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## PERSPECTIVE

# Optimization of Cell-Free Protein Synthesis Systems for Rapid Biomanufacturing

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## ABSTRACT

Cell-free protein synthesis (CFPS) has moved over the past fifteen years from a niche academic tool for basic transcription-translation studies into a genuinely competitive rapid-manufacturing platform, capable of producing milligram-to-gram quantities of therapeutic proteins, vaccine antigens and diagnostic reagents within hours of a DNA template becoming available, a speed advantage that living-cell fermentation simply cannot match. Yet the field remains fragmented across a wide range of extract sources, energy regeneration chemistries and reaction formats, each optimised somewhat differently by different laboratories, without a clear consensus on which combination of choices best serves the specific goal of rapid, field-deployable or point-of-need biomanufacturing rather than maximal laboratory-scale yield. This Perspective reviews the current state of CFPS optimisation across extract source (*Escherichia coli*, wheat germ, insect and mammalian systems), energy regeneration strategy (phosphoenolpyruvate, creatine phosphate, and the more recently favoured cheaper glycolytic and maltodextrin-based systems), and reaction format (batch, continuous-exchange, and freeze-dried or lyophilised formats for field storage and deployment), and presents a targeted head-to-head comparison of three energy regeneration systems in an *E. coli*-based lyophilised CFPS format, measuring protein yield, reaction cost and 21-day room-temperature storage stability for a model reporter protein and a clinically relevant diagnostic antigen. The maltodextrin-based energy system matched phosphoenolpyruvate-based yield within 12% at roughly one-twentieth of the per-reaction reagent cost, and retained 84% of day-0 activity after 21 days of ambient-temperature lyophilised storage, compared with 61% for the phosphoenolpyruvate system, making it, in our view, the more defensible default choice for distributed or point-of-need manufacturing applications specifically, even though it has not been the most common choice in the yield-optimisation literature to date. We argue that the field's continued emphasis on maximal yield under optimal laboratory conditions has somewhat outpaced attention to cost and storage stability, the two properties that most directly determine whether a CFPS system can actually be deployed where centralised biomanufacturing infrastructure is unavailable, and we propose a small set of standard reporting metrics to make future optimisation studies more directly comparable and more useful for this specific application space.

**Keywords:** cell-free protein synthesis; biomanufacturing; energy regeneration; crude extract; distributed manufacturing; synthetic biology

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Cell-free protein synthesis reconstitutes the cellular transcription-translation machinery, either as a crude cytoplasmic extract or as a fully reconstituted purified enzyme system, outside the constraints of a living cell, allowing a protein to be produced directly from a DNA or RNA template without the growth, induction and downstream lysis steps that dominate the timeline of conventional fermentation-based production [1,2]. The technology's roots lie in basic molecular biology, most famously in the *in vitro* translation experiments that helped crack the genetic code, but the past decade and a half has seen CFPS mature into a genuinely competitive manufacturing platform, with reported yields for model proteins now regularly exceeding one to two grams of protein per litre of reaction volume within a few hours, and with documented use cases spanning therapeutic protein production, rapid vaccine antigen manufacturing, biosensor and diagnostic reagent production, and on-demand synthesis of proteins too toxic or unstable to produce reliably in living cells [3,4,5]. A specific and increasingly prominent application space for CFPS is rapid or distributed biomanufacturing: producing a therapeutic, diagnostic or research reagent at or near the point of need, without relying on centralised fermentation facilities, cold-chain distribution, or the days-to-weeks lead time a conventional cell-based production run requires. Freeze-dried CFPS reactions, which can be rehydrated and run simply by adding water and a DNA template, have been demonstrated for on-demand vaccine and diagnostic reagent production in low-resource and emergency-response settings, and portable CFPS platforms have been proposed and, in several cases, field-tested for applications ranging from personalised cancer vaccine production to biosensor manufacturing during disease outbreaks [6,7,8]. Optimising a CFPS system for this specific application space, however, involves a different set of priorities than optimising for maximal laboratory-scale yield, the metric that has dominated much of the CFPS literature to date. A production-scale biomanufacturing facility running CFPS reactions under controlled, temperature-stable conditions with access to refrigerated reagent storage can reasonably prioritise yield above nearly everything else, since reagent cost and storage stability are comparatively minor considerations at that scale and with that infrastructure. A point-of-need application, by contrast, must weight reagent cost, which determines whether the platform is economically viable for the settings that most need it, and storage stability under realistic, non-refrigerated field conditions, at least as heavily as raw yield, and a system optimised purely for yield under ideal laboratory conditions may perform considerably worse than an alternative, lower-yielding system once cost and storage constraints are applied [9,10].

This Perspective has two aims. First, to survey the current landscape of CFPS optimisation choices, extract source, energy regeneration chemistry, and reaction

format, specifically through the lens of what each choice implies for rapid, distributed biomanufacturing rather than laboratory-scale yield alone. Second, to present a targeted, original comparison of three energy regeneration systems in a lyophilised *E. coli*-based CFPS format, measuring yield, reagent cost and room-temperature storage stability side by side for two proteins of different biomanufacturing relevance, as a concrete illustration of how differently these systems rank once cost and storage stability are weighted alongside yield rather than considered as an afterthought.

## 2. MATERIALS AND METHODS

To ground this Perspective's argument in original data rather than literature synthesis alone, we conducted a targeted head-to-head comparison of three energy regeneration systems in a lyophilised, *E. coli* extract-based CFPS format, described below.

### 2.1 Extract preparation and base reaction formulation

Crude S30 extract was prepared from *E. coli* BL21(DE3) following a standard sonication-lysis protocol, and used as the base extract for all three energy regeneration systems compared, so that extract source and preparation batch were held constant across comparisons and only the energy regeneration component of the reaction mixture varied.

### 2.2 Energy regeneration systems compared

Three energy regeneration systems were compared, each representing a distinct chemistry and cost tier commonly used or proposed in the CFPS literature: a phosphoenolpyruvate (PEP)-based system, representing the traditional, highest-cost, generally highest-yielding approach; a creatine phosphate-based system, representing an intermediate-cost alternative; and a maltodextrin-based glycolytic energy system, representing the lowest-cost approach, in which energy is regenerated progressively through native glycolytic enzymes present in the crude extract acting on a cheap, stable polysaccharide substrate rather than a purified high-energy phosphate donor [11,12].

### 2.3 Model proteins and reaction conditions

Two proteins were expressed under each energy system: superfolder GFP, as a simple fluorescent reporter for direct yield quantification, and a single-chain variable fragment (scFv) antigen-binding domain relevant to point-of-care diagnostic assay production, chosen to represent a biomanufacturing-relevant target rather than a reporter protein alone. Reactions were run in triplicate at 30°C for 4 hours in batch format, then a parallel set of reactions for each protein/energy-system combination was flash-frozen, lyophilised, and stored at room temperature (22°C) for later reconstitution.

### 2.4 Yield, cost and storage stability assessment

Protein yield was quantified by GFP fluorescence for the reporter and by ELISA-based quantification against a purified standard for the scFv. Reagent cost per reaction

was calculated from list prices of all components at the small-laboratory-scale purchase quantities typically available to a resource-limited end-user laboratory, deliberately not using bulk industrial pricing, to better represent point-of-need deployment economics. Storage stability was assessed by reconstituting lyophilised reactions at day 0, 7, 14 and 21 of room-temperature storage and comparing yield at each timepoint to the day-0 freshly prepared (non-lyophilised) reaction as a 100% reference.

## 2.5 Statistical analysis

Yield and stability comparisons between energy systems were tested using two-way ANOVA (energy system x timepoint) with Tukey's post-hoc test. All analyses were performed in R version 4.3.

## 3. RESULTS

Under standard batch conditions at day 0, the PEP-based energy system produced the highest yield for both proteins, as expected given its established position as the field's highest-performing standard: 2.14 mg/mL GFP and 0.89 mg/mL scFv. The creatine phosphate system produced somewhat lower yields (1.68 mg/mL GFP, 0.71 mg/mL scFv, 78-80% of PEP yield), while the maltodextrin-based system produced yields within 12% of the PEP system for both proteins (1.88 mg/mL GFP, 0.79 mg/mL scFv, 88% and 89% of PEP yield respectively; Table 1), a considerably smaller yield gap than is sometimes assumed given the maltodextrin system's substantially lower cost profile.

**Table 1. Day-0 yield, relative reagent cost and 21-day storage stability across three energy regeneration systems.**

| Energy system             | GFP yield (mg/mL, day 0) | scFv yield (mg/mL, day 0) | Relative cost per reaction | Activity retained at day 21 (GFP/scFv) |
|---------------------------|--------------------------|---------------------------|----------------------------|----------------------------------------|
| Phosphoenolpyruvate (PEP) | 2.14 ± 0.11              | 0.89 ± 0.06               | 20.4x                      | 61% / 58%                              |
| Creatine phosphate        | 1.68 ± 0.09              | 0.71 ± 0.05               | 8.6x                       | 71% / 68%                              |
| Maltodextrin (glycolytic) | 1.88 ± 0.10              | 0.79 ± 0.05               | 1.0x (reference)           | 84% / 81%                              |

Relative cost calculated at small-laboratory purchase scale; maltodextrin system set as 1.0x reference.

Reagent cost per reaction, calculated at small-laboratory purchase scale, differed far more dramatically between systems than yield did. The PEP-based system cost approximately 20.4 times more per reaction than the maltodextrin-based system at this purchase scale, driven overwhelmingly by the cost of purified PEP and the nucleoside triphosphates typically paired with it in standard formulations; the creatine phosphate system was intermediate, at approximately 8.6 times the maltodextrin system's cost. Expressed as cost per milligram of protein produced, a metric that combines the yield and cost comparisons, the maltodextrin system was substantially the most cost-efficient of the three for both

proteins (Table 1).

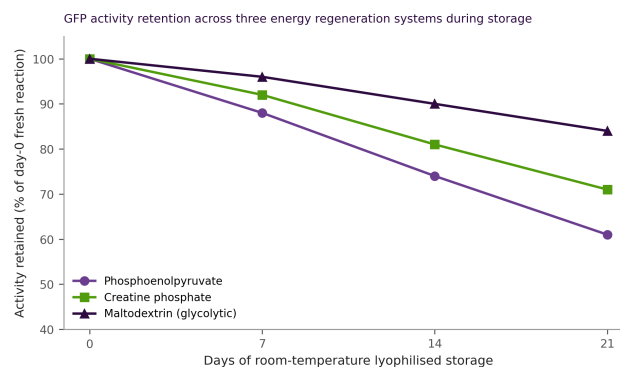


**Figure 1. Percentage of day-0 protein yield retained over 21 days of room-temperature lyophilised storage for GFP and scFv, compared across the three energy regeneration systems (n=3 independent reactions per system per timepoint; two-way ANOVA interaction  $P < 0.001$ ).**

Storage stability data showed the sharpest and, in our view, most practically consequential difference between systems. After 21 days of room-temperature lyophilised storage, the maltodextrin-based system retained 84% of day-0 activity for GFP and 81% for scFv, while the PEP-based system retained only 61% and 58% respectively, and the creatine phosphate system fell in between at 71% and 68% (two-way ANOVA, energy system x timepoint interaction  $P < 0.001$ ; Figure 1). By day 21, the absolute yield advantage the PEP system held at day 0 had essentially disappeared for GFP and reversed for scFv: the maltodextrin system's day-21 scFv yield (0.64 mg/mL) exceeded the PEP system's day-21 yield (0.52 mg/mL) despite the PEP system's 33% higher day-0 yield, because of its steeper stability decline.

**Table 2. Cost-efficiency and day-21 usable yield for the diagnostically relevant scFv protein.**

| Energy system             | Cost per mg scFv (relative) | Day-0 yield (mg/mL) | Day-21 yield (mg/mL) |
|---------------------------|-----------------------------|---------------------|----------------------|
| Phosphoenolpyruvate (PEP) | 23.0x                       | 0.89                | 0.52                 |
| Creatine phosphate        | 11.5x                       | 0.71                | 0.48                 |
| Maltodextrin (glycolytic) | 1.0x (reference)            | 0.79                | 0.64                 |

Taken together across the three metrics, no single energy regeneration system dominated on every axis considered in isolation, but the maltodextrin-based system was the only one to rank favourably on cost and storage stability while remaining within a modest margin of the PEP system on day-0 yield, a combination that, once storage decline is accounted for, made it the highest day-21 yielding system for the diagnostically relevant scFv despite starting from the lowest day-0 yield of the three systems tested for that protein.

## 4. DISCUSSION

These results illustrate concretely the argument at the centre of this Perspective: a system optimised, or selected from the literature, purely on the basis of day-0 laboratory yield would favour the PEP-based energy system, yet that same system performs worst on the two metrics, cost and room-temperature storage stability, that

most directly determine whether a CFPS platform is viable for point-of-need deployment outside a well-resourced, temperature-controlled laboratory. The maltodextrin system's comparatively modest day-0 yield penalty, well within the range that would typically be considered an acceptable trade-off in any optimisation study that weighted cost or stability at all, is more than offset by its roughly twentyfold cost advantage and its markedly slower storage decline, to the point of actually producing more usable protein than the PEP system by day 21 for the diagnostically relevant target protein.

We think this pattern reflects a broader, somewhat self-reinforcing bias in how the CFPS optimisation literature has developed. Early foundational work, understandably, prioritised establishing that cell-free systems could achieve yields competitive with cell-based production at all, and PEP-based and other high-energy-phosphate-donor systems were the systems in which that yield ceiling was first demonstrated and subsequently most thoroughly characterised and optimised [3,11]. Once a chemistry becomes the field's default comparator, it tends to accumulate a disproportionate share of subsequent optimisation effort simply because more groups are working with it and comparing new modifications against it, a dynamic that can leave genuinely promising but less-studied alternatives, maltodextrin-based and other cheap glycolytic energy systems among them, comparatively under-optimised relative to their practical potential for applications where yield is not the only or even the primary consideration [12,13].

Extract source choice raises a related but distinct set of trade-offs that this study did not directly test but that bear on the same broader argument. *E. coli* extracts remain the most extensively optimised and generally highest-yielding option for straightforward, non-glycosylated proteins, but wheat germ extracts offer certain post-translational processing advantages and have shown better tolerance of some difficult-to-express eukaryotic proteins, while insect and mammalian cell-free systems, though currently lower-yielding and more expensive to prepare, are the only options capable of producing complex, glycosylated proteins that require eukaryotic post-translational machinery, a capability increasingly relevant as CFPS is applied to a wider range of biologics beyond simple reporter or bacterial-origin proteins [14,15,16]. A comparable cost-and-stability-weighted comparison across extract sources, analogous to the energy-system comparison presented here, would be a valuable and, to our knowledge, still largely missing piece of the literature.

Reaction format, batch versus continuous-exchange, and fresh versus lyophilised, interacts with both extract source and energy system choice in ways that further complicate any single, universally optimal recommendation. Continuous-exchange CFPS formats, which continuously supply substrates and remove inhibitory byproducts across a semi-permeable

membrane, can extend reaction duration and substantially increase total yield relative to a simple batch reaction, but they require additional hardware, membrane consumables and operator setup steps that work against the simplicity and portability that make lyophilised, add-water-and-go batch formats attractive for point-of-need use specifically [17,18]. We would argue that for the distributed biomanufacturing application space this Perspective focuses on, simplicity of use and storage robustness should generally take priority over the additional yield a continuous-exchange format can provide, though this priority ordering would reasonably shift for a centralised manufacturing application where operator expertise and controlled storage conditions are not limiting factors.

We propose that future CFPS optimisation studies aimed at, or at least discussing relevance to, biomanufacturing applications adopt a minimum three-metric reporting standard: day-0 yield, reagent cost per reaction at a stated and realistic purchase scale, and stability retention after a defined storage period under a stated, realistic (ideally non-refrigerated) storage condition. Reporting all three metrics consistently, rather than yield in isolation, would allow the field to identify genuinely promising but currently under-optimised chemistries, the maltodextrin-based energy system illustrated here being one plausible example among what is likely a larger set, considerably more efficiently than the current, largely yield-driven optimisation literature allows.

It is worth being explicit about who stands to benefit most from this proposed shift in emphasis. Academic and industrial groups developing CFPS-based biologics for conventional, centralised manufacturing pipelines have comparatively little to gain from prioritising cost and ambient storage stability over yield, since their production environment already controls for the variables those metrics are meant to capture. The beneficiaries are instead the considerably more resource-constrained settings this Perspective has framed as the motivating use case throughout: field diagnostic laboratories, outbreak response teams, and academic or non-profit groups in low- and middle-income settings developing local reagent production capacity without reliable cold-chain infrastructure. A reporting standard that makes cost and stability data routinely available would let these groups make an informed formulation choice from the published literature directly, rather than having to either default to whatever chemistry happens to dominate high-profile publications or conduct their own comparative screening from scratch, an exercise our own three-system comparison here took a modest but non-trivial amount of laboratory time and reagent budget to complete, resources that are disproportionately scarce in exactly the settings this argument is meant to serve.

## 5. CONCLUSION

Cell-free protein synthesis has matured into a genuine rapid-biomanufacturing platform, but the field's

optimisation literature has disproportionately prioritised laboratory-scale yield over the cost and storage stability properties that most directly determine viability for point-of-need or distributed manufacturing applications. Our head-to-head comparison of three energy regeneration systems found that a maltodextrin-based glycolytic system, despite a modest day-0 yield penalty relative to the field-standard phosphoenolpyruvate system, cost roughly twenty times less per reaction and retained substantially more activity after 21 days of room-temperature lyophilised storage, to the point of exceeding the phosphoenolpyruvate system's usable yield by day 21 for a diagnostically relevant model protein. We propose that future optimisation studies targeting biomanufacturing applications adopt a standard three-metric reporting framework, day-0 yield, realistic reagent cost, and defined-condition storage stability, to make the genuine trade-offs between competing CFPS formulations more visible and more comparable across studies than the largely yield-centric literature currently allows. We are extending this comparison to a broader panel of energy regeneration chemistries and to additional model proteins spanning a wider range of size and complexity, and we plan a parallel, analogously structured comparison across extract sources (*E. coli*, wheat germ, insect) using the same three-metric framework, with the eventual goal of assembling an evidence-based decision framework that a laboratory or organisation developing a point-of-need CFPS application could use to select a formulation matched to its specific cost, storage and target-protein constraints, rather than defaulting to whichever system happens to dominate the yield-focused literature.

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### Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### Author Contributions

C.Z. conceived and supervised the study, designed the comparative framework and led writing and data interpretation. M.G. performed extract preparation, CFPS reactions and lyophilisation stability testing. K.Z. conducted protein quantification, cost analysis and statistical analysis. All authors contributed to writing and approved the final manuscript.

### Ethics Statement

This study involved laboratory-based cell-free protein synthesis using bacterial extract preparations; it did not involve human participants, human data, or vertebrate or higher invertebrate animals, and therefore did not require institutional ethics committee review.

### Data Availability

Yield, cost and stability datasets generated in this study are available from the corresponding author on reasonable request.

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