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Impact of Process Interruptions in the Production of Lysates for Cell-Free Expression Systems

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ABSTRACT

Cell-free gene expression systems offer cell-like functionalities outside the confines of the cell, garnering increasing interest for applications from biomanufacturing to sensing. As applications expand, the need to implement economically scaled processes to produce cellular lysates grows. The protocols to produce these cellular lysates are complex, and the impact of altering many of the process variables remains understudied. Here, we set out to evaluate the effect of extended incubations at several points in the extract preparation process with the goal of identifying breakpoints that would enable flexibility in process implementation. As a model, we prepared lysates from 50 L cultures instead of typical 1 L volumes. We produced 72 lysates, 36 that were incubated overnight before and after culture centrifugation, and 36 that were incubated with and without a run-off reaction, each across different temperatures. We found that incubations before and after culture centrifugation substantially increased variability between culture replicates but did not reduce cell-free protein synthesis activity, contrary to conventional wisdom that materials should be kept cold as much as possible throughout the process. We also observed that omitting the run-off reaction reduced yields but resulted in lysates that were robust to incubation up to room temperature overnight. When a run-off reaction was included, activity dropped both as a function of duration and temperature, and the overall variability increased. Our work offers potential options for flexibility in implementing lysate production processes and motivates further investigation into how key processing steps relate to cell-free expression activity.

1 | Introduction

Cell-free expression (CFE) systems are a suite of tools to recapitulate cellular processes such as transcription, translation, and metabolism in a non-living format (Carlson et al. 2012; A. C. Hunt et al. 2024; Silverman et al. 2020). These systems have applications spanning basic research to biomanufacturing, including decentralized production of medicines (Adiga et al. 2018; Borhani et al. 2023; Pardee et al. 2016; Stark

et al. 2021; Warfel et al. 2023; Williams et al. 2023), genetic component design and engineering (Ekas et al. 2024a, 2024b; Karim et al. 2020; Karim and Jewett 2016; Kightlinger et al. 2018, 2019; McManus et al. 2021), portable diagnostics (Amalfitano et al. 2021; Beabout et al. 2021, 2022; Carr et al. 2022; J. P. Hunt et al. 2022; Jung et al. 2020; Pardee et al. 2014; Thavarajah et al. 2020), incorporation into materials (Blum et al. 2021; Lee et al. 2020, 2024; Nguyen et al. 2021;

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Whitfield et al. 2019), and education (Collins et al. 2024; Huang et al. 2018; Jung et al. 2023; Rybnicky et al. 2022; Stark et al. 2018, 2019). Some CFE approaches rely on combining purified versions of the components necessary for gene expression (Shimizu et al. 2001), while the majority utilize cellular lysates, especially those derived from *Escherichia coli* (Gregorio et al. 2019).

The general process for preparing *E. coli* lysate for CFE includes culturing, culture centrifugation, cell lysis, lysate centrifugation, incubation (known as the run-off reaction), and dialysis (Figure 1). Researchers have explored the impact of changing the many process variables, optimizing the Nobel-prize-winning method used to unravel the genetic code (Nirenberg and Matthaei 1961), to the more streamlined protocols used today (Cole et al. 2020). Some changes have been practical, such as culture centrifugation reducing 90 min with three washes to 10 min without loss of activity (Cole et al. 2020). In other cases, the picture is more complex. Most methods harvest cells during mid-exponential phase as cells are thought to be most translationally active, though some methods harvest at later points (Failmezger et al. 2017; Levine et al. 2019). The run-off reaction, thought to exhaust any ongoing endogenous expression activity at the time of lysis, has impacts that vary with strain and native versus exogenous polymerase activity (Kwon and Jewett 2015; Silverman et al. 2019). These examples highlight gaps in our understanding of basic principles underpinning CFE, despite significant progress in their production and applications.

One area of the process that has not been explored thoroughly is the sensitivity of the material to varied handling at potentially convenient breakpoints. To date, protocols offer only one breakpoint: multiple studies have shown that cell pellets can be frozen and stored at -80°C before lysis without loss of activity (Dopp and Reuel 2018; Silverman et al. 2019), though some protocols in use today do not even include that option (Sun et al. 2013). Additional breakpoints are especially relevant at large-scale production, where handling materials can take much longer. In particular, the conventional wisdom that materials should be kept cold as much as possible throughout the

process is more difficult and costly at large scales, yet data to support the necessity of chilling is lacking in the literature. Moreover, these extended durations mean that without incorporating breakpoints, the full process can take upwards of 24 h at the 1000 L scale, creating challenges with respect to normal working hours. For small-scale production, breakpoints are less critical; indeed, one protocol offers a 24 h process completed only during normal working hours (Levine et al. 2019). Regardless of scale, improved understanding of the limitations of the process offers opportunity for improvements.

Here, we explored the effect on CFE yields for varying temperatures and durations at alternative process breakpoints. We found that extended incubations before and after culture centrifugation at varying temperatures did not necessarily reduce activity; however, we did observe substantially increased variability, limiting the practical utility of observations on activity. We further found that excluding the run-off reaction lowered activity but resulted in lysates that were robust to incubations up to overnight at room temperature (RT). With the run-off reaction, we observed a clear loss of activity as a function of both duration and temperature of incubation. In general, given the conventional wisdom to maintain chilled material throughout the process, the observations here suggest that the process is likely more robust than previously thought. These results provide new options for flexible process implementations and highlight a need for a deeper understanding of the relationship between process variables and yields.

2 | Materials and Methods

2.1 | Lysate Preparation

Lysate production was based on the method described by Silverman et al. (2019) and modified for larger scales. To start, four fresh plates of 2× YTP agar were inoculated from a glycerol stock of *E. coli* Rosetta 2(DE3) $\Delta lacZ\alpha$ and incubated at 37°C for 16–18 h. Using a large loop, all material was scraped from two starter plates and transferred into 750 mL of 2× YTP broth in a

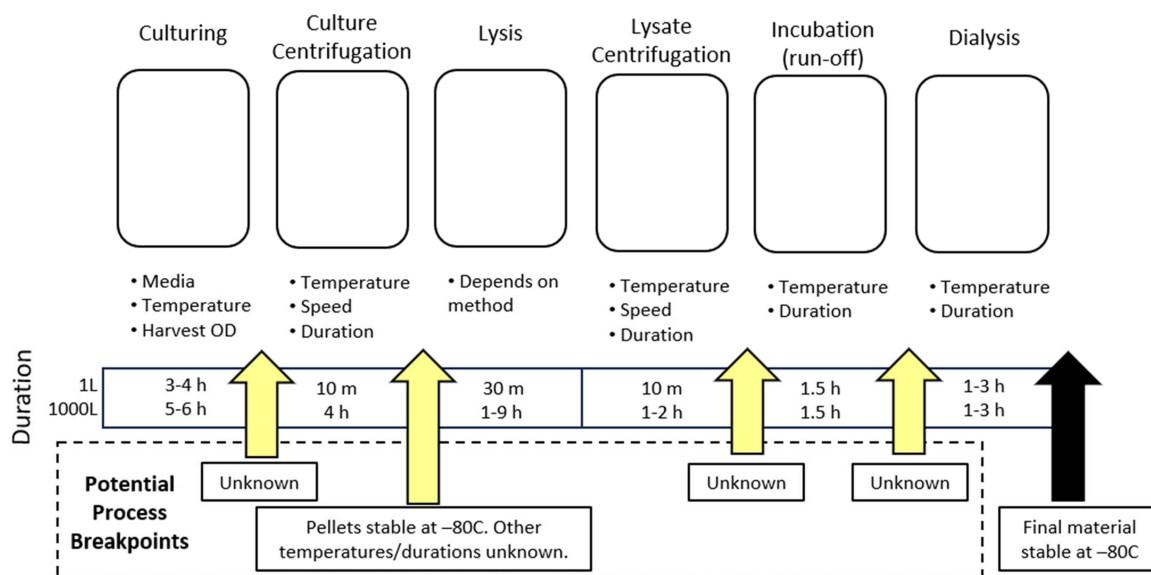


FIGURE 1 | Lysate production process workflow. Arrows indicate potential process breakpoints. Yellow arrows indicate stages where the impact of incubation at various temperatures for extended durations has not been explored.

2.8 L Erlenmeyer flask; this step was repeated for the additional two starter plates for a total of 1.5 L of inoculated media. The cultures were inoculated 16 h before the start of the final culture and incubated at 37°C with a shaking speed of 200 rotations per minute (rpm). An additional incubation in the Micros 30 L (New Brunswick Scientific) fermenter is required for the 1000 L scale, so the flask material was inoculated into 20 L of 2× YT media prepared using 600 g of 2× YT media powder, 140 g of potassium phosphate dibasic (Sigma Cat, P3786), 60 g of potassium phosphate monobasic (Sigma, P5655) and 1 mL of antifoam 204 (Sigma, A8311) to 20 L of deionized (DI) water the day before inoculation. This material was inoculated at a starting OD₆₀₀ of 0.05, 12 h before the final culture scale up to 1000 L. For 100 L fermentations, the medium was prepared in the IF 150 L (New Brunswick Scientific) fermenter by adding 3.1 kg of 2× YT media powder, 700 g of potassium phosphate dibasic (Sigma Cat, P3786), 300 g of potassium phosphate monobasic (Sigma, P5655) and 5 mL of antifoam 204 (Sigma, A8311) to 100 L of DI water the day before inoculation. The media was allowed to aerate overnight with a rotor speed of 100 rpm, airflow of 20 standard liters per minute (SLPM), and the temperature set to 37°C. The majority of the experimental preparations in this paper were 50 L (Figures 3–6) and followed an identical protocol to the 100 L scale (Figure 2 only), but with exactly half of the volumes and weight for all inoculations, materials, and reagents. For 1000 L fermentations, the media was prepared in the IF 1500 L (New Brunswick Scientific) fermenter by adding 31 kg of 2× YT media powder, 7 kg phosphate dibasic (Sigma Cat, P3786), 3 kg of potassium phosphate monobasic (Sigma, P5655) and 50 mL of antifoam 204 (Sigma, A8311) to 1000 L of DI water the day before inoculation.

The morning of inoculation for both 50 and 100 L preparations, the overnight cultures were combined, and OD₆₀₀ was measured using a spectrophotometer (Genesys 20, Fisher Scientific). All fermentation volumes are seeded at a starting density of approximately 0.05 OD₆₀₀ using a 2 L pre-sterilized transfer

bottle or direct connections to the smaller scale fermenter seed culture, to inject the media with the appropriate amount of overnight culture based on starting optical density. The fermenter settings were adjusted to 300 rpm, 50 SLPM air flow, and the dissolved oxygen (DO) meter was calibrated to 100%. The OD₆₀₀ was measured every hour until 0.6–1.0, at which point the culture was induced with 100 mL of 1 M Isopropyl β-D-1-thiogalactopyranoside (IPTG) (GoldBio, I2481C) for a final concentration of 1 mM. The fermentation continued and when DO levels fell below 50%, the rotation speed was increased to 500 rpm (this usually occurred around OD₆₀₀ of 2–2.5 at around 3 h into the process). Upon reaching OD₆₀₀ of 3.5 around 4.5 h after the start of the run, the culture was harvested by emptying the material into 50 L carboys for storage and distribution. While higher harvest ODs are generally possible and desirable when scaling lysate production, we chose to stick to induction and harvest ODs typical of smaller scales to reduce the number of variables when adapting to larger scales (Figure 2) and to maintain consistency across the study (Figures 3–6). Efforts to minimize the cost of lysate production would undoubtedly benefit from higher harvest ODs and other optimizations, such as lower-cost culture media.

To test each temperature and condition, the culture material was prepared for incubation as follows: for culture storage, 3 L of culture was aliquoted into 4 L glass Erlenmeyer flasks and covered with aluminum foil and/or parafilm. To test pellet storage, 3 L of culture was spun at 4°C and 5000 × g for 10 min in 1 L centrifuge bottles. The supernatant was poured off and discarded, while the pellet material was scraped into a clean 250 mL centrifuge bottle. The slurry material was prepared using the same conditions as the pellet, except it was then partially resuspended by vortexing for 1 min in approximately 50 mL of DI water. The control material was also prepared exactly like the pellet material and then flash frozen and placed in storage at –80°C.

For each lot of testing, the specified material type was stored at the indicated temperature of 4°C, 10°C, or RT for 4–18 h as indicated in the results. After incubation, the culture samples were centrifuged as described for the pellet case above, then immediately lysed; the pellet and slurry cases were processed into a lysate immediately after incubation. Each condition was processed with a control lysate to account for any variability in the processing procedures and/or equipment. Pellets and lysates were kept on ice in between processes for short durations when necessary. Pellets were resuspended in S30B buffer (10 mM Tris-acetate [pH 8.2], 14 mM magnesium acetate, 60 mM potassium acetate, 1 mM DTT) at a ratio of 1 mL per 1 g of pellet weight. Samples classified as slurry were processed with only the water used to create the slurry. All lysates were lysed using the M110P microfluidizer at 20,000 psi and then spun at 12,000 × g at 4°C for 10 min in 250 mL Beckman centrifuge bottles. For reactions including a run-off reaction, the supernatant was transferred to a 50 mL conical tube and heated with shaking at 37°C for 90 min. The material was spun again at 12,000 × g at 4°C for 10 min in the 50 mL conical tube, and the supernatant was aliquoted into PCR strip tubes in 100 μL aliquots and flash frozen using liquid nitrogen. For the lysates without a run-off reaction, cells were lysed and centrifuged as described above, incubated for the described temperatures and durations, and aliquoted and flash frozen.

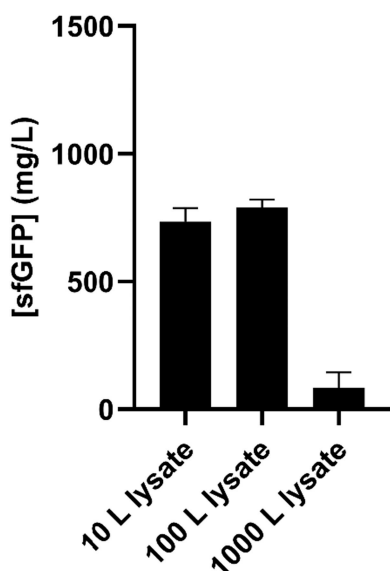


FIGURE 2 | Reduced CFE yields of superfolder green fluorescent protein (sfGFP) observed for lysates produced from 1000 L culture compared to 10 L and 100 L. Data represent $n = 10$ CFE reactions with error bars showing one standard deviation.

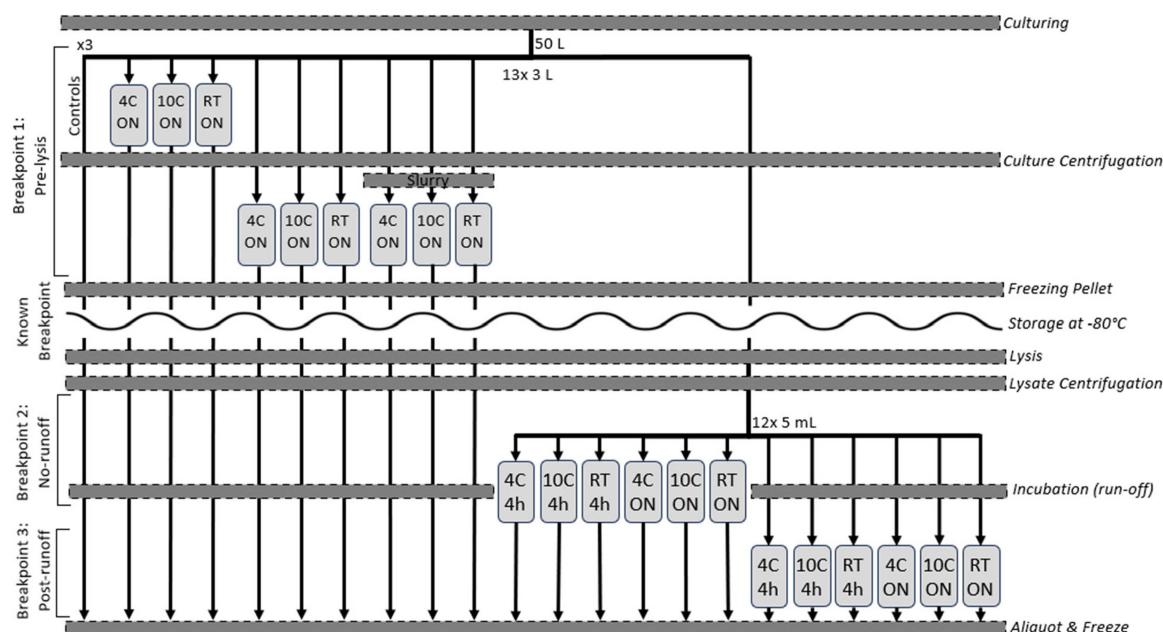


FIGURE 3 | Experimental Design. Starting from 3×50 L cultures, 39×3 L cultures were processed with varying incubations before and after centrifugation (Pre-lysis). One set of replicate lysates processed normally up through lysis was subsequently split and incubated at different temperatures and durations without a run-off reaction (No-runoff) or afterwards (Post-runoff). In total, $72 \times$ distinct lysate preparations were evaluated.

2.2 | CFE Reactions

All reagents were prepared as described previously (Silverman et al. 2019) to create a combined reagent mix that was divided into $200 \mu\text{L}$ and flash frozen. To avoid batch-to-batch variability of reagent mix preps (Cole et al. 2019), all reactions in this study came from the same batch of reagent mix. Final concentrations of each reaction component in the mix are provided in the [Supporting Data file](#). Plasmid DNA, pJL1-sfGFP, was added to the reagent mix at a final total reaction volume concentration of 5 nM and used as a “master-mix” for comparison across all lysate reactions. Each CFE reaction consisted of $5 \mu\text{L}$ of lysate material and $5 \mu\text{L}$ of master mix. Each condition used for comparison consisted of three separate biological replicates run in technical replicates of four. The plasmid DNA was prepared using a ZymoPURE II Gigaprep kit, and all DNA was eluted in molecular-grade water. All reactions were carried out in 96-well clear round-bottom plates in a BioteK H1 plate reader, reading every 3 min with an excitation and emission of 488 and 535, respectively. Reaction plates ran for 16 h, with constant orbital shaking, at 30°C . Resultant relative fluorescent units (RFU) values were converted to mg/L via a standard curve generated from measuring the concentration of sfGFP for six randomly-selected samples from the study via radiolabeling using ^{14}C -leucine incorporation. ^{14}C -leucine incorporation was carried out as reported previously (Jewett et al. 2008; Jewett and Swartz 2004; Olsen et al. 2025).

2.3 | Statistical Analysis

Data analysis was performed using the software IBM SPSS V27.0.1.0. Pre-lysis yield, pre-runoff yield, and post-runoff yield were analysed using univariate linear mixed models that included technical replication as an unstructured random

variable, with duration, temperature (and technical replication) fitted as categorical fixed variables. Each of these models included all combinations of interactions. Model assumptions were verified to hold true by examination of residual and diagnostic plots accordingly.

3 | Results and Discussion

The study was motivated by a production run for CFE lysates from a 1000 L fermentation that resulted in > 5 -fold lower production of superfolder green fluorescent protein (sfGFP) compared to reactions using lysates produced by 10 or 100 L fermentations (Figure 2). The centrifuge model that was available for processing the 1000 L scale fermentation (Alfa Laval BTPX 205) is intended for proteins and other biomolecules rather than cells, which we suspect was the likely reason for the decreased yields. However, we also had challenges with our chilling equipment, leaving materials at elevated temperatures for extended periods (e.g., 37°C cultures remaining above 30°C by the end of the 4 h centrifugation process). As discussed in the introduction, such issues highlight knowledge gaps for how the time and temperature of handling material at various steps impact yield, in addition to the utility of process breakpoints at larger scales.

To probe the dependency of CFE yields on these incubations, we devised an experimental plan as outlined in Figure 3. Starting from 50 L fermentations, the cultures were divided into 13×3 L batches that were processed as described in the figure and following text. The entire procedure was repeated three times, resulting in 39 total lysates, of which 3 were further divided into 12 post-lysis conditions, resulting in an additional 36 lysates. The first set of process variables explored, referred to collectively as ‘Pre-lysis’ conditions, were intended to address

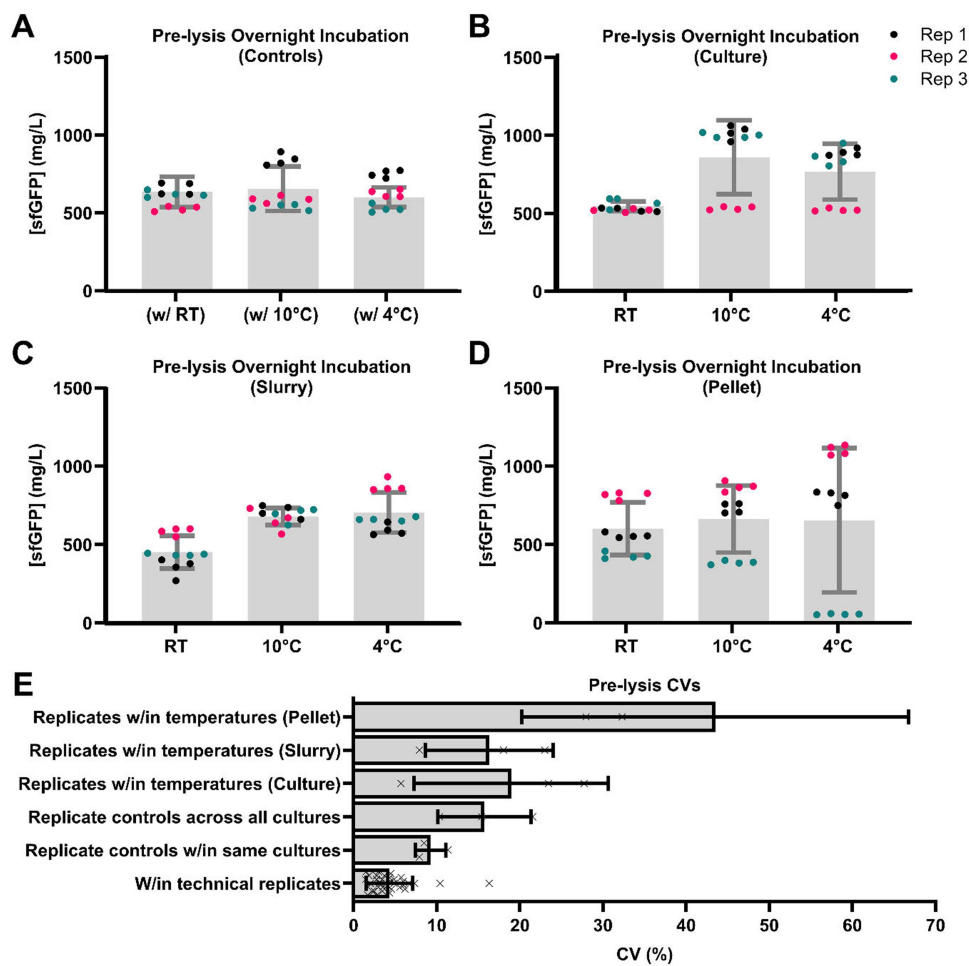


FIGURE 4 | Impact of Pre-lysis incubations. (A) Yields of Control lysates. Material for each temperature across the Culture, Slurry, and Pellet cases was defrosted and further processed in parallel, and a distinct control lysate was included in each case. Different temperatures and different culture replicates were processed on different days. (B) Yields of lysates from Culture incubations. (C) Yields of lysates from Slurry incubations. (D) Yields of lysates from Pellet incubations. (E) CV values for different groupings. For (A–D), colors correspond to $n = 3$ lysates produced with corresponding incubation conditions from each of 3×50 L fermentations with $n = 4$ technical replicates in each case. Error bars represent one standard deviation of the combined lysate and technical replicates ($n = 12$).

slow chilling of cultures that remain at elevated temperatures while portions are gradually piped off and centrifuged (~4 h for 1000 L in our facility), as well as centrifuged cells that remain at elevated temperatures for extended periods while the remaining cells are centrifuged. While we did not measure these temperatures carefully during our 1000 L run, we observed that the temperature of the culture did not go below 30°C. Furthermore, centrifuged material came off the instrument warm (not measured) and then incubated at RT for up to ~4 h, while the remainder of the material was centrifuged. In addition, while it is known that cell pellets can be frozen and stored at –80°C before lysis, both freezing and thawing of the cells is more challenging at higher scales, so knowledge of robustness to storage at other temperatures would be useful. To test the sensitivity of CFE yields to this set of process variables, cells were incubated overnight at 4°C, 10°C, and RT before centrifugation (Culture) or after; cells incubated after centrifugation were incubated either in pellet form (Pellet) or resuspended as a slurry that mimics the material that results from the centrifuge we used in our 1000 L production run (Slurry). Typical protocols have users immediately proceed from culturing to centrifugation and then either directly from centrifugation to lysis or

storage as a frozen pellet. At larger scales, some cells will sit for extended periods before/after each processing step compared to smaller scales, which is consistent with the conventional wisdom that keeping cells cool is important to achieve high-yielding lysates. Our experiments were designed to test the hypothesis that not chilling cells before or after centrifugation results in decreased activity.

Notably, activity similar to the controls was retained even after overnight incubation at RT; however, high variability between replicate cultures in some cases obscures results (Figure 4). To examine the data further, we fitted a generalized linear model with temperature, replicate, and stage (Control, Culture, Pellet, and Slurry) as dependent variables. Consistent with the observed high variability, the most explanatory effect (F score = 56, $p < 0.001$) was a three-way interaction between variables, which does not provide insight into the impact of process changes. The only other statistically significant effect is the two-way interaction between stage and replicate ($p = 0.001$), suggesting that extended incubations before and after centrifugation may amplify small differences between replicate cultures that are not apparent in the controls. For the

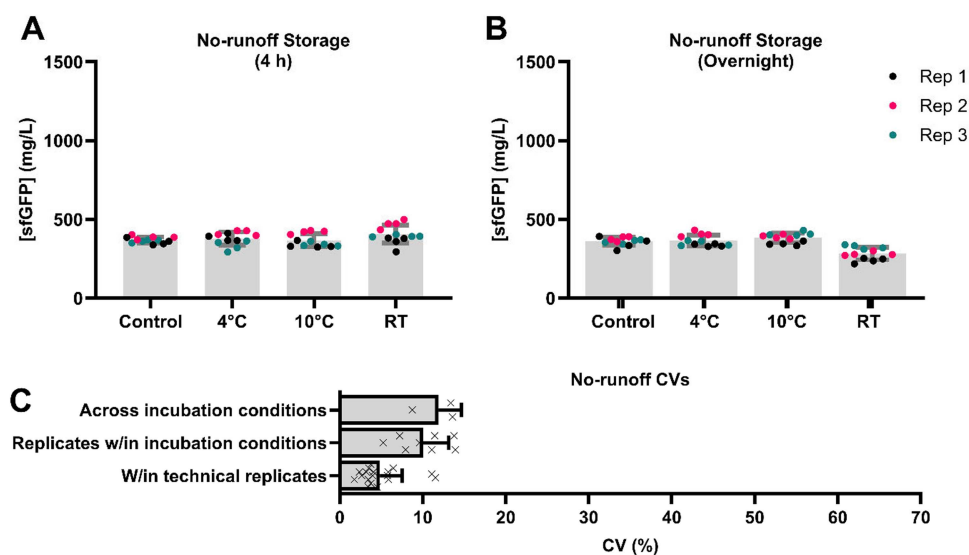


FIGURE 5 | Impact of No-runoff incubations. (A) Yields of lysates when incubated at different temperatures for 0 or 4 h. (B) Yields of lysates when incubated at different temperatures for 0 h or overnight. (C) CV values for different groupings of data are shown in panels (A and B). For (A and B), colors correspond to $n = 3$ lysates produced with corresponding incubation conditions from each of 3×50 L fermentations with $n = 4$ technical replicates in each case. Error bars represent one standard deviation of the combined lysate and technical replicates ($n = 12$).

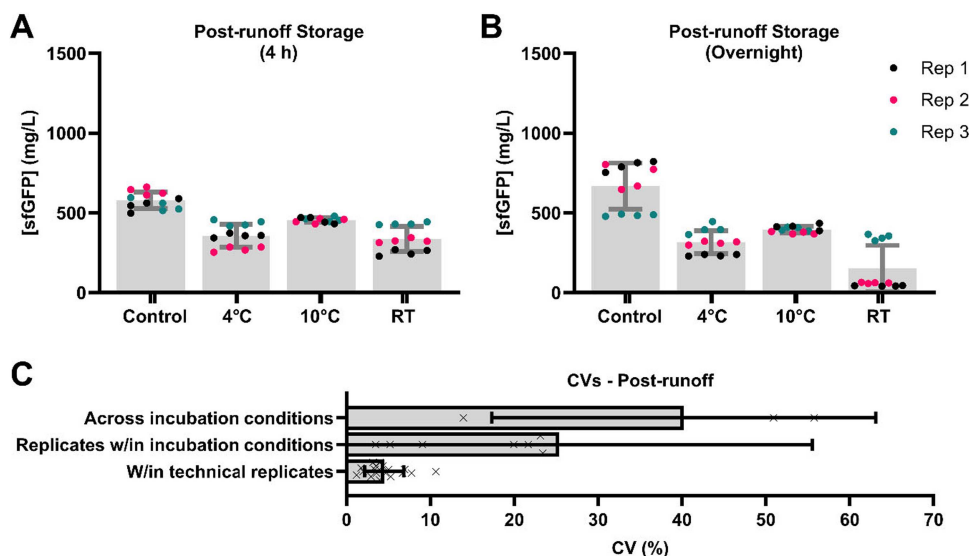


FIGURE 6 | Impact of Post-runoff incubations. (A) Yields of lysates when incubated at different temperatures for 0 or 4 h. (B) Yields of lysates when incubated at different temperatures for 0 h or overnight. (C) CV values for different groupings of data are shown in panels (A and B). For (A and B), colors correspond to $n = 3$ lysates produced with corresponding incubation conditions from each of 3×50 L fermentations with $n = 4$ technical replicates in each case. Error bars represent one standard deviation of the combined lysate and technical replicates ($n = 12$).

Culture case, several of the replicates had much higher yields than the controls. Since these incubations are effectively extended culturing with the temperature gradually reducing towards RT, the cells presumably moved beyond the exponential phase (not measured). While our data does not offer any conclusions with respect to the growth phase, it does complement other studies showing that other harvest points besides mid-exponential are viable (Failmezger et al. 2017; Levine et al. 2019). Moreover, these observations challenge the conventional wisdom that cells must be kept cool as much as possible before and after centrifugation to achieve active lysates.

We further used the coefficient of variation (CV) as an intuitive and unitless metric to examine variability across groupings (Figure 4E). The average CV of 4.3% within technical replicates across conditions is slightly smaller than previous reports (Cole et al. 2019), indicating that preparation of the CFE reactions themselves is not a major factor. For replicate controls across replicate cultures, the variability rises to 16.6%, which is slightly higher than previously reported CVs of ~6%–11% for replicate lysates produced using different methods (Cole et al. 2019; Silverman et al. 2019), indicating that the core process is comparably repeatable to other methods. When considering the CV across replicates within each incubation condition, the average

CVs across temperature for the Culture and Slurry cases rise slightly to 19.0% and 16.3%, while the Pellet case rises to 43.5%. It is difficult to explain this large jump for the Pellet case. One possibility is that the large pellets (19–23 g of cells) form inconsistent morphologies that lead to differential effects of temperature during incubation, though this idea is speculative.

The second and third breakpoints (No-runoff and Post-runoff, respectively) occur after lysate centrifugation. We did not include a breakpoint after lysis and before lysate centrifugation because lysate is kept cool during lysis by the instrumentation in this work and with other methods (Kwon and Jewett 2015; Sun et al. 2013). Run-off reactions have been shown to have different impacts for different strains (Kwon and Jewett 2015) and can be eliminated without loss of activity in some cases (Silverman et al. 2019). We tested the impact of incubating supernatants from lysate centrifugation at 4°C, 10°C, or RT for 4 h or overnight before aliquoting and storing with no run-off reaction. We found that CFE yields dropped substantially without the run-off reaction (365 vs. 631 g/L for the controls in the Pre-lysis experiments; $p < 0.001$, though we note that these experiments were run on different days). Prior results using a very similar strain and supplement mixture but lysing by sonication have shown conflicting results: decreased yields with increased run-off reaction times (Kwon and Jewett 2015), and no effect on yields from the run-off reaction (Silverman et al. 2019); here we found that yields improved with the run-off reaction, highlighting that the role of the run-off reaction and its interdependence with other factors requires further investigation. We found that incubation conditions had little impact on yields for the lysates without a run-off reaction (Figure 5A,B). This observation may shed additional light on the run-off reaction, as the incubations are effectively extended run-off reactions at lower temperatures, which would likely impact the putative role of the reaction to exhaust any ongoing endogenous expression activity at the time of lysis. A generalized linear model again indicated that the largest effect was a two-way interaction between duration and temperature ($F = 11.1$, $p = 0.007$), demonstrating a small but statistically significant loss of yield as a function of both duration and temperature. The CV values again showed that within technical replicates the error is small (4.8%), and the variability only rose to 10.0% for replicate cultures within the same condition and 11.9% within individual culture replicates across incubation conditions (Figure 5C). These results suggest that, in the absence of a run-off reaction, after lysate centrifugation is an option as a process breakpoint for flexible implementations.

The final breakpoint, Post-runoff, evaluated whether materials could be incubated for extended periods after the run-off reaction but before aliquoting and freezing. Lysates were incubated at 4°C, 10°C, or RT for 4 h or overnight. The control reactions retained similar activity to the Pre-lysis controls, confirming the impact of the run-off reaction. Unlike the No-runoff case, however, these lysates were clearly impacted by incubation (Figure 6). A generalized linear model in this case indicated statistically significant effects due to temperature (F -score = 9.0, $p = 0.023$) and duration (F -score = 41.7, $p = 0.012$) and a marginally significant two-way interaction between the two (F -score = 3.77, $p = 0.078$). Consideration of CV values again confirmed small average variations within technical replicates (4.5%) and showed large increases in

variability across replicate cultures within each incubation condition (25.4%) and, unsurprisingly, within each culture replicate across conditions (40.2%) (Figure 6C). The increased CV values and the wide range of CV values for replicates within incubation conditions may suggest a link between small differences in replicate culturing and lysis, the run-off reaction, and post-lysis incubations, again highlighting that the role of run-off reactions warrants further investigation. Practically, these results indicate that when including a run-off reaction, lysates should be aliquoted and stored immediately to avoid loss of activity.

4 | Conclusions

Overall, we found that it is possible to pause the lysate production process at several points with little or no loss of activity, even overnight at RT. The exception was incubation of material after the runoff reaction, where activity was clearly impacted by both temperature and duration. Conclusions about the degree of robustness of the process are complicated by the observed increase in variability for samples incubated at the pre-centrifugation stage and especially for centrifuged material stored as a pellet, which we suspect may result from differences in pellet morphology. Nonetheless, the degree of robustness observed here is surprising for what is typically considered to be a sensitive process. Our results suggest a higher degree of flexibility for developing processes to produce lysates for CFE that can accommodate a wider range of workflows that are amenable to different labs, schedules, and equipment. We further note that in some cases, the incubations increased rather than decreased yields. While these cases showed high variability between biological replicates and were not statistically significant, they point to a potential opportunity to find new process implementations that increase yields, particularly in the culturing phase. In combination, this study provides new insights into key process variables for producing lysates for CFE and points to additional avenues that warrant future investigation.

Acknowledgments

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Conflicts of Interest

M.C.J. is a co-founder and has financial interest in Ridge Bio, Pearl Bio, Gauntlet Bio, and Synolo Therapeutics. These interests are reviewed and managed by Northwestern University and Stanford University in accordance with their conflict-of-interest policies. The other authors declare no conflicts of interest.

Data Availability Statement

All data and analyses are provided in the Supporting Information S1 and S2.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supplement: data. Supplement: stats analysis.