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Technical Note

Cell Extracts from Bacteria and Yeast Retain Metabolic Activity after Extended Storage and Repeated Thawing

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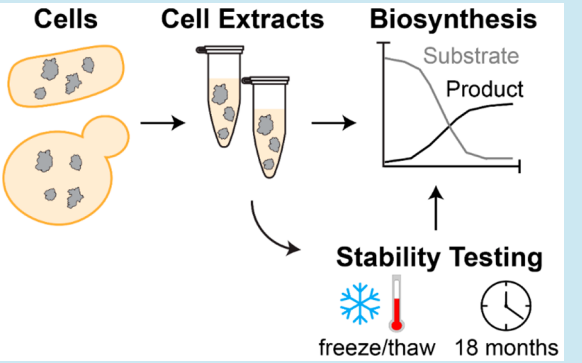
ABSTRACT: Cell-free synthetic biology enables rapid prototyping of biological parts and synthesis of proteins or metabolites in the absence of cell growth constraints. Cell-free systems are frequently made from crude cell extracts, where composition and activity can vary significantly based on source strain, preparation and processing, reagents, and other considerations. This variability can cause extracts to be treated as black boxes for which empirical observations guide practical laboratory practices, including a hesitance to use dated or previously thawed extracts. To better understand the robustness of cell extracts over time, we assessed the activity of cell-free metabolism during storage. As a model, we studied conversion of glucose to 2,3-butanediol. We found that cell extracts from *Escherichia coli* and *Saccharomyces cerevisiae* subjected to an 18-month storage period and repeated freeze−thaw cycles retain consistent metabolic activity. This work gives users of cell-free systems a better understanding of the impacts of storage on extract behavior.



KEYWORDS: *cell-free, biocatalysis, metabolism, extract, in vitro, stability*

INTRODUCTION

Cell-free systems are derived from the soluble components of crude cell lysates (often referred to as cell extracts) that contain active biological machinery for metabolism, transcription, and translation without homeostasis or growth constraints faced by living cells. These systems have emerged as powerful tools for studying and harnessing protein and metabolite synthesis.[1−4] Applications span prototyping genetic parts or metabolic pathways,[5−14] biomanufacturing small molecules,[15−18] synthesis of proteins,[19,20] molecular diagnostics,[21−23] and educational kits.[24−29]

Cell-free gene expression has demonstrated prolonged transcription/translation activity with reactions >48 h using prokaryotic[30,31] and eukaryotic[32,33] cell extracts. Extract metabolism (including glycolysis, cofactor regeneration, and heterologous anabolic pathways) also remains active over 72 h for the synthesis of terpenes[9] and styrene.[18] Although cell extracts are preserved by ultralow temperature storage (−80 °C)[34] or lyophilized for long-term storage,[20,24,35−41] aliquots without cryo- or lyo-protectants remain capable of consistent protein synthesis yields after 5 freeze−thaw cycles.[42] However, to our knowledge, there is not clear evidence on the consistency of thawed extracts and on how long extracts can be safely stored, especially while maintaining high metabolic activity. A rigorous analysis of the impact of prolonged storage on cell-free metabolism would address these open questions.

Here, we report a long-term study of metabolic stability in cell extracts derived from *Escherichia coli* and *Saccharomyces cerevisiae* enriched with enzymes for 2,3-butanediol (BDO) synthesis from glucose. Extracts were prepared following conventional protocols[43,44] from the lysates of cells engineered for BDO synthesis,[43,45] and then cell-free biosynthesis reactions were run periodically over an 18-month storage period to track substrate, pathway intermediate, and product profiles. Cell-free metabolism was also assessed after 5 cycles of freezing and thawing the extracts. Both stability tests demonstrated robust metabolic profiles from the bacterial and yeast cell extracts, supporting the ability to use batches of cell extract for extended periods of time in prototyping, biomanufacturing, or educational applications.

RESULTS AND DISCUSSION

We assessed the metabolic stability of extracts from *E. coli* and *S. cerevisiae* enriched with enzymes for 2,3-BDO synthesis[43,44] after storage at −80 °C for up to 18 months and after 5 freeze−thaw cycles (Figure 1a). First, we expressed *alsD* and

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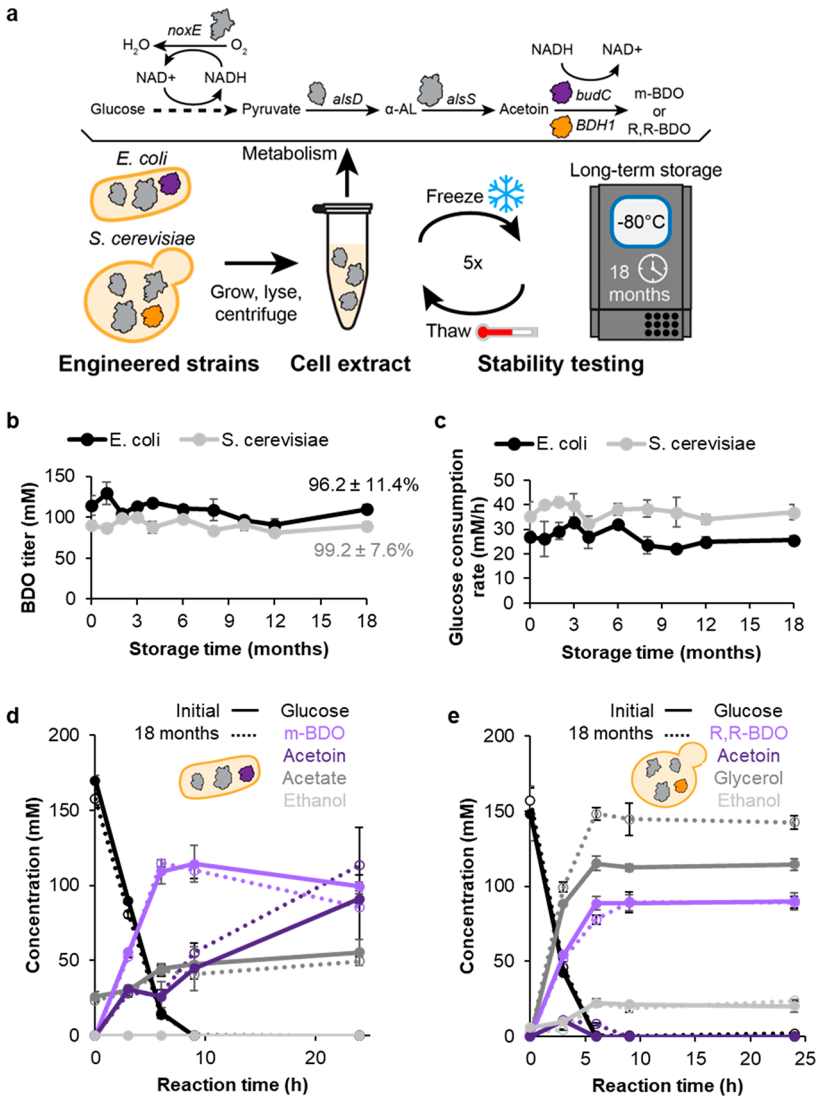

Figure 1. Cell-free metabolism remains consistent over 18 months of storage. (a) Schematic of extract preparation, cell-free metabolism, and stability testing reported here. (b) BDO titers in cell-free reactions performed over the course of an 18-month storage period. (c) Glucose consumption rates for cell-free reactions over the course of extended storage. (d) Metabolite time courses for cell-free reactions with *E. coli* extract immediately after preparation (solid) and after 18 months of storage (dotted). (e) Metabolite time courses for cell-free reactions with *S. cerevisiae* extracts before (solid) and after storage (dotted). Data represent mean ± standard deviation of 3 technical replicates.

alsS from *Bacillus subtilis* in strains of both *E. coli* and *S. cerevisiae* along with *budC* from *Klebsiella pneumoniae* producing meso-BDO in *E. coli* and *BDH1* in *S. cerevisiae* producing R,R-BDO. Additionally, the *S. cerevisiae* strain expressed *noxE* from *Lactococcus lactis* for NAD recycling. Cells were grown, harvested, and lysed to produce extracts using standard methods.[34,43,44,46] Aliquots of these extracts were flash frozen in liquid nitrogen and then stored at −80 °C for up to 18 months. At ten time points over the 18 months, extracts were thawed and used for cell-free biosynthesis of 2,3-BDO from glucose using enzymes present in the extracts (Figure 1b and c). Cell-free biosynthesis reactions were performed by adding 160 mM glucose, 1 mM NAD, 2 mM ATP, glutamate salts, and buffer to enzyme-enriched extracts and incubating reactions at 30 °C for 20 h (full materials and methods available in the Supporting Information). *E. coli*-based reactions produced 114.3 ± 12.4 mM m-BDO immediately after extract preparation, which decreased insignificantly to 110 ± 5.4 mM m-BDO after 18 months of storage, representing <4% change within error (Figure 1b). Similarly, the activity of *S. cerevisiae*-based reactions remained nearly constant with 90 ± 5.6 mM R,R-BDO synthesized by fresh extract and 89.3 ± 4.0 mM R,R-BDO synthesized by extract stored for 18 months.

We also sought to assess the impact of storage on glycolysis and the rate of BDO formation (Figure 1c). Glucose consumption rates remained comparable over the extended storage window, changing from 26.8 ± 1.2 mM/h to 25.6 ± 1.5 mM/h for *E. coli*-based reactions and 35.2 ± 6.1 mM/h to 36.9 ± 3.0 mM/h for *S. cerevisiae*-based reactions. This kinetic consistency over storage time was also observed for BDO synthesis rates, which were proportional to BDO titers (Supp. Tables S1−2). In addition, kinetic traces of key metabolites retained similar profiles after 18 months of storage for both *E. coli*-based reactions (Figure 1d) and *S. cerevisiae*-based reactions (Figure 1e). These profiles did not show significant differences at 3 months, 6 months, or 12 months of storage (Supp. Figures S1−2). *E. coli*-based reactions produced more BDO than *S. cerevisiae*-based reactions, while the *S. cerevisiae*-

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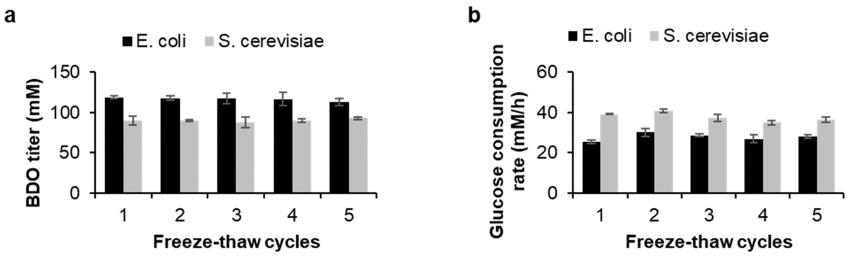

Figure 2. Cell-free metabolism remains consistent after 5 freeze−thaw cycles. BDO titers (a) and glucose consumption rates (b) for cell-free reactions after sequential thawing and refreezing. A single freeze−thaw cycle is considered standard, as cell extracts are flash-frozen in aliquots immediately after lysis and centrifugation. Data represent mean ± standard deviation of 3 technical replicates.

based reactions had higher glucose consumption rates despite containing a lower extract concentration (~6 mg total protein/mL *S. cerevisiae* reactions versus ~13 mg total protein/mL *E. coli* reactions)[43,44] (Supp. Figure S3). Polyacrylamide gel electrophoresis suggests that *E. coli* extracts have a higher proportion of BDO pathway enzymes (Supp. Figure S4).

Similar robustness in cell-free metabolism was observed after sequentially thawing extracts at room temperature and flash-freezing them 5 times (Figure 2). BDO titers averaged 116.6 ± 2.2 mM for *E. coli*-based reactions and 90 ± 1.9 mM for *S. cerevisiae*-based reactions (Figure 2a). Glucose consumption rates averaged 27.8 ± 1.8 and 37.7 ± 2.3, respectively (Figure 2b). The tight distribution of titers and rates throughout 5 freeze−thaw cycles indicates enzyme stability in crude cell extracts without exogenous cryo-protectants. However, lyophilized cell-free reactions have well-documented activity for both protein synthesis[20,24,38] and metabolism,[47] providing an additional layer of stability without the need for ultralow temperature storage.

After validating extract stability, we confirmed the metabolic activity of both *E. coli*-based and *S. cerevisiae*-based reactions after lyophilizing in tubes and on paper.[48] Lyophilized reactions exhibit similar BDO synthesis capacity after rehydration in either format (Supp. Figure S5). This method can be investigated further for the deployment of cell-free biosynthesis or larger-scale preparation of cell extracts without requiring extensive freezer space.[24,38,49]

The overall consistency of cell-free metabolism indicates that a single batch of extract can provide reliable information for pathway prototyping, enzyme characterization, and biochemical conversions over many months before a new batch is required. Variability in extract batches has been documented for cell-free protein synthesis,[50−53] which often leads researchers to utilize a single batch of extract for a given set of experiments for greater consistency between conditions. However, the metabolic dynamics of cell extracts stored for extended periods had not, to our knowledge, been well documented in prokaryotic and eukaryotic cell-free systems. The consistency between BDO synthesis reactions performed immediately after extract preparation and reactions performed 18 months later provide confidence when utilizing dated extracts. We suspect the minor deviations in metabolic profiles are due to variability in reagent lots rather than extracts. Reagent lots were not held consistent for this study to ensure extract stability was the primary variable and would not be confounded by potential reagent instability during long-term storage. Different reagent lots explained 35% of the variability in cell-free protein synthesis reactions observed in a separate study of interlaboratory consistency,[53] indicating that differences in reagent lots can be more significant than extract batches or storage time based on the present study. With these data in mind, researchers can better prepare for large-scale or collaborative studies in which cell-free reaction variability may be a consideration.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.2c00685.

Materials and Methods, Supp. Figure S1 (representative time courses from reactions with *E. coli* extract that was stored for 3 to 18 months), Supp. Figure S2 (representative time courses from reactions with *S. cerevisiae* extract that was stored for 3 to 18 months), Supp. Figure S3 (reactions require higher concentration of *E. coli* extract for complete glucose consumption), Supp. Figure S4 (SDS-PAGE comparing wildtype and enriched cell extracts), Supp. Figure S5 (lyophilized reactions retain metabolic activity), Supp. Table S1 (relative metrics for *E. coli* extract performance over 18 months), and Supp. Table S2 (relative metrics for *S. cerevisiae* extract performance over 18 months) (PDF)

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Author Contributions

B.J.R. designed the study and performed the experiments. B.J.R., A.S.K., and M.C.J. wrote the manuscript. A.S.K., H.S.A., and M.C.J. supervised the research and secured the funding.

Notes

The authors declare no competing financial interest.

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