MBP-SATURDAY-658

June 17, 2023

The Importance of Standardizing Lyophilization Techniques in Microbial Preservation

Jyoti K. Jha, PhD;* Rahul Tevatia, PhD; Lukas Underwood, PhD; Quinn Osgood, BSE; Emmanuel Boafo-Asare, MS; Nilay Chakraborty, PhD

ATCC, Manassas, VA 20110

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Abstract

Storage of microorganisms in a quantitative format is essential for modern industrial quality control and other related applications. Lyophilization is one of the most widely used techniques for preserving microorganisms in a desiccated format as it allows lab workers to avoid expensive ultra-cold chain storage and related distribution challenges. However, lyophilization is a complex process that involves phase changes of the extracellular environment, and the outcome is highly influenced by the compositional, thermal, and pressure changes of the materials. In this study, we evaluate how changes in lyophilization formulation affect microbial viability and protein expression characteristics using *Escherichia coli* (ATCC® 8739™) as a model. Our results highlight the need for optimization of lyophilization formulation for desiccated storage of microorganisms. Further studies are required to identify the standardized lyophilization processes.

Introduction

Lyophilization is a well-established method for supporting the long-term storage of bacterial strains and it is by far the most frequently used method for achieving long-term shelf life.[1,2] In this process, the addition of a selected buffer formulation to the biomass and subsequent lyophilization are vital steps for maximizing the viability of a microorganism.[3]

In this study, we used *E. coli* (ATCC 8739) as model organism for evaluating viability optimization and long-term storage in a novel buffer formulation. This bacterial strain is a WT version of *E. coli,* and it has wide applications as a quality control strain in testing antimicrobial handwashing formulations, media, efficacy, and bio-resistance. Further, it is cited as a reference material in multiple United States Pharmacopeia (USP) methods, namely USP <51> and <62>.[4,5] Here, we used global proteomic analysis to provide evidence that may co-relate for long-term viability of *E. coli* in our novel lyophilization formulation.

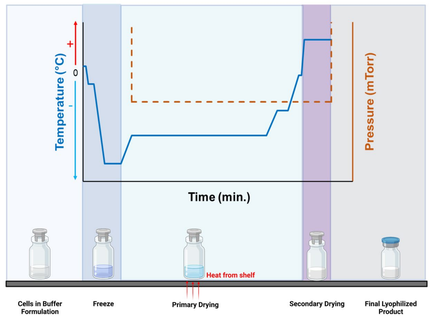

Figure 1: Overview of microbial lyophilization. Lyophilization, or freeze drying, is the process of removing water from a product in its frozen state under a vacuum. This process allows ice to change from a solid to a vapor without going through a liquid phase. This process comprises three major steps: freezing the sample, primary drying (ice sublimation), and secondary drying (unfrozen water desorption).

References

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Materials and Methods

Lyophilization Formulation for *E. coli* Preservation: Initial viability screening was performed using a combination of multiple sugars, polyols, proteins. The viability of *E.coli* preserved in buffer #1 (B#1; our optimized mixture of carbohydrates, polyols, and proteins) was compared to a commonly used lyophilization buffer, buffer #2 (B#2; a mixture of carbohydrate and proteins).
Bacterial Viability Assay: Bacterial viability was determined using a plate-based dilution method. Viable bacteria were quantitated via colony formation units (CFU).
Quantitative Proteomics Analysis (TMT-Multiplex Labeling and LC-MS/MS): To identify potential protein profile change related to different buffer and storage conditions, a comparative proteomic analysis was performed. A total of 12 samples from four conditions were analyzed in triplicate using the TMT-13plex labeling-based quantitative proteomics method and liquid chromatography-tandem mass spectrometry (LC-MS/MS). In this study, 2658 proteins were quantitatively identified. About 22 of the 2658 proteins significantly changed in abundance (expression) (≥2 fold; p<0.05; n=3) in at least one condition compared to another.

Results

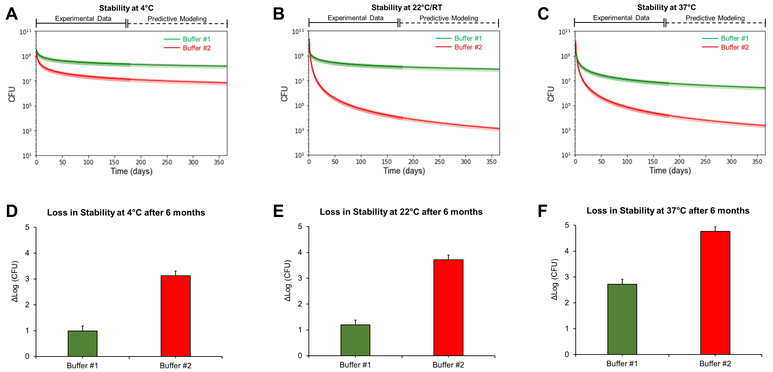


Figure 2: Stability of *E. coli* in two different formulations. (A-C) Stability of *E.coli* in B#1 and B#2 at 4°C, 22°C, and 37°C, respectively, for one year. Experimental data was collected for 6 months, and predictive modeling was performed to extrapolate stability up to a year. (D-F) Viability loss (ΔLog CFU) for *E. coli* in B#1 and B#2 at 4°C, 22°C, and 37°C, respectively, after 6 months of storage.

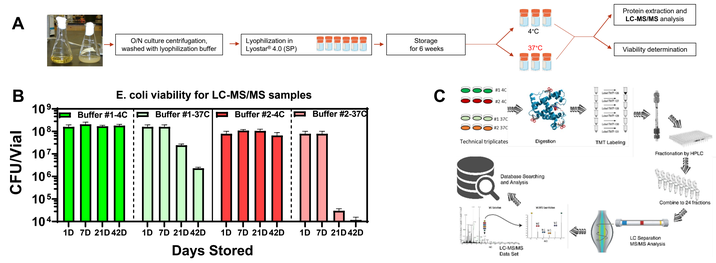
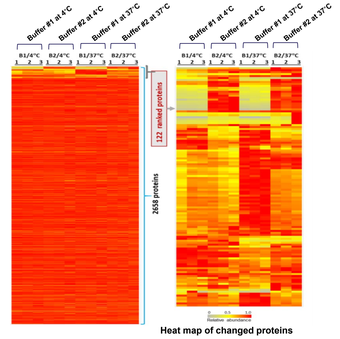
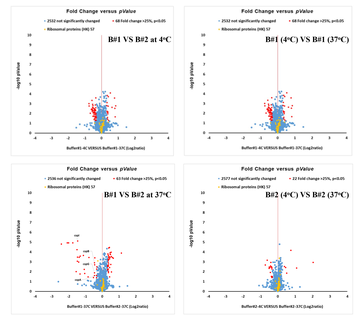


Figure 3: Sample preparation for lyophilization, viability determination, and LC-MS/MS. (A) Flow cart of sample processing for LC-MS/MS and viability of *E coli*. (B) Viability determination for lyophilized samples of *E. coli* in B#1 and B#2 and incubation at respective temperatures (4°C and 37°C) for 6 weeks. (C) Mass spectrometry-based quantitative proteomic profiling workflow (lysate preparation, trypsin digestion, TMT-13plex labeling, purification, and analysis).

A Proteomic characterization of *E. coli* lyophilized and stored in different buffers



B Volcano plot demonstrating the fold change of protein abundance



C Relative protein abundance of *E. coli* stored in B#2 and B#1 at 4°C

Accession	Gene	Protein name	Fold Change B#2/B#1 at 4C Mean	SD	Cellular function
P12364	mod	Type III restriction-modification system EcoP15I enzyme mod	2.61	0.27	DNA cleavage
Q59900	pet	Serine protease pet autotransporter	2.49	0.63	Enterotoxic effect
P0A910	cspA	Cold shock protein CspA	4.45	0.36	Cold Shock Proteins induction dramatically reduce global protein synthesis
P36995	cspB	Cold shock-like protein CspB	3.88	0.13	
P0A978	cspG	Cold shock-like protein CspG	4.50	0.29	
P0A987	cspI	Cold shock-like protein CspI	5.85	0.61	
P0A987	cspI	Cold shock-like protein CspI	5.85	0.61	
P64610	yhbL	Uncharacterized protein YhbL	2.61	0.38	
P64476	ydiH	Uncharacterized protein YdiH	1.85	0.16	
P14175	proW	Glycine betaine/proline betaine transport system permease	1.73	0.32	Modulation of transport
P64476	ydiH	Uncharacterized protein YdiH	2.61	0.38	
P0AFM2	proX	Glycine betaine/proline betaine-binding periplasmic protein	2.91	0.45	
P0AB40	bhsA	Multiple stress resistance protein BhsA	3.07	0.72	Stressed Condition
P76073	ynaE	Uncharacterized protein YnaE	4.52	1.77	
P14175	proV	Glycine betaine/proline betaine transport system ATP-bindir	3.33	0.20	
Q47129	feaR	Transcriptional activator FeaR	0.55	0.09	Abnormal cell production
P0A9P7	deaD	ATP-dependent RNA helicase DeaD	2.98	0.24	
Q8X9W7	lamB	Maltoporin	1.71	0.34	Membrane Integrity loss
P68653	nohA	P21 prophage-derived terminase small subunit	3.92	0.42	
P06134	ada	Bifunctional transcriptional activator/DNA repair enzyme Ad	0.55	0.13	Methylated DNA repair
P0ABS1	yciH	Uncharacterized protein YciH	0.63	0.20	

D Relative protein abundance of *E. coli* stored in B#2 and B#1 at 37°C

Accession	Gene	Protein name	Fold Change B#2/B#1 at 37C Mean	SD
P12364	mod	Type III restriction-modification system EcoP15I enzyme mod	2.78	0.35
Q59900	pet	Serine protease pet autotransporter	2.65	0.47
P0A910	cspA	Cold shock protein CspA	4.26	0.36
P36995	cspB	Cold shock-like protein CspB	3.38	0.25
P0A978	cspG	Cold shock-like protein CspG	4.00	0.40
P0A987	cspI	Cold shock-like protein CspI	5.41	1.18
P0A987	cspI	Cold shock-like protein CspI	5.85	0.61
P64610	yhbL	Uncharacterized protein YhbL	1.74	0.17
P14176	proW	Glycine betaine/proline betaine transport system permease	2.10	0.47
P64476	ydiH	Uncharacterized protein YdiH	2.18	0.37
P0AFM2	proX	Glycine betaine/proline betaine-binding periplasmic protein	2.49	0.24
P0AB40	bhsA	Multiple stress resistance protein BhsA	2.65	0.57
P76073	ynaE	Uncharacterized protein YnaE	2.80	1.03
P14175	proV	Glycine betaine/proline betaine transport system ATP-binding	2.83	0.26
Q47129	feaR	Transcriptional activator FeaR	0.45	0.07
P0A9P7	deaD	ATP-dependent RNA helicase DeaD	2.76	0.22
P22426	cbi	Colicin-B immunity protein	1.79	0.80
P0AEL5	feoA	Fe(2+) transport protein A	2.05	0.63
B1LDT4	kptA	Probable RNA 2'-phosphotransferase	2.51	1.51
P56614	yhcN	Uncharacterized protein YhcN	2.94	1.45
P77766	ycfV	5'-3' exoribonuclease	0.69	0.12

Figure 4: Proteomic characterization of *E. coli* in different storage condition and formulation. (A) A heat map showing the relative abundance of 2658 proteins identified across 12 samples from 4 group conditions depicting a total of 122 changed proteins (fold change >=25% (p<0.05, n=3). (B) Volcano plot demonstrating the fold change of protein abundance. [Note that the sample comparison in (A) and (B) is among B#1@4°C and B#2@4°C, B#1@4°C and B#1@37°C, #B1@37°C and #B2@37°C, or B#2@4°C and B#2@37°C.] (C) Select changed proteins between B#1@4°C and B#2@4°C (>2 fold). (D) Select changed proteins between B#1@37°C and B#2@37°C (>2 fold). Orange highlighted proteins are common changes in B#2 vs B#1 at both 4°C and 37°C. Change of other proteins are related to their conditions. No protein was found changed (>2 fold) within the same formulation (B#1@4°C vs B#1@37°C and B#2@4°C vs B#2@37°C).

Conclusions

Here, we have identified a formulation for stabilizing the lyophilized product of *E. coli* at 4°C, 22°C, (room temperature), and 37°C storage. The viability of the lyophilized *E. coli* was found to be 2 to 3 log better for up to 6 months in our optimized formulation (B#1) as compared to a conventional formulation (B#2). We exploited LC-MS/MS to understand the proteomic variances that might have caused reduced viability of lyophilized *E. coli* in B#2. Interestingly, we found that the cold shock proteins were induced three times more in formulation B#2 as compared to B#1, which indicates a reduction in global protein synthesis[6] and may point toward lower viability after lyophilization. Also, downregulation of the 'Ada' protein[7] and transcriptional activator 'FaeR' in B#2 will lower the capability of DNA methylation repair and increase the chance of abnormal cell production. Further, multiple stress response proteins (BhsA) were overexpressed, which indicated that the *E. coli* cells in formulation B#2 are unstable. Elevated expression of the type III restriction modification enzyme (mod) in formulation B#2 indicated DNA cleavage and hence may be another factor that contributed to lower viability.[8] Global mass spectrometry analysis indicates several biomolecular mechanisms contributing to variations in the viability of lyophilized *E. coli* in formulations B#1 and B#2. Further investigation is required to determine the direct impact of these proteomic differences on lyophilization stability.

ATCC 10801 University Boulevard, Manassas, Virginia 20110-2209 **Phone:** 800.638.6597 **Email:** sales@atcc.org **Web:** www.atcc.org