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2 Biotechnology Progress – Note:
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4 # Creating a Completely "Cell-free" System for Protein Synthesis
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Abstract

Cell-free protein synthesis is a promising tool to take biotechnology outside of the cell. A cell-free approach provides distinct advantages over *in vivo* systems including open access to the reaction environment and direct control over all chemical components for facile optimization and synthetic biology integration. Promising applications of cell-free systems include portable diagnostics, biotherapeutics expression, rational protein engineering, and biocatalyst production. The highest yielding and most economical cell-free systems use an extract composed of the soluble component of lysed *Escherichia coli*. Although *E. coli* lysis can be highly efficient (>99.999%), one persistent challenge is that the extract remains contaminated with up to millions of cells per mL. In this work, we examine the potential of multiple decontamination strategies to further reduce or elimiate bacteria in cell-free systems. Two strategies, sterile filtration and lyophilization, effectively eliminate contaminating cells while maintaining the systems' protein synthesis capabilities. Lyophilization provides the additional benefit of long-term stability at storage above freezing. Technologies for personalized, portable medicine and diagnostics can be expanded based on these foundational sterilized and completely "cell-free" systems.

Keywords

- cell-free
- protein synthesis
- sterilization
- lyophilization
- *in vitro* protein synthesis

1 # Introduction

2 Cell-free protein synthesis (CFPS) is a robust *in vitro* transcription/translation platform that has
3 become increasingly useful in the bioengineers' toolkit.[1] The open and accessible nature of the
4 cell-free environment allows for direct manipulation, monitoring, and optimization. The features
5 of CFPS make it a compelling platform for diverse biotechnology applications, such as protein
6 engineering, biotherapeutics development, and synthetic biology.[2-6]
7 The most robust and highest yielding CFPS systems are based on the soluble portion of cell
8 lysates from *Escherichia coli,* outperforming the expensive systems with individually purified
9 components by >50% in protein production.[7-9] To produce these systems, *E. coli* is grown,
10 harvested by centrifugation, lysed, and finally centrifuged to remove superfluous cellular debris.
11 The resulting supernatant is collected as the final cell-extract. To create high-yielding extracts,
12 lysis is best accomplished by physical methods, predominantly using high-pressure
13 homogenization or sonication.[10, 11] These methods can be extremely efficient, exceeding
14 99.999% lysis of cells.[10, 12] Repeated lysis treatments increases lysis efficiency, however,
15 increased treatment can damage the activity of the extract .[10, 11]
16 In this sense, the most robust cell-free systems are not completely free of cells and can be
17 contaminated with millions of residual cells per mL of cell-extract. The inability to achieve
18 100% lysis poses a complicating obstacle for some CFPS technologies, inclduing applications in
19 industrial biomolecule production, commercial biodiagnostics, and portable CFPS systems. For
20 industrial applications, residual bacterial contamination can exponentially bloom and be
21 deleterious in any scale-up or bioreactor conditions.[13] For commercial biodiagnostics,
22 contamination can impact consistency and shelf-life of the system.[12] Furthermore, some
23 promising applications of CFPS include portable and personalized technologies, such as

pharmacy- and lab-on-a-chip.[14] Bacterial contamination of such devices raises potential ethical and regulatory issues regarding the possible discharge of recombinant microorganisms into the environment.[15, 16]

Thus, future CFPS technologies would benefit if the high-yielding nature of cell-free systems could be maintained while eliminating residual bacterial contamination. In this study, we examine the feasibility of traditional and non-traditional sterilization techniques towards robust and decontaminated cell-free systems. We highlight two successful techniques for removing residual bacterial contamination – sterile filtration and lyophilization – to create completely cell-free systems from high performing extracts.

Materials and Methods

Cell-free Protein Synthesis

Cell extract was prepared from *Escherichia coli* strain BL21 Star™ (DE3) (Life Technologies, Carlsbad, CA) as previously described with the following specifications.[12] Cells were grown, harvested, and lysed using an Avestin EmulsiFlex B-15 Homogenizer with 3 passes at 21,000 psi. Lysate was centrifuged at 16,000 xg, 4 ^{o}C for 30 minutes and the supernatant was collected, aliquotted, flash frozen and stored at -80 ^{o}C until use. CFPS was performed using the PANOxSP system using the gene pY71-sfGFP (green fluorescent protein) as previously reported.[12]

Sterilization and Contamination Assay

Lyophilization was performed as previously reported.[12] For antibiotic treatment, extracts were incubated with freshly prepared ampicillin (0.1-0.8 mg per mL cell extract) for 30 minutes at 25 ^{o}C while rotating end-over-end. For lysozyme treatments, extracts were incubated with chicken egg white lysozyme (EC 3.2.1.17, Sigma Aldrich) (1-8 mg per mL cell extract) for 30 minutes at

room temperature while rotating end-over-end. For sterile filtration, extracts were sterile filtered by syringe or vacuum filtration through a Thermo Scientific Nalgene filter (syringe: 25 mm diameter, 0.2 micron; vacuum: 50 mm diameter, 0.2 micron; surfactant-free cellulose acetate low protein binding). For UV treatments, extracts were aliquotted into 96-well plates (60-240 µL) and irradiated for 20-40 minutes at room temperature using a Spectroline® Germicidal EF-140C placed directly atop the plate (254 nm, 4 watts). Dilutions to extracts caused by treatment effects were accounted for in the final CFPS reaction mixtures. Contamination levels were assayed as previously reported by plating cell extracts on LB agar Miller culture dishes and measured in colony forming units (CFU) per µL extract, as previously reported.[10, 12] Cost analysis is based on the best performing treatment in a given technique and prices from the Sigma Aldrich 2015 online catalogue.

Results and Discussion

Cell-extracts for CFPS were prepared by high-pressure homogenization of BL21 Star™ (DE3) *Escherichia coli* harvested during late log phase. For extracts prepared in this work, high-pressure homogenization had an efficiency consistently exceeding 99.999% lysis and results in highly active S16 extracts (GFP yields > 0.8 mg per mL).[10] Prior to lysis, the cell slurry contains approximately 600 billion cells per mL. Therefore, at 99.999% lysis, the concentration of residual contaminating cells after lysis and centrifugation can be as large as 6 million cells per mL, although contamination is typically lower and may be reduced by further processing. This contamination persists after freezing and during storage below freezing.[12]

The CFPS reaction environment is similar to cell fermentation condition, containing buffering salts, high-energy small molecules, and protein-rich lysates. Furthermore, CFPS reactions are typically performed at 30-37 ^{o}C with high levels of oxygenation. Thus, contaminating cells have

1 the potential to flourish and eventually dominate the reaction given sufficient time. Indeed, even
2 extracts stored at room temperature for less than 1 hour exhibited about 30% increase in cell
3 contamination based on increases in colony forming units (CFU) (Supporting Information Figure
4 S1). This problem would be exacerbated with semi-batch reactions, a popular method to increase
5 protein yields, as reaction time and nutrient availability are increased.

6 In efforts to effectively sterilize the cell extracts, we considered multiple methods of treatment:
7 1) lyophilization, 2) sterile filtration, 3) UV irradiation, 4) antibiotics, and 5) lysozyme (Table 1).
8 The techniques were selected based on their ubiquity to biological labs and previous uses as
9 cytotoxic or cytostatic techniques.

10 *1 Lyophilization*: Previously, we fortuitously discovered that extracts could become stable and
11 free of contamination after lyophilizaiton and incubation above freezing (4 ^{o}C or room
12 temperature).[12] A potential mechanism of cell destruction is the change in salinity of the solution
13 during lyophilization, which increases greater than 60 times (upwards of 10 M of salt ions–
14 Supporting Information Figure S2). Such high salinity levels can cause total die-off of *E. coli*
15 that are not held in stasis – i.e. not frozen.[17] Thus, lyophilization coupled with 1-14 days above
16 freezing is sufficient to destroy residual bacteria.[12]

17 Lyophilized extract retains greater than of 80% protein synthesis activity after lyophilization and
18 storage above freezing (Figure 1). This method requires some additional labor (~3 hours) and
19 access to standard relatively inexpensive shell freezer and lyophilizer equipment.[12] However,
20 overall this method is inexpensive, easily replicated, and does not require additional reagents.

21 *2 Sterile Filtration*: Sterile filtration is an ubiquitous method for sterilizing buffers and other
22 fluids by filtering the material through submicron membranes to remove micron-sized bacteria.
23 We hypothesized sterile filtration would provide a straightforward, facile alternative to extract

1 sterilization by allowing proteins, nucleic acids, and other biomolecules essential for CFPS to
2 pass through while excluding residual bacteria. Extract was filtered using a syringe filter or a
3 vacuum filtration device. Both methods achieved complete extract sterilization (Figure 1,
4 Supporting Information Figure S3). The resulting extracts retained up to 95% protein synthesis
5 activity of the untreated control extract. However, the filtered extracts were highly variable in
6 protein synthesis activity, ranging from 56-95% of the control's activity. The reduction in
7 synthesis activity may be due to the incidental removal of molecules important to the
8 performance of CFPS, such as inverted lipid vesicles that are elemental to oxidative
9 phosphorylation pathways.[18]

10 While sterile filtration effectively removed contaminating bacteria, filters rapidly clog and the
11 method would likely only be practical in small-scale formats or after development of an
12 optimized, multistep filtration process to mitigate blockages. For example, 25 mm syringe filters
13 clog after filtering less than 2 mL of extract. Our vacuum filtration setup clogs after only a few
14 drops passed through the filter. Without augmented filtration processes, rapid clogging could
15 restrict potential industrial and scalable applications. Furthermore, the rapid clogging makes for
16 relatively high treatment costs of up to 1000 USD per L extract treated. On the other hand, the
17 method is straightforward, inexpensive on the bench scale, and the tools for implementation are
18 ubiquitous in the biological laboratory.

19 *3 UV Irradiation*: UV sterilization is another standard in decontamination, particularly common
20 in biological research settings to prevent bacterial contamination during manipulation of
21 eukaryotic cells. Extract treated with UV-254 (~1100000 μW/cm^2) had reduced contamination
22 and maintained >60% protein synthesis activity (Figure 1, Supporting Information Figure S4).
23 However, significant levels of contamination remained (>30% of control), even in the best case

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tested with a treatment depth of <2 mm (60 µL in a 96-well plate). Although UV-254 can be potently cytotoxic, there are two probable reasons why it is not more successful in extract decontamination. First, UV254 damages nucleic acids, which can lead to cell death or repair through native pathways in the cell. The result is that some cells can remain viable even after high doses of UV irradiation.[19] Second, UV254 may not sufficiently penetrate the extract to cause cell death. Cell extract contains high concentrations of proteins (approximately 70 mg per mL), which can attenuate the intensity of the light by up to 90% in approximately 0.2 mm (Supporting Information Figure S5). The significant attenuation of light hampers the utility of the UV-treatment method. However, with more advanced equipment and higher power UV bulbs, UV treatment might provide a more effective treatment alternative for extract decontamination, similar to UV-pasteurization used in food technologies.[20] However, the rapid attenuation of UV-254 intensity due to protein density and the potential to overheat extracts with long-term exposure pose significant challenges.

4 Antibiotics: Antibiotics are frequently used to selectively pressure and screen bacterial cultures due to the cytotoxic and cytostatic properties. Thus, many antibiotics would be readily available and easily applied for extract treatments in a typical microbiology laboratory setting. To select an appropriate antibiotic, we eliminated the majority of the most effective lab-available antibiotics due to mechanisms that target essential components for transcription/translation (e.g. ribosomes). Ampicillin was chosen as the antibiotic, as it targets the cell-membrane production pathway.[21] The addition of freshly prepared ampicillin did not negatively impact CFPS levels (Figure 1, Supporting Information Figure S6). Also, ampicillin lowered contamination levels by up to 55% at a cost of <4 USD per L extract. However, ampicillin's indirect mechanism of killing by cell membrane depletion was found to be insufficient to achieve a completely cell-free environment

within the given treatment time of 30 minutes. Increased treatment times led to decreased protein synthesis yields. Other antibiotics may provide sufficient and more rapid killing to be effective for decontamination. However, selections must be made carefully to avoid deleterious effects to the transcription/translation machinery. In addition, the use of common relatively inexpensive laboratory antibiotics holds the risk of contamination from antibiotic resistant strains.

5 Lysozyme: Chicken egg white lysozyme directly attacks bacterial cell membranes by cleaving peptidoglycans, leading to cell lysis and death. This inexpensive technique (<20 USD per L cells) is frequently used in biological laboratories as an alternative to chemical and physical cell disruption. Furthermore, lysozyme's mechanism does not target transcription/translation machinery. Thus, we hypothesized treatment with lysozyme may reduce bacterial contamination without deleterious effects on CFPS activity.

In all treatment cases, lysozyme significantly lowered contamination with the best case (2 mg lysozyme per mL extract) reducing bacterial levels by greater than 70% (Supporting Information Figure S7). Increasing lysozyme content up to 8 mg per mL did not improve decontamination efforts. Optimal lysozyme treatment (2 mg per mL) did not significantly affect total CFPS yields (Figure 1). However, increasing lysozyme content caused visible precipitation and CFPS yields dropped by >98% (Supporting Information Figure S7).

Stable Storage of Sterile Extracts

Of the five methods tested here, only sterile filtration and lyophilization were effectively decontaminated of viable bacterial cells. Previously, we demonstrated that lyophilzation also increased shelf life of the extract up to 90 days at storage temperatures up to 25 ^{o}C.[12] Extract stability above freezing would be fundamental in creating robust portable cell-free systems. We were interested to see if the decontamination by sterile filtration provided similar benefits of

1 storage stability by reducing or removing the impacts of cell growth during storage. After
2 preparation, extracts were tested for CFPS activity, stored at room temperature for 14 days, and
3 tested again. Standard untreated control extract lost more than 85% its original activity (Figure
4 2). Surprising to us, sterile filtered extract lost effectively 100% of its CFPS activity, more than
5 standard aqueous extract stored at room temperature. The loss of activity despite sterile filtration
6 indicates contamination may only play a minor role in extract stability above freezing.
7 Considering the additional loss in activity suffered by sterile filtered extracts, it is possible that
8 sterile filtration removes biomolecules important to protein, tRNA, other biomolecule
9 stabilization above freezing, contributing further to the rapid decline of extract activity.

10 Conclusion

11 The potential to make cell-free systems sterile reduces or eliminates many potential ethical and
12 biosafety concerns while making promising steps towards cGMP technologies. The simplicity
13 and relatively ubiquitous nature of sterile filtration and lyophilization make their implementation
14 a straightforward process. These techniques provide a promising framework from which to
15 enhance current and build future cell-free biotechnologies.

16 Acknowledgements

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18 Award (#1254148) and a DARPA Young Faculty Award (#D13AP000037). Lyophilization data
19 reported in Figures 1 and 2 is reproduced with permission from Smith et al, Biotechniques, 2014
20 (Ref 12).

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1 # Figures and Legends

2 **Table 1 – Comparison of methods for cell-contamination reduction or elimination in cell-**
3 **free protein synthesis**

Technique	Advantages	Disadvantages
Lyophilization	1. Completely cell-free 2. CFPS Activity for up to 90 days[12] 3. No additional reagents required 4. Readily scalable	1. Equipment required 2. 3 h additional labor
Sterile Filtration	1. Completely Cell-free 2. Rapid extract sterilization 3. Ubiquitous equipment	1. Filters clog easily 2. Difficult scale-up 3. Unstable for long-term storage above 0 ^{o}C 4. Variable protein synthesis yields 5. Expensive due to rapidly clogged filters
Lysozyme	1. Straight-forward treatment 2. Low cost per volume treated	1. Residual cell contamination 2. Increased treatment reduces yields
UV-254	1. Ubiquitous biology lab equipment 2. No additional reagents required	1. Residual cell contamination 2. Protein density limits treatment volume 3. Specialized equipment may be necessary for more effective decontamination
Ampicillin/Antibiotics	1. Ubiquitous biology lab reagents 2. Low cost per volume treated	1. Residual cell contamination 2. Antibiotics often target CFPS machinery

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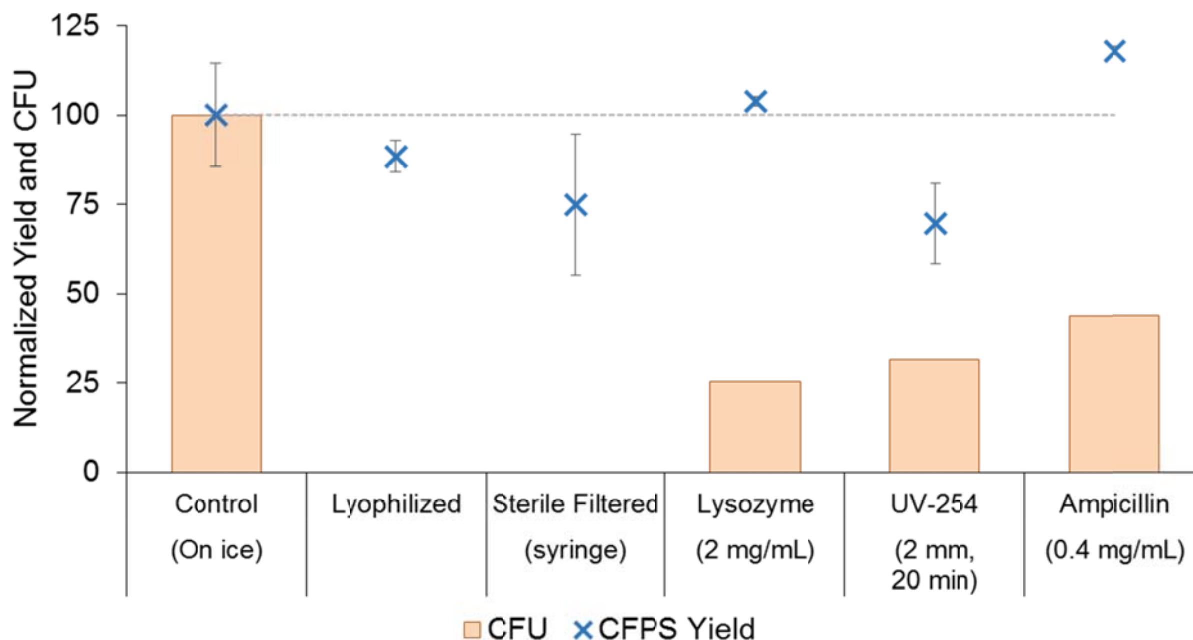

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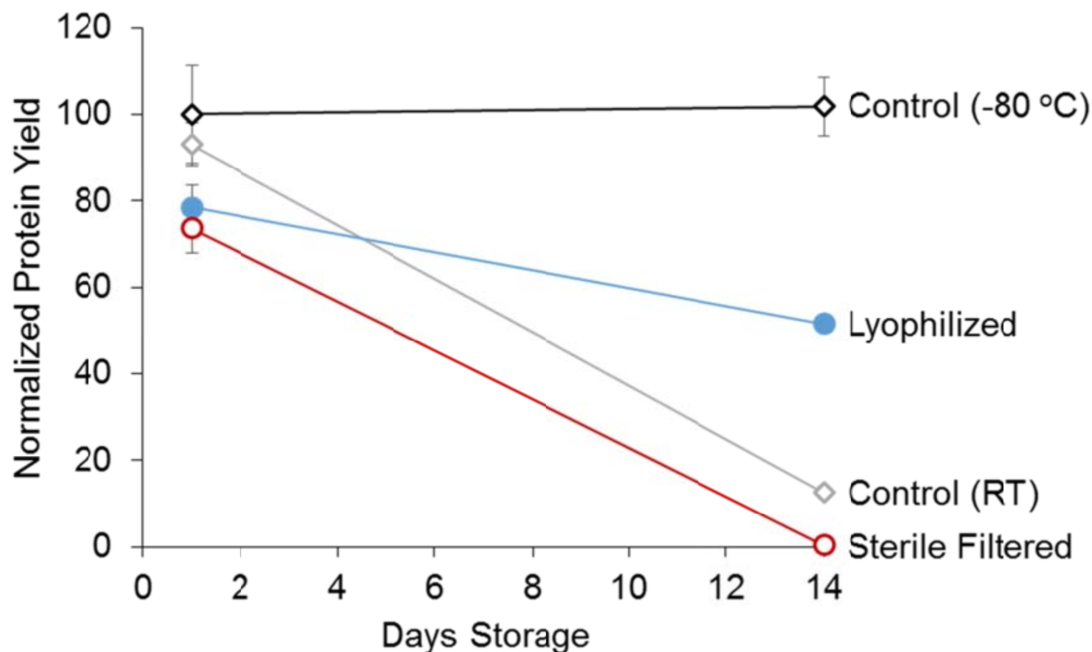
2 **Figure 1 – Normalized Decontamination Levels (bars) and CFPS Yields (x). Data**
3 **represents highest decontamination levels achieved for a given method. Contamination**
4 **levels are reported in normalized colony forming units (CFU) normalized to untreated**
5 **extract controls stored on ice. Average contamination levels in control extracts was 665000**
6 **CFU per mL extract. Protein synthesis yield is normalized to CFPS with untreated extract**
7 **stored on ice during treatment (Control). Average protein synthesis levels from control**
8 **extracts was 0.63 mg per mL CFPS. In order of decontamination efficiency, the conditions**
9 **were: lyophilized extract stored at 4 ^{o}C. sterile filtered extract filtered by syringe (0.2**
10 **micron), extract treated with 2 mg per mL lysozyme for 20 minutes, extract treated with**
11 **UV-254 at a depth of 2 mm for 20 minutes, and extract incubated with 0.4 mg per mL**
12 **ampicillin. Yield error bars = 1 standard deviation, n≥3.**
13



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2 **Figure 2 – Extract CFPS Activity after Treatment and Storage at Room Temperature.**
3 **Extracts were prepared, tested for protein synthesis, and stored at room temperature (RT,**
4 **24-27 ^{o}C), except for the standard control which was stored at -80°C. After 14 days of**
5 **storage, the extract was again tested for protein synthesis. n=3, error bars = 1 standard**
6 **deviation.**
7
8

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1 # Supporting Information:

2 ## Creating a Completely “Cell-free” System for Protein Synthesis

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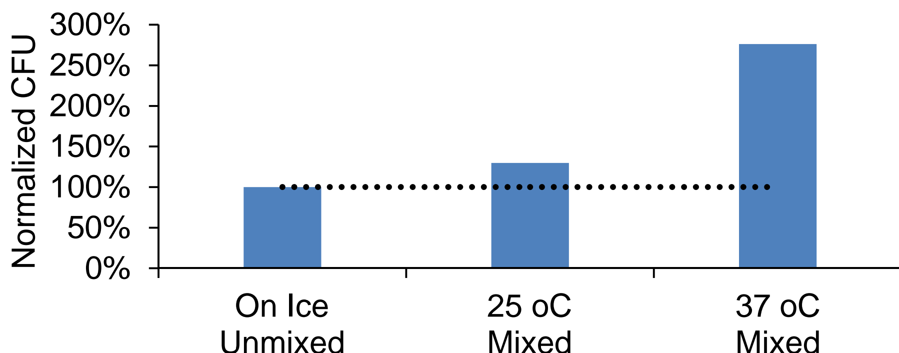
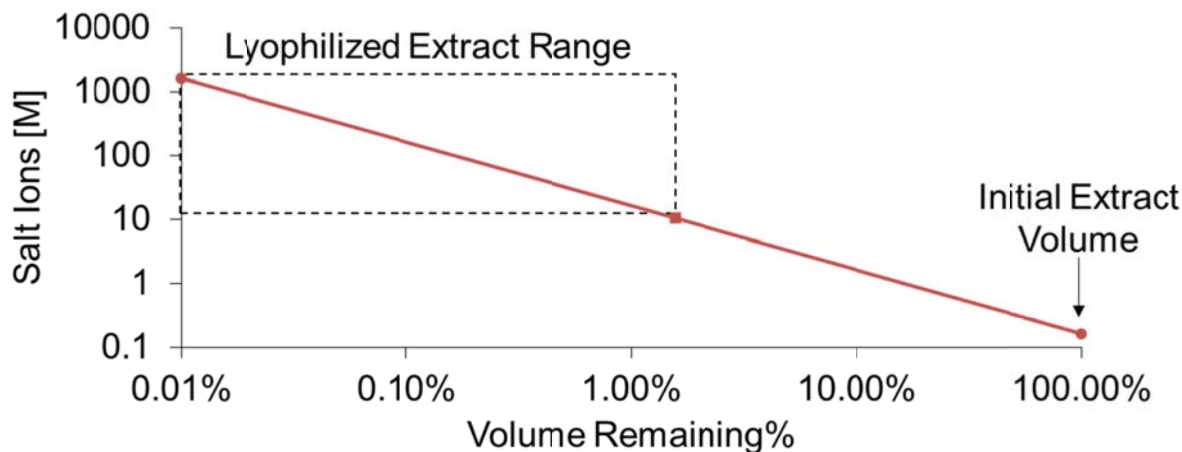
16

1 Untreated Extract at Incubated at Elevated Temperatures



2

3 **Figure S1: Extract Contamination Change from Storage at 25 and 37 °C for 30 minutes.**
4 Bacteria are known to have a relatively short doubling time, typically ranging from 20-45
5 minutes for BL21 Star™ (DE3) under optimal fermentation conditions. End-over-end mixing of
6 the cell extracts at room temperature was sufficient for cells to proliferate and increase
7 contamination by 30%. At 37 °C, the cells more than doubled while mixed.
8
9 Changes in Salinity Caused by Lyophilization



10

11 **Figure S2: Extract Salt Ion Concentration as Aqueous Volume is Removed by Lyophilizaiton**
12 High salinity is known to be deleterious to the viability of cells. For example, Hrenovic and
13 Ivankovic reported that a 48 hour incubation of *E. coli* with approximately 5M NaCl in solution
14 was sufficient to effectively eliminate all viable bacteria, a greater than 12 log-fold reduction.[1]
15 Lyophilization of cell extracts removes upwards of 98.5% of the aqueous volume, thus increases
16 salt concentration by more than 60 times.[2] Based on the ions in the buffer solution alone, this
17 suggests an increase from ~0.16 M to greater than 10.3 M after lyophilization. This conservative
18 estimate does not account the ions already contained in the lysed material, which would increase
19 overall salinity. The post-lyophilization storage of cell extracts above freezing keeps the
20 contaminating bacteria active in an extremely salty solution, and likely causes cell death.[2]

Vacuum and Syringe Filtration

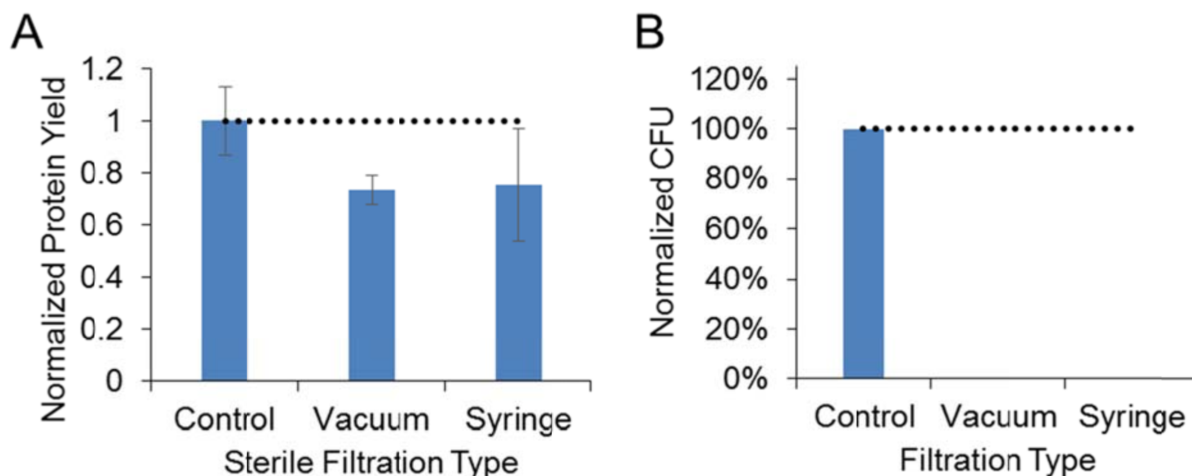


Figure S3: CFPS Yields of Filtered Extracts: Vacuum verses Syringe. A) Normalized Protein Yields. B) Normalized Contamination

Both formats of filtration (vacuum and syringe) were effective at eliminating detectable bacterial contamination. On average, each performed equivalently well as the other. However, the syringe-filtered extracts provided less consistency than the vacuum filtered extracts. Yield error bars = 1 stdev, n=3.

UV Treatment – Normalized Yields and CFU

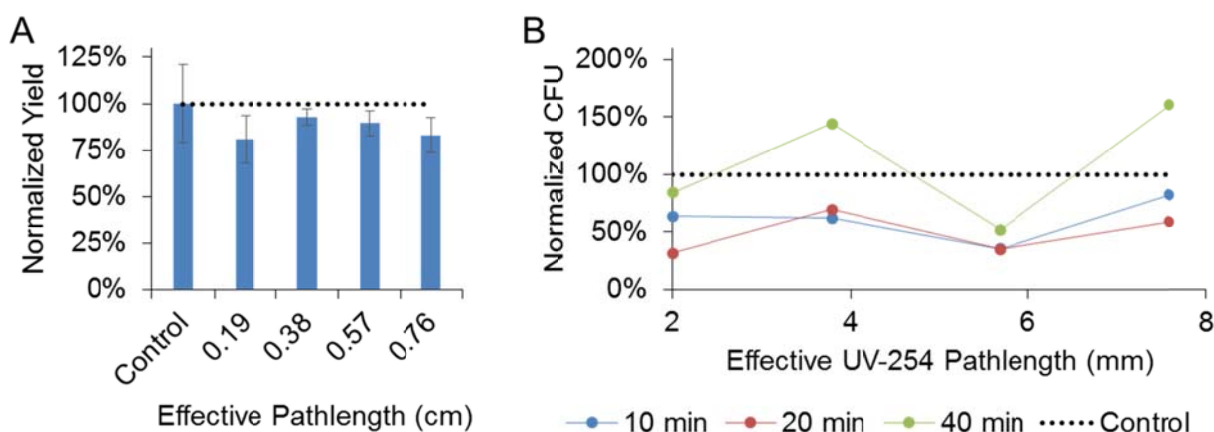
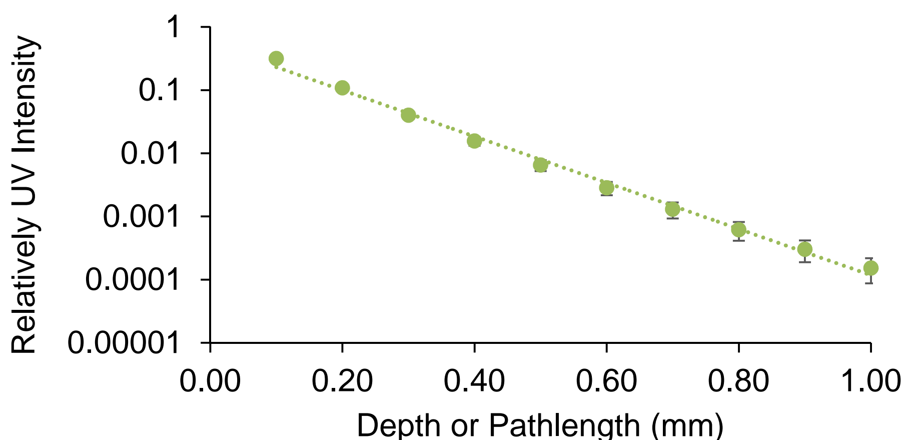


Figure S4: UV Treatment Effects. A) Normalized protein yield after 40 min UV-254 Treatment. B) Normalized contamination at given pathlengths and treatment times.

Increasing the pathlength of the treatment led to weak overall increase in bacterial contamination (regression p-value = 0.26). The weakness of the trend may be due to the extremely attenuated

1 UV intensity that approached 90% at 2 mm and exceeds 96% attenuation by 3 mm (Figure S5).
2 At these treatment depths, the difference in attenuation effects is limited. However, increasing
3 incubation time led to a strong increase in contamination levels (regression p-value < 0.01). The
4 combination of poor UV-penetration and the increased incubation time allows for the cells to
5 propogate and increase contamination. UV is a promising technique, but implementation of this
6 method for extract sterilization will likely require more advanced equipment and higher power
7 UV bulbs. Yield error bars = 1 stdev, n=3.
8
9 UV-254 nm Intensity Attenuation



10

11 **Figure S5: Model of UV-254 Intensity Attenuation through Extract.**
12 Cell extract predominantly consists of protein, with an average of 68 mg protein per mL extract.
13 To model the impacts of this dense solution on UV-254 intensity, we predicted the average
14 extinction coefficient of the solution based on known parameters: 1) average length of *E. coli*
15 protein: ~300 amino acids, 2) statistical probability of given amino acid based on codon bias in
16 *E. coli* randomly assigned to 896 model proteins, and 3) individual extinction coefficients of
17 amino acids that absorb UV-254 (Tyr=383, Trp=2861, His=18, Phe=143 $cm^{-1}M^{-1}$). The resulting
18 average extinction coefficient modeled was 0.75 ± 0.19 $cm^{-1}(mg/mL)^{-1}$. At the high concentration
19 of protein in extract, the UV-254 intensity would decrease by about 90% within 0.2 mm. In the
20 shallowest depth tested in this work, the theoretically predicted pathlength was 1.8 mm, which
21 corresponds to a predicted attenuation to UV-254 intensity greater than 99.9999%. This level of
22 reduction suggests that the 1100000 $\mu W/cm^2$ is reduced to 1.1 $\mu W/cm^2$ at the bottom of the
23 sample, which is insufficient to reduce the residual bacteria in the exposure times tested (up to 40
24 mins).

Ampicillin Treatment – Normalized Yields and CFU

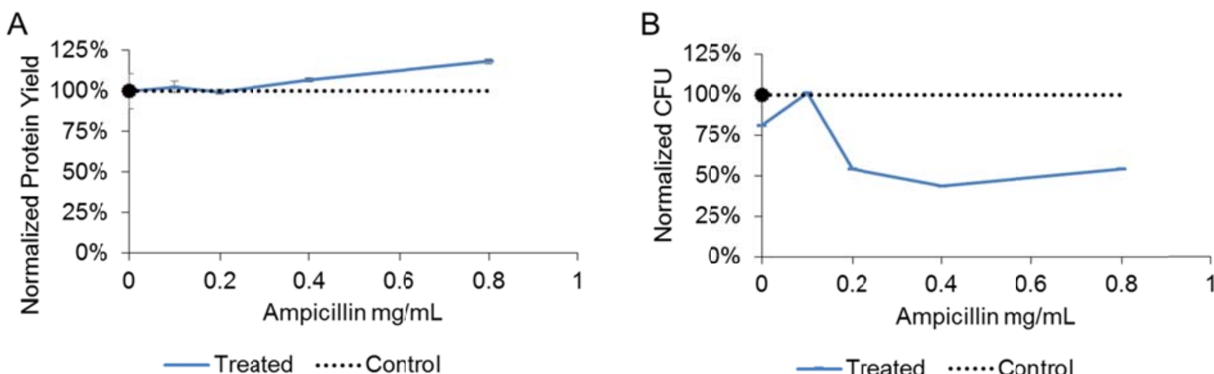


Figure S6: Ampicillin Treatment Effects. A) Normalized CFPS yields with treated extract compared to an untreated control on ice. B) Normalized CFU with treated extracts compared to an untreated control on ice.
Ampicillin was added to extracts at doses at and higher than typical culture concentration (>0.1 mg per mL). The mixtures were incubated at room temperature for 30 minutes. Extracts were subsequently assayed for contamination and protein synthesis viability. The addition of ampicillin led to a mild improvement of protein yield while reducing contamination. However, under the conditions tested, ampicillin was insufficient to effectively eliminate cell contamination. This inability to eliminate contamination may be due to factors such as insufficient treatment time. Increased treatment duration at temperatures above freezing is known to reduce protein synthesis viability. (n=3 for CFPS yields, error bars represent 1 standard deviation).

Lysozyme Treatment – Normalized Yields and CFU

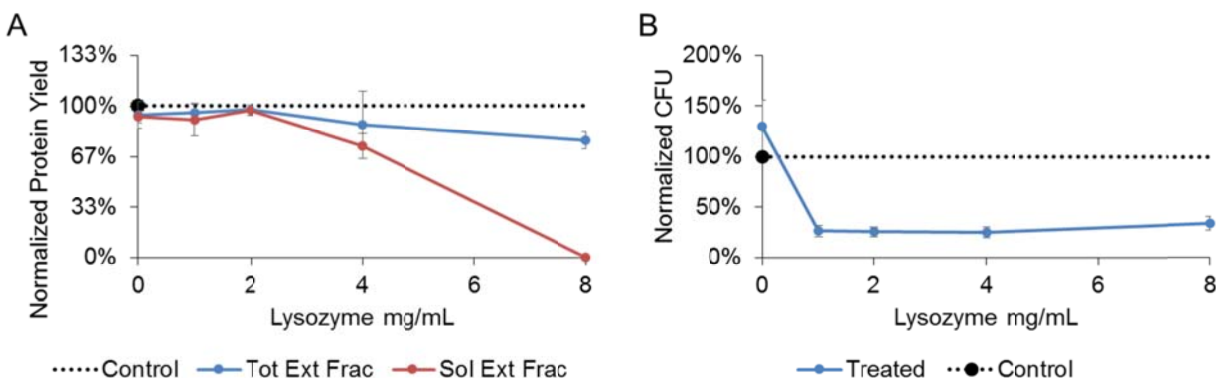


Figure S7: Lysozyme Treatment Effects. A) Normalized CFPS yields with total extract fraction and soluble extract fraction compared to an untreated control on ice. B) Normalized CFU with treated extracts compared to an untreated control on ice.
Increasing amounts of lysozyme in the extract caused a visible increase in the formation of aggregates. Therefore, we considered the total extract fraction and the soluble extract fraction after lysozyme treatment for CFPS viability. Notably, the formation of aggregates decreased yields slightly, but the centrifugal removal of aggregates virtually eliminated protein synthesis

1 when treated with 8 mg/mL lysozyme. Lysozyme is effective at removing >70% of the bacterial
2 contamination in the extract. However, the treatments described here are insufficient for multiple
3 log-fold reductions in contamination.
4

5 References

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