre-made wash, elution, and regeneration buffers from the manufacturer.

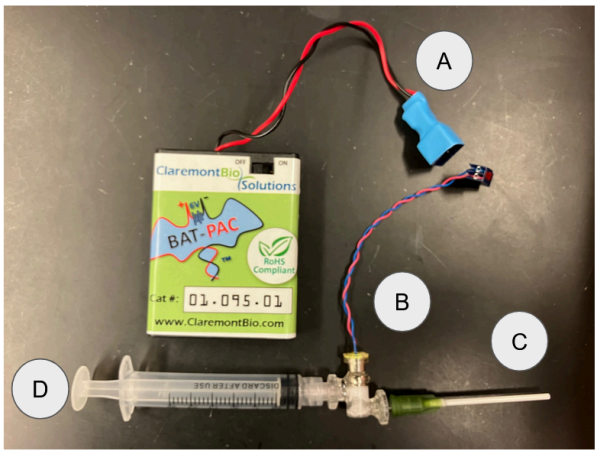

Fig. 2. OmniLyse (ClaremontBio) cell lysis system. Contains a battery pack (A), lysis chamber packed with glass beads (B), tip to aspirate and dispense fluid (C), and a syringe (D).

2.6 FOM analysis

The FOM analysis started by identifying materials and equipment needed to grow, lyse, and purify a 50 mL culture of *B. subtilis* strain AK45 expressing eforRED using either traditional laboratory methods or using the spaceflight hardware prototypes. This strain was used for experiments as eforRED protein is highly visible to the naked eye, making it easier to compare yields and troubleshoot issues. The mass of each required material was measured using Quintix2101-1S scale (Sartorius, Gottingen, Germany). Due to the irregular shape of many materials, the volume was estimated from measured length, width, and thickness, assuming rectangular prism shape, which provided a conservative estimate. For traditional laboratory methods, all liquids are stored in fixed-volume containers. Thus, only mass of the liquids was reported in the FOM analysis, while their volume was accounted for in the FOM for the storage container. For spaceflight hardware prototypes,

liquids are stored in bags that expand in volume when the liquid is added. Thus, the volumes of the liquid and the storage bags were reported separately. When available, the mass and volume of the equipment were determined from manufacturer's specifications. The power consumption by the incubators was measured using an industrial energy monitor installed between the wall outlet and incubator. Otherwise, power consumption in kWh was estimated from maximum power consumption in W specified by the manufacturer, multiplied by hours of operation.

The scenarios and assumptions described in sections 2.6.1 and 2.6.2 below were used to measure or estimate FOM for the standard laboratory methods and the spaceflight hardware prototypes, respectively. The administration step is expected to be the same between traditional laboratory methods and the hardware prototypes and the FOM for syringe, needle, and alcohol wipe were measured. However, required assays for critical quality attributes (CQAs) have not been defined yet.

2.6.1 FOM analysis of standard laboratory methods

Growth/expression step: 50 mL of media is transferred from a 50 ml Falcon tube to a 250 mL flask. 500 µL of overnight culture is used to inoculate the culture, which is then incubated at 37°C, 200 rpm for 5 hours.

Lysis step: The culture is collected into 2 mL tubes (to fit into 5424R benchtop refrigerated centrifuge from Eppendorf, Hamburg, Germany) and centrifuged at 15,000 rcf for 30 seconds. Most of the supernatant is aspirated and the remaining supernatant is used to resuspend pellets and consolidate cells into a single 2 mL tube. The tube is centrifuged again and the remaining supernatant is discarded. The resulting pellet is resuspended in 2 mL of lysis buffer made of Milli-Q® water containing Halt™ protease inhibitor cocktail (Thermo Fisher Scientific) at 1x concentration and 5 mg/mL lysozyme. The cells are incubated in lysis buffer on a rotating mixer for 1 hour at 4°C. The lysate is then centrifuged at 16,000 rcf for 10 min and the clarified soluble lysate fraction is collected.

Purification step: 2 mL of clarified lysate are purified using MagStrep® Strep-Tactin®XT beads (IBA Lifesciences), following manufacturer's instructions with the following modifications: 200 µL of 5% bead suspension used, wash buffer volume increased to 1 mL per equilibration or wash, elution buffer volume increased to 200 µL. Invitrogen™ Dynabeads™ magnetic rack (Thermo Fisher Scientific) for 1.5 mL tubes was used to retain magnetic beads.

2.6.2 FOM analysis of spaceflight hardware prototypes

Growth/expression step: 50 mL of media stored in a bioprocess bag are transferred through tubing into a 70 mL FEP bag using a peristaltic pump. The culture is inoculated from spores of engineered *B. subtilis* dried onto a piece of paper. The culture is incubated at 37°C for 5 hours, with manual shaking by the crew every hour.

Lysis step: Using a peristaltic pump, the culture is pulled from the FEP bag and passed through 5 OmniLyse devices positioned in series and circulated through the OmniLyse devices for a total of 2 times. The lysate is then centrifuged at 16,000 rcf for 10 min and the clarified soluble lysate fraction is collected into a bioprocess bag.

Purification step: Using a peristaltic pump, ~20 mL of clarified lysate are pumped into a spin column 500 µL of 50% suspension of Strep-Tactin®XT 4Flow® resin (IBA LifeSciences), while the outlet tubing is clamped closed. The lysate is incubated with the resin for 10 min. The tubing clamp is then removed to allow the lysate to flow into a bioprocess bag for waste collection. The step is repeated to process the remaining clarified lysate. Next, 20 mL of wash buffer are pumped through the spin column and into the waste collection bag. Next, 2 mL of elution buffer are pumped into the spin column and incubated with the resin for 5 min, before being collected into a collection bag. This step is repeated for a total of 3 times. Next, 5 mL of regeneration buffer, followed by 10 mL of wash buffer are pumped through the spin column and into the waste collection bag. Lastly, 500 µL of 20% ethanol are added to the beads and the column is stored until next use.

3. Results and Discussion

3.1 Spaceflight hardware prototype development and testing

3.1.1 Culture growth and target protein expression comparison between different culture vessels

Culture growth and peptide expression of GM-CSF-expressing *B. subtilis* strain AK29 were compared between the 25 mL and 50 mL 3D-printed prototypes and traditional 125 mL and 250 mL laboratory flasks (25 mL and 50 mL working culture volume, respectively) at 37°C, 28°C, and room temperature (~18 to 22°C). As expected, the highest culture growth in the traditional flasks was observed at 37°C at both culture volumes (Fig. 3A, i & ii), with both cultures reaching stationary phase ~5h after inoculation. The culture density in the prototype was reduced ~4-fold in the 50 mL culture and ~2.5-fold in the 25 mL

culture (Fig. 3A, i & ii). As expected, culture growth was reduced at 28°C as the flask culture reached OD600 of 0.6 and did not appear to reach the stationary phase by 8 hours (Fig. 3A, iii). On the other hand, the culture in the prototype appeared to reach a stationary phase at OD600 of ~ 0.25 after 5h (Fig. 3A, iii). Flask and prototype cultures at room temperature did not grow sufficiently over 8h to observe any differences (Fig. 3A, iv). This is attributed to both lower temperature and the fact that these cultures were not agitated like 37°C and 28°C cultures.

While 37°C is commonly used to maximize biomass, lower temperatures are often beneficial for protein expression [15, 20]. In the traditional laboratory flasks, peak peptide expression at 37°C was reached after 3 hours of culture in both culture volumes, followed by an immediate decline in the measured HiBiT in the supernatant (Fig. 3B, i & ii). This could be due to the degradation of the target peptide at 37°C. In the prototypes at 37°C, protein expression was ~1.5-fold lower at the 3h peak, compared to traditional flasks. At 28°C, peptide expression was lower, but the produced peptide remained in the supernatant for longer (Fig. 3B, iii). Due to limited culture growth at RT, peptide expression was greatly reduced in both the flask and the prototype (Fig. 3B, iv).

To interpret the impacts of the prototype on both the culture growth and peptide expression, we also considered productivity, as a ratio of measured HiBiT luminescence to the culture density. The results showed similar productivity between the flask and the prototype cultures across all conditions (Fig. 3C), with the highest overall productivity after 3h at 37°C (Fig. 3C, i). We hypothesized that reduced culture growth in the prototype was due to reduced oxygenation, as the prototype had limited headspace, compared to the traditional flask (Fig. 1). Indeed, when only 10 mL of culture was cultivated in the 50 mL prototype to increase the headspace and promote oxygenation, the resulting culture growth was similar to that in the flask (Fig. 4A). Another parameter that contributes to oxygenation is agitation. Culture growth was reduced when 10 mL were cultivated in the 50 mL prototype without agitation (except manual agitation at the times of sampling) (Fig. 4B).

To maximize volume utilization while improving oxygenation, we also compared culture growth and expression in gas-permeable FEP culture bags. When the culture bag was filled to the maximum with 100 mL of culture and cultured agitated, the growth was reduced compared to 70 mL of culture in 125 mL flask (Fig. 4C), but performed better than 50 mL culture in the 50 mL 3D-printed prototype. The culture growth was greatly reduced without agitation (Fig. 4C). Peptide expression measured based on HiBiT luminescence was similar between the flask control and the agitated bag, with the maximum HiBiT reached between 2 to 3 hours after inoculation (Fig. 4D). Despite greatly reduced culture growth, the non-agitated bag reached similar HiBiT levels by 4 hours of culture (Fig. 4D), indicating higher productivity, similar to the results observed in the 50 mL culture in the 3D-printed prototype. To check whether the observed culture growth results were unique to *B. subtilis*, we also compared growth of *Escherichia coli* culture in the flask or an agitated culture bag. Similarly to the results observed in *B. subtilis*, the *E. coli* culture density was ~1.5-fold lower in the bag compared to the control flask.

Overall, when selecting preferred culture and expression conditions, it is important to consider the trade-offs between culture growth, productivity, peptide yields and the required input power, culture duration, and equipment. This highlights the need to define the FOM for these different systems and conditions.

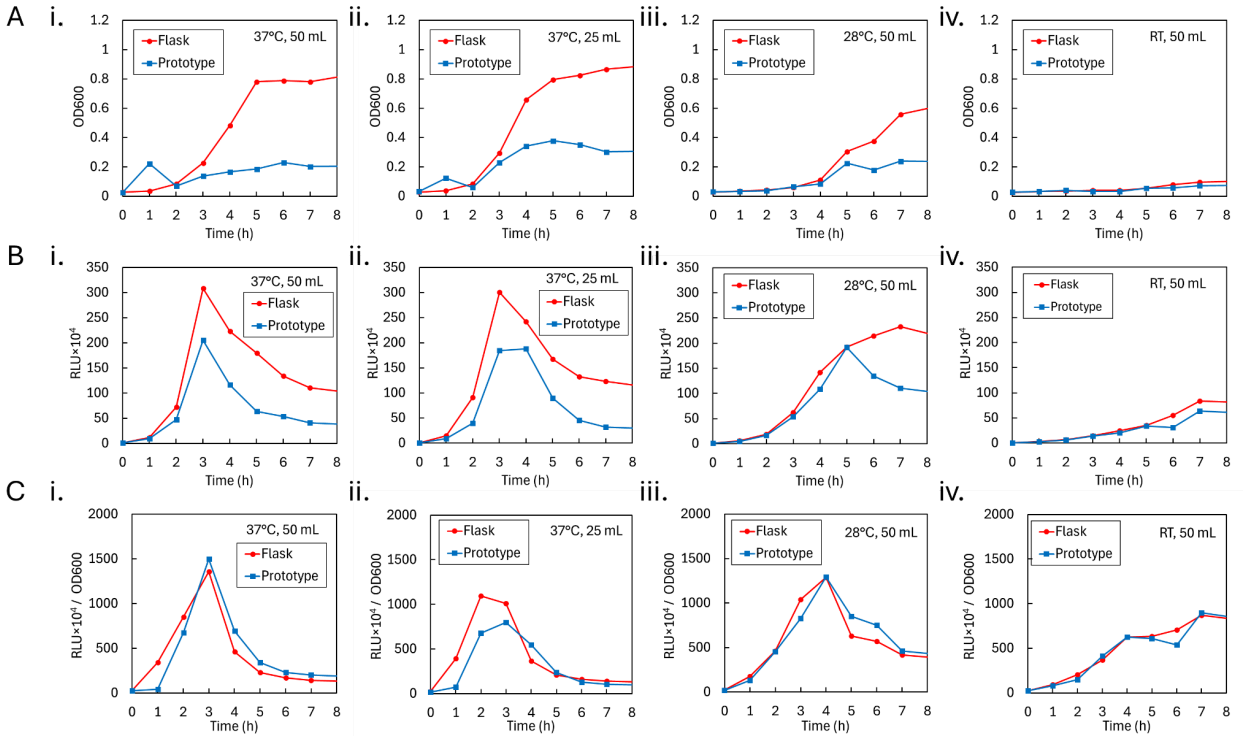

Fig. 3. Microbial culture growth and target peptide expression comparison between traditional laboratory flasks and 3D-printed growth/expression chamber prototypes. A) Culture growth (OD600) at i) 37°C, 50 mL cultures, ii) 37°C, 25 mL cultures, iii) 28°C, 50 mL cultures, and iv) room temperature, 50 mL cultures. B) Target peptide expression (HiBiT luminescence) at i) 37°C, 50 mL cultures, ii) 37°C, 25 mL cultures, iii) 28°C, 50 mL cultures, and iv) RT, 50 mL cultures. C) Productivity expressed as a ratio of HiBiT luminescence to OD600 for i) 37°C, 50 mL cultures, ii) 37°C, 25 mL cultures, iii) 28°C, 50 mL cultures, and iv) RT, 50 mL cultures.

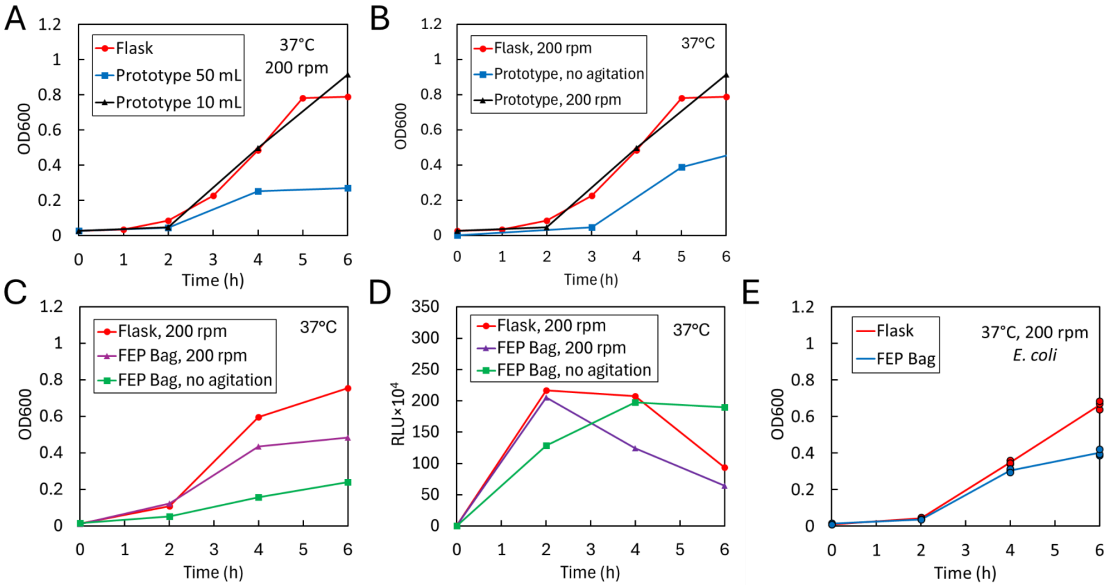

Fig. 4. Further investigations of microbial culture growth and target protein expression. A) Culture growth with or without extra headspace (10 mL or 50 mL culture, respectively) in the 50 mL prototype. B) Growth of 10 mL culture in 50 mL prototype with and without agitation. C & D) Culture growth and peptide expression, respectively, in FEP bags, and E) *E. coli* culture growth in the FEP bag. All compared to a traditional laboratory flask control (50 mL culture in 250 mL flask).

3.1.2 Lysis testing with OmniLyse device

An eforRED-expressing culture of *B. subtilis* was used to qualitatively compare lysis yields using the standard chemical lysis protocol (with lysozyme) and the OmniLyse device. As cells are lysed, eforRED protein is released into the soluble lysate fraction. When more cells are lysed, the red color of the soluble (liquid) lysate fraction will become more intense, while the color of the insoluble (solid pellet) lysate fraction will contain less red color.

The traditional chemical lysis protocol resulted in a bright red soluble lysate fraction (Fig. 5A), with little to no red color in the pellet (not shown). As a reference for comparison, we used this as a control with a lysis score of 10 (i.e., most cells lysed). When the cell suspension was passed through the OmniLyse device once, some lysis was observed, but to a much lower extent, as seen from the fainter red color (Fig. 5B). This was assigned a lysis score of 2. The lysis yields were greatly improved when the cell suspension was passed back and forth through the OmniLyse device 10 times, with the assigned lysis score of 7 (Fig. 5C).

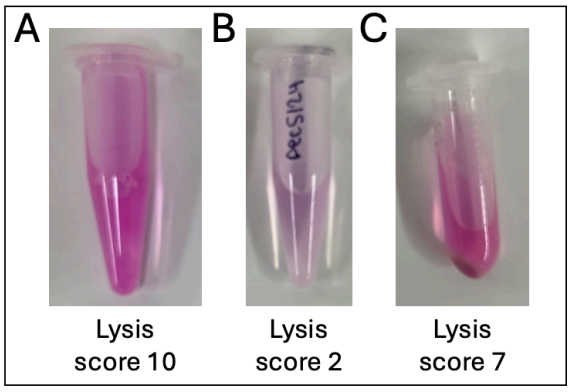

Fig. 5. Comparison of cell lysis methods. A) Chemical lysis using lysozyme. B) A single pass of the cell suspension through the OmniLyse device. C) 10 passes of the cell suspension through the OmniLyse device.

Although the OmniLyse device achieved lower lysis yields, it was a shorter procedure that took ~0.5 h compared to 1.5 h for traditional chemical lysis and did not introduce a contaminant (lysozyme) that can complicate downstream purification. While the OmniLyse protocol will need to be optimized to achieve similar lysis yields, these results demonstrate that the use of this device is a feasible option for the spaceflight hardware. While visual detection of eforRED is useful for rapid troubleshooting and prototyping, protein concentration in the soluble lysate fraction can be quantified using Bicinchoninic Acid (BCA) assay in the future.

3.1.3 Purification prototype testing

As a proof of concept, we tested purification of eforRED from lysate using the set-up described in sections 2.5 and 2.6.2. The spin column that contains a filter to retain the purification resin was manually loaded with Strep-Tactin®XT 4Flow® resin (Fig. 6A). The lysate was then added to the column (Fig. 6B) and allowed to incubate with the resin for 10 min. Once the lysate fraction was allowed to flow through the spin column, we observed successful binding of the eforRED protein to the purification resin (Fig. 6C). During washes, only a small amount of eforRED came out from the column, while the majority remained bound and retained inside the spin column (not shown). After 2 elution fractions were collected, a visibly lower amount of eforRED was still bound to the column (Fig. 6D). Following resin regeneration, only a small amount of eforRED remained on the resin (Fig. 6E). Additional elutions and regeneration optimization may be needed to fully remove bound proteins to minimize contamination when resin is reused.

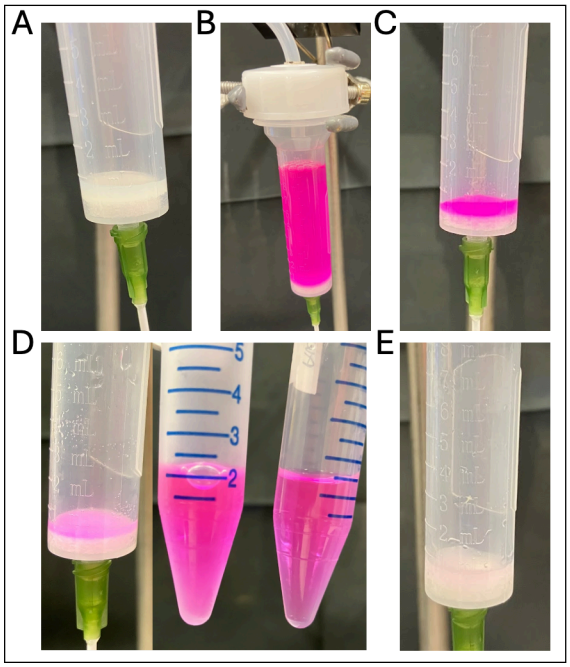

Fig. 6. Testing of purification system prototype with eforRED protein. A) Spin column loaded with purification resin. B) Lysate containing eforRED protein loaded onto the spin column. C) eforRED protein bound to the purification resin. D) Less eforRED remains bound to the resin (left) after two elution fractions (right) were collected. E) After elutions and regeneration, most eforRED was removed from the resin.

These results demonstrate the feasibility of the proposed approach for purification of target peptides during deep space missions. Further development of the purification system will focus on increasing automation to minimize required crew time, optimizing purification parameters to maximize yields and purity, and ensuring flexibility of operation at varied gravity environments.

3.2 FOM comparison between traditional laboratory methods and spaceflight hardware prototypes

The FOM for producing a peptide from 50 mL of culture using traditional laboratory methods, as described in section 2.6.1, are reported in Table 1. The mass of required materials and equipment was found to be ~122 kg, occupying a volume of ~440 L and consuming ~1 kWh. The biggest contribution to the mass and volume is the incubator for culture growth, which accounts for 80% of the mass and 88% of the volume. The incubator size is much larger than needed for this culture to accommodate other cultures in the lab and standard laboratory equipment is, of course, not optimized for spaceflight.

The start-to-finish process is expected to take ~8.5 hours, including 47 min of crew time. It should be noted, however, that the purification procedure includes 14 two-minute incubations (28 min total). The short duration of these incubations would make it challenging to utilize this time on other tasks. Thus, the crew time for purification may be underestimated. The calculated time also does not currently include the time needed for CQA assays and administration of the pharmaceutical.

Table 1. FOM for traditional laboratory methods. Normalized to 50 mL of culture.

Process Steps	Materials & Equipment	QTY	Mass (kg)	Volume (L)	Power (kWh)	Crew time (h:mm)	Total time (h:mm)
Growth and Expression	Microbes	500 µL	5×10^{-4}	5×10^{-4}	-	0:08	5:08
	Media	50 mL	0.05	-	-		
	Falcon tube, 50 mL	1	0.01	0.12	-		
	Flask, 250 mL	1	0.13	0.3	-		
	Incubator	1	98	387	0.63		
	Pipette tips, 1000 µL	1	6×10^{-4}	1.5×10^{-3}	-		
	Pipette-Aid, manual	1	0.13	0.71	-		
	Serological pipette	1	0.5	4.8	-		
Lysis	Tubes (1.5 mL)	1	9×10^{-4}	0.01	-	0:17	1:30
	Tubes (2 mL)	27	0.03	0.29	-		
	Lysis buffer	2 mL	0.01	-	-		
	Protease inhibitor	100 µL	5×10^{-4}	-	-		
	Pipette tips, 1000 µL	5	3×10^{-3}	0.01	-		
	Rotator mixer	1	1.2	12	0.02		
Purification	Falcon tube, 15 mL	1	0.01	0.05	-	0:22	1:55
	Magnetic rack	1	0.28	0.03	-		
	Tubes (1.5 mL)	5	4.5×10^{-3}	0.06	-		
	Beads	200 µL	2×10^{-4}	-	-		
	Wash buffer	9 mL	0.01	-	-		
	Elution buffer	200 µL	2×10^{-4}	-	-		
	Regeneration buffer	1 mL	1×10^{-3}	-	-		
	Pipette tips, 1000 µL	28	0.02	0.04	-		
	Buffer exchange / protein concentration	1	3.3×10^{-3}	0.01	-		
Adminis-tration	Assays for CQA	TBD	TBD	TBD	TBD	TBD	TBD
	Alcohol wipe	1	1.5×10^{-3}	4×10^{-3}	-		
	Syringe w/ needle	1	4×10^{-3}	0.02	-		
Shared across steps	P1000 Pipettor	1	0.09	0.36	-	NA	NA
	Refrigerated centrifuge	1	21.8	36	0.36		
		TOTAL:	**122.3**	**441.8**	**1.01**	**0:47**	**8:33**

CQA = critical quality attributes, TBD = to be determined, NA = not applicable

The FOM for producing a peptide from 50 mL of culture using spaceflight hardware prototypes, as described in section 2.6.2, are reported in Table 2. The mass of required materials and equipment was found to be ~23 kg, occupying a volume of ~44 L and consuming ~0.4 kWh. Most of the weight (94%) and volume (83%) are attributed to the refrigerated centrifuge. The significant reduction in mass and volume compared to the traditional laboratory methods was largely attributed to the removal of the incubator, which is not suitable for spaceflight. If the incubator mass and volume are excluded from the traditional laboratory method, the spaceflight hardware prototype is ~1 kg lower in mass and occupies ~11 L less volume. This is likely attributed to the use of bioprocess bags with reduced storage volumes instead of rigid containers (e.g., flasks, tubes, bottles) and a small peristaltic pump with tubing instead of pipetting. The start-to-finish process is expected to take 7 h hours, including 20 min of crew time. Compared to traditional laboratory methods, the overall time is reduced due to shower lysis and purification procedures.

There remains a need to develop a small scale heating system, as an alternative to the laboratory incubator. For example, the culture bag can be enclosed between two heating pads controlled by the Arduino microcontroller. While this is not currently accounted for in the FOM, such a system is expected to be <0.5 kg and <0.5 L. A way to provide continuous automated agitation could also improve microbial growth. Additional experiments can help determine the lowest needed temperature and agitation that would achieve sufficient culture growth and product yields.

A potential limitation of the proposed spaceflight process is that the lysate volume is equal to the culture volume. Compared to the traditional laboratory method, this results in lower concentration of the target peptide, which can impact purification efficiency and require more buffers and protease inhibitors.

Table 2. FOM for spaceflight hardware prototypes. Normalized to 50 mL of culture.

Process Steps	Materials & Equipment	QTY	Mass (kg)	Volume (L)	Power (kWh)	Crew time (h:mm)	Total time (h:mm)
Growth and Expression	Dried microbe spores	1 sheet	1×10^{-4}	1×10^{-4}	-	0:09	5:09
	Media	50 mL	0.05	0.05	-		
	Bioprocess bag	1	0.09	0.65	-		
	FEP culture bag	1	0.02	0.36	-		
	Heating pad	2	TBD	TBD	TBD		
Lysis	OmniLyse	5	0.02	0.07	-	0:05	0:45
	Battery pack	5	0.35	0.29	-		
	Tubes (2 mL)	25	0.03	0.29	-		
	Bioprocess bag	2	0.18	1.3	-		
	Protease inhibitor	5 mL	5×10^{-3}	5×10^{-3}	-		
Purification	Spin column	1	0.01	0.54	-	0:06	1:06
	Bioprocess bag	5	0.43	3.25	-		
	Tubes (2 mL)	2	2.4×10^{-3}	0.02	-		
	Resin	500 µL	5×10^{-4}	5×10^{-4}	-		
	Wash buffer	30 mL	0.03	0.03	-		
	Elution buffer	6 mL	6×10^{-3}	6×10^{-3}	-		
	Regeneration buffer	5 mL	5×10^{-3}	5×10^{-3}	-		
	Buffer exchange / protein concentration	1	3.3×10^{-3}	0.01	-		
Adminis-tration	Assays for CQA	TBD	TBD	TBD	TBD	TBD	TBD
	Alcohol wipe	1	1.5×10^{-3}	4×10^{-3}	-		
	Syringe w/ needle	1	4×10^{-3}	0.02	-		
Shared across steps	Tubing	1 pkg	0.09	0.36	-	NA	NA
	Peristaltic pump	1	0.11	0.12	TBD		
	Arduino MEGA	1	0.05	0.02	TBD		
	Circuit board/wiring	1	0.04	0.1	-		
	Refrigerated centrifuge	1	21.8	36	0.36		
	TOTAL:		**23.3**	**43.5**	**~0.4**	**0:20**	**7:00**

CQA = critical quality attributes, TBD = to be determined, NA = not applicable

3.5 Limitations and next steps

The impacts of agitation in the spaceflight hardware prototypes have only been investigated at 200 rpm or no agitation at all, while culture growth in the FEP bags has not yet been investigated at temperatures other than 37°C. Future experiments utilizing Design of Experiments methodologies can investigate a wider range of conditions to determine optimal temperature and agitation to maximize yields and productivity, while minimizing input power and process duration. The FOM can be derived for the different agitation and temperature conditions to evaluate the trade-offs between process duration, required input power, and product yields.

The results of cell lysis and purification tests of the spaceflight hardware prototypes remain to be confirmed quantitatively and with *B. subtilis* strains expressing pharmaceutical peptides. In particular, quantifying the resulting purity will help determine whether additional purification steps may be needed. Additionally, future experiments will evaluate regeneration of purification resin and determine how many times the resin can be reused. Lastly, some purification components (e.g., resin) is currently stored at 4°C and its stability at RT remains to be determined.

The FOM analysis was conducted based on the current state of the spaceflight hardware prototypes and is expected to be updated as new iterations of the prototypes are developed with the goal of minimizing the FOM, while producing desired amounts of pharmaceutical peptides. For both production methods, the volumes were overestimated based on simplified rectangular prism shape. Instead, all required materials can be packaged into a box for a better estimate on the total occupied volume. The required assays for CQA and syb-systems such as heating of the FEP bag remain to be determined and to be included in the FOM.

Most importantly, the FOM presented here were normalized to peptide production from 50 mL of culture for comparison. However, there remains a need to quantify production of pharmaceutical peptides using the two methods and to normalize the FOM accordingly. Nevertheless, the baseline FOM determined here should be easily adjustable as we confirm required culture volumes to produce a therapeutic dose of the target peptide.

4. Conclusions

This study discussed the current developments and testing of spaceflight hardware prototypes to produce pharmaceutical peptides from engineered *B. subtilis*. In particular, FEP culture bags were selected as a low mass/volume alternative to traditional laboratory flasks, cell lysis was demonstrated with the OmniLyse devices previously flown on the WetLab-2 mission, and purification of eforRED was successfully demonstrated with Strep-Tactin®XT 4Flow® resin, as required buffers were circulated through a spin column using an Arduino-controlled peristaltic pump. The FOM for traditional laboratory methods were 122 kg, 442 L, 1 kWh, and 8.5 h total process duration, including 47 min of crew time. For spaceflight hardware prototypes, the FOM were reduced to 23 kg, 44 L, 0.4 kWh, and 7 h total process duration, including 20 min of crew time.

The FOM analysis provides a useful metric to compare different approaches and technologies, as we continue to develop the Astropharmacy hardware with the goal of minimizing the FOM. Overall, this is a stepping stone to establishing capabilities for on site, on demand pharmaceutical production to enable human exploration of deep space.

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