Biotechnology Journal
www.biotechnology-journal.com

Biotechnol. J. 2014, 9, 630–640

DOI 10.1002/biot.201300383

Research Article

Substrate replenishment and byproduct removal improve yeast cell-free protein synthesis

Jennifer A. Schoborg[1,2], C. Eric Hodgman[1,2]*, Mark J. Anderson[1,2] and Michael C. Jewett[1,2,3,4]*

[1] Department of Chemical and Biological Engineering, Northwestern University, Evanston, IL, USA
[2] Chemistry of Life Processes Institute, Northwestern University, Evanston, IL, USA
[3] Member, Robert H. Lurie Comprehensive Cancer Center, Northwestern University, Chicago, IL, USA
[4] Institute for BioNanotechnology in Medicine, Northwestern University, Chicago, IL, USA

Cell-free protein synthesis (CFPS) platforms are now considered a powerful tool for synthesizing a variety of proteins at scales from pL to 100 L with accelerated process development pipelines. We previously reported the advancement of a novel yeast-based CFPS platform. Here, we studied factors that cause termination of yeast CFPS batch reactions. Specifically, we characterized the substrate and byproduct concentrations in batch, fed-batch, and semi-continuous reaction formats through high-performance liquid chromatography (HPLC) and chemical assays. We discovered that creatine phosphate, the secondary energy substrate, and nucleoside triphosphates were rapidly degraded during batch CFPS, causing a significant drop in the reaction's energy charge (E.C.) and eventual termination of protein synthesis. As a consequence of consuming creatine phosphate, inorganic phosphate accumulated as a toxic byproduct. Additionally, we measured amino acid concentrations and found that aspartic acid was rapidly consumed. By adopting a semi-continuous reaction format, where passive diffusion enables substrate replenishment and byproduct removal, we achieved over a 70% increase in active superfolder green fluorescent protein (sfGFP) as compared with the batch system. This study identifies targets for the future improvement of the batch yeast CFPS reaction. Moreover, it outlines a detailed, generalized method to characterize and improve other CFPS platforms.

Received	06 SEP 2013
Revised	11 NOV 2013
Accepted	06 DEC 2013
Accepted article online	10 DEC 2013

Supporting information available online

Keywords: Cell-free biology · Cell-free protein synthesis · In vitro translation · Protein expression · *Saccharomyces cerevisiae*

1 Introduction

Protein production is a highly active area of research spanning applications from industrial enzyme synthesis to the production of next generation therapeutics. From its roots as a research tool designed to study translation, cell-free protein synthesis (CFPS) has emerged as one strategy for cost-effective and scalable protein production [1]. While *Escherichia coli* extract-based CFPS is now recognized as a promising disruptive technology [2], eukaryotic CFPS systems currently suffer from lower product yields, expensive reagents, smaller reaction scales, and laborious extract preparation procedures. There is a great potential payoff for the development of a eukaryotic CFPS system that can address these limitations and expand the scope of traditional manufacturing practice for proteins that are difficult to produce in vivo.

From a practical standpoint, in vivo eukaryotic protein expression systems are widely used and have shown advantages for producing recombinant proteins that are difficult to actively express in *E. coli* [3]. Similar advantages have been seen with regards to eukaryotic cell-free systems as well [4]. *Saccharomyces cerevisiae* extract is a

Correspondence: Dr. Michael C. Jewett, Department of Chemical and Biological Engineering, Northwestern University, 2145 Sheridan Road, Tech E-136, Evanston, IL 60208-3120, USA
E-mail: m-jewett@northwestern.edu

Abbreviations: CFPS, cell-free protein synthesis; **E.C.,** energy charge; **FMOC,** fluorenylmethyl chloroformate; **HEPES,** 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid; **IRES,** internal ribosome entry site; **sfGFP,** superfolder green fluorescent protein; **Tx/Tl,** transcription and translation

* These authors contributed equally to this work.

prime candidate to harness the benefits of both a eukaryotic- and microbial-based system. Similar to the *E. coli* extract platform, yeast CFPS benefits from cost-effective microbial fermentation, inexpensive and robust lysate preparation, as well as detailed knowledge from its use as an established protein production platform and model organism. Recently, our lab has developed improved extract preparation methods and optimized conditions for *S. cerevisiae* extract-based CFPS reactions capable of automated high-throughput combined transcription and translation (Tx/Tl) [5]. In addition, the rich literature with respect to developing CFPS systems has allowed us to evaluate and determine several guiding principles that we can implement for the advancement of our nascent yeast cell-free protein expression platform. Of note are the efforts pursued by the Swartz lab when significantly advancing the development of *E. coli* extract CFPS [6].

As described by Swartz [6], the most obvious focus with respect to developing a CFPS platform would initially be placed on the product (i.e., protein). However, careful consideration reveals that focusing on the substrates allows one to better understand the underpinnings of the reaction [6]. For example, the Swartz lab placed a focus on characterizing individual biochemical reactions and pathways and discovered an exhaustion of the arginine, cysteine, and tryptophan supply was initially tied to the termination of the reaction [7]. Future changes to the genome of the *E. coli* host strain were implemented to stabilize non-productive amino acid metabolism and extend cell-free reaction lifetimes [8, 9]. In a similar effort here, we aimed to characterize the amino acid, nucleotide, creatine phosphate (used as a secondary energy source), and mRNA supply during the course of the protein synthesis reaction in order to leverage this information to improve its productivity. It should not be overlooked that our approach to characterize the yeast CFPS system is applicable to any CFPS platform.

Previously, we reported active firefly luciferase yields in 2 h yeast CFPS batch reactions of 7.7 ± 0.5 μg mL^{-1} [5]. Although protein synthesis ceased after 2 h, this did not appear to be caused by a limitation of the catalytic potential of the extract itself, as demonstrated through "pre-incubation" experiments [5]. Here, we seek to understand the root cause of reaction termination. By taking advantage of the open reaction environment, a benefit of cell-free systems, straightforward measurement of the reaction conditions is possible. Through time-course assays and fed-batch reactions in combination with HPLC, chemical assays, and radioactivity incorporation assays; we have determined that the exhaustion of the nucleoside triphosphate pool, along with the accumulation of the inhibitory small molecule inorganic phosphate, are tied to termination of protein synthesis in a batch reaction.

Ultimately, our results led to the adoption of a semi-continuous reaction format where the protein synthesis yields improved and reaction duration lengthened from 8.2 ± 0.1 μg mL^{-1} superfolder green fluorescent protein (sfGFP) in a 4 h batch reaction to 17.0 ± 3.8 μg mL^{-1} sfGFP in a 10 h semi-continuous reaction. Additionally, several targets were identified that can be used as the focus of future work to improve yeast CFPS batch productivity.

2 Materials and methods

2.1 Linear DNA template

Linear DNA template encoding for sfGFP was amplified from the pY71sfGFP plasmid [10]. A two-stage polymerase chain reaction (PCR) method was adopted to amplify the sfGFP gene. Primer sequences and PCR workflow can be found in Supporting information, Table S1. Transcription was assumed to terminate via run-off of the T7 bacteriophage polymerase.

2.2 Extract preparation

Extracts were prepared by the same method developed in Hodgman and Jewett [5] with fermentation for cellular growth, high-pressure homogenization for cell lysis, and dialysis for buffer exchange. *S. cerevisiae* strain MBS was used as the source strain for extract preparation [11].

2.3 Cell-free protein synthesis batch reaction

CFPS batch reactions were carried out according to the method for combined Tx/Tl reactions in Hodgman and Jewett [5] with the following working concentrations: 22 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES)–KOH pH 7.4, 120 mM potassium glutamate, 4.5 mM magnesium glutamate, 1.5 mM each of adenosine triphosphate (ATP), guanosine triphosphate (GTP), cytidine triphosphate (CTP), and uridine triphosphate (UTP), 0.08 mM each of 20 amino acids, 25 mM creatine phosphate, 1.7 mM dithiothreitol (DTT), 1 mM putrescine, 0.5 mM spermidine, 11% v/v glycerol, 0.27 mg mL^{-1} creatine phosphokinase (from rabbit muscle; Sigma–Aldrich, St. Louis, MO), 0.027 mg mL^{-1} T7 RNA polymerase (made in house following the protocol developed by Swartz et al. [12]), 6.67 μg mL^{-1} Ω-sfGFP-A_{50} PCR amplified DNA, and 50% v/v yeast extract. The concentration of proteins in the yeast extract was 18.2 ± 0.9 mg mL^{-1}, as determined by Bradford Assay using commercially available assay reagents (Bio-Rad, Hercules, CA) compared to a bovine serum albumin protein standard. All other reagents were purchased from Sigma–Aldrich unless otherwise noted.

2.4 sfGFP assay

The amount of active sfGFP produced was determined by adding 2 μL of CFPS sample to 100 μL of sfGFP assay buffer (100 mM HEPES–KOH pH 7.6, 14 mM magnesium

acetate, 60 mM potassium acetate) in a black 96-well half-area plate. Samples were allowed to incubate in the assay buffer for 1 h before measuring total fluorescence to allow for complete protein folding during the time course assays. The total fluorescence was read using a BioTek Synergy 2 plate reader (Winooski, VT) with excitation/emission of 485/528 nm. Relative fluorescent units (RFUs) were recorded for each cell-free reaction. RFUs were compared to a linear standard curve made in house by expressing ^{14}C-leucine labeled sfGFP in an *E. coli* PANOx CFPS reaction and relating trichloroacetic acid (TCA) precipitable soluble protein yield to RFUs [13].

2.5 Fed-batch CFPS reactions

For fed-batch reactions, 15 μL CFPS batch reactions were prepared as described above. At the specified time, the reactions were removed from the incubator, supplied with 1 μL of feeding solution containing the appropriate concentration of the desired substrate(s), mixed with a pipette, and returned to the incubator. All reactions were incubated at 21°C for 8 h total and assayed using the sfGFP assay.

2.6 Semi-continuous CFPS reactions

For semi-continuous CFPS reactions, a 10 mL beaker with 2 mL of dialysate was placed on a stir plate with a stir bar. A membrane tube (1 kDa MWCO Tube-O-Dialyzer; G-Biosciences, St. Louis, MO) was placed in the foam support with the membrane cap facing downward in the beaker such that the membrane contacted the surface of the dialysate with minimal submersion. The non-membrane end of the tube was removed for adding the reaction mixture and sampling. The beaker was covered with aluminum foil when not sampling. Initial reaction volumes totaled 100 μL, with the same composition as the batch reactions. The dialysate imitated the final reaction mixture: 37 mM HEPES–KOH pH 7.4, 170 mM potassium glutamate, 5.5 mM magnesium glutamate, 1.5 mM each of ATP, GTP, CTP, and UTP, 0.08 mM each of 20 amino acids, 25 mM creatine phosphate, 1.9 mM DTT, 1 mM putrescine, 0.5 mM spermidine, 11.0% glycerol, 0.05 mM phenylmethanesulfonyl fluoride (PMSF), and 115 mM mannitol. Differences in concentrations between the batch reaction mixture and the dialysate are due to contributions from the lysis buffer in the reaction. The reactions occurred at room temperature (23–24°C) and were compared to a 100 μL batch reaction occurring under the same conditions. At 0, 0.5, 1, 2, 3, 4, 6, 8, and 10 h of incubation time, 5 μL samples were removed from the batch and semi-continuous reactions, frozen on liquid nitrogen to quench the reaction, and later thawed and assayed concurrently. The remainder of the reaction mixture after 10 h of incubation was saved for small molecule assays and stored at –80°C until use.

2.7 Creatine and inorganic phosphate quantitative assays

The quantitative analysis of creatine was performed using the EnzyChrom™ Creatine Assay Kit (BioAssay Systems, Hayward, CA). The quantitative analysis of inorganic phosphate was performed using the EnzChek® Phosphate Assay Kit (Life Technologies, Carlsbad, CA). For each kit, the CFPS samples were frozen on liquid nitrogen to quench the reaction and later thawed and assayed concurrently. To measure creatine concentration, samples were diluted 30-fold with water and compared to a linear creatine standard ranging from 0 to 1 mM. Absorbance was measured at 570 nm, following manufacturer's instructions with 100 μL reactions in a 96-well plate. To measure phosphate concentration, samples were diluted 500-fold with water and compared to a linear phosphate standard ranging from 0 to 150 μM. Absorbance was read at 360 nm following manufacturer's instructions.

2.8 Nucleotide and amino acid concentration quantitative HPLC assay

HPLC analysis was used to measure nucleotide and amino acid concentrations. For both assays, 5% v/v TCA was added to the cell-free reaction mixture in a 1:1 volumetric ratio. Samples were centrifuged at 12 000 g for 15 min at 4°C. The supernatant was collected and samples were analyzed using an Agilent 1260 series HPLC system (Agilent, Santa Clara, CA).

For amino acid analysis, a ZORBAX Eclipse Plus column (4.6 mm × 100 mm, 1.8 μm particle size; Agilent) was used with a Rapid Resolution HT derivitization method using *o*-phthalaldehyde (OPA) and fluorenylmethyl chloroformate (FMOC). Separation was carried out at a flow rate of 1.0 mL min^{-1} for 20 min. Mobile phase A contained 10 mM sodium borate, 10 mM sodium phosphate dibasic, and 5 mM sodium azide (pH 8.2 with HCl) and mobile phase B contained acetonitrile:methanol:water in a 45:45:10 volumetric ratio. The buffer gradient for B was as follows: 0 min, 2%; 0.35 min, 2%; 16.4 min, 57%; 16.5 min, 100%; 17.7 min, 100%; 17.8 min, 2%; 20 min, end. Amino acids were detected at 262 and 338 nm. Amino acid concentrations were determined by comparison to a standard calibration.

For nucleotide analysis, a BioBasic AX column (4.6 mm × 150 mm 5 μm particle size; Thermo Scientific, West Palm Beach, FL) was used for analysis. Separation was carried out at a flow rate of 0.75 mL min^{-1}. The method started with a mobile phase of 100% 5 mM Na_2HPO_4 (mobile phase A) and 0% 750 mM Na_2HPO_4 (mobile phase B), both adjusted to pH 3.2 with phosphoric acid. The buffer gradient for B was as follows: 0 min, 0%; 10 min, 40%; 40 min, 80%; 45 min, 100%; 47 min, 0%; 50 min, end. Nucleotides were detected at 254 nm.

Nucleotide concentrations were determined by comparison to a standard calibration.

2.9 Denaturing RNA gel electrophoresis

The denaturing RNA gel was prepared with 1.5% agarose containing 2.2 M formaldehyde and 2× GelRed in 1× MOPS Buffer (2 M 3-(*N*-morpholino) propanesulfonic acid (MOPS) pH 7.0, 20 mM sodium acetate, 10 mM EDTA pH 8.0) under RNase free conditions to a final volume of 50 mL. CFPS samples were quenched by placing on ice and supplying with 1 μL Qiagen RNase Inhibitor (Qiagen, Valencia, CA) per 15 μL reaction. Samples were immediately purified using Qiagen RNeasy MinElute Cleanup Kit (Qiagen). Total RNA was quantified using ThermoScientific NanoDrop 2000c (ThermoScientific, Waltham, MA). Twenty micrograms of total RNA, the bulk of which is ribosomal RNA, was mixed with 1× denaturing mix (1.3× loading buffer, 1.3× MOPS, 42% v/v deionized formamide, and 8.2% v/v formaldehyde) and adjusted to a final volume of 20 μL. Samples were heated to 70°C for 10 min to denature any secondary structure, after which they were immediately placed on ice for 5 min. In addition, the denaturing gel was supplied with 100 V for 10 min before loading the RNA samples. After loading the RNA samples, the gel was supplied with 60 V for 2 h. The buffer was recirculated every 30 min to prevent ion/pH gradients from forming. The gel was imaged with BioRad GelDoc XR+ (BioRad, Hercules, CA) for GelRed staining.

3 Results

3.1 Characterizing the batch reaction

To initially assess the CFPS batch reaction, we carried out a 15 μL combined Tx/Tl reaction for 8 h at 21°C. Samples were taken at 0, 0.5, 1, 2, 3, 4, 6, and 8 h to profile sfGFP production during the course of the reaction. From Fig. 1A, we can see the synthesis of sfGFP shows a slight lag during the first 0.5 h and then progresses linearly from 0.5 to 2 h at a rate of 4.06 ± 0.15 μg sfGFP h^{-1}. Between 2 and 4 h sfGFP synthesis slows and after 4 h minimal additional sfGFP is synthesized.

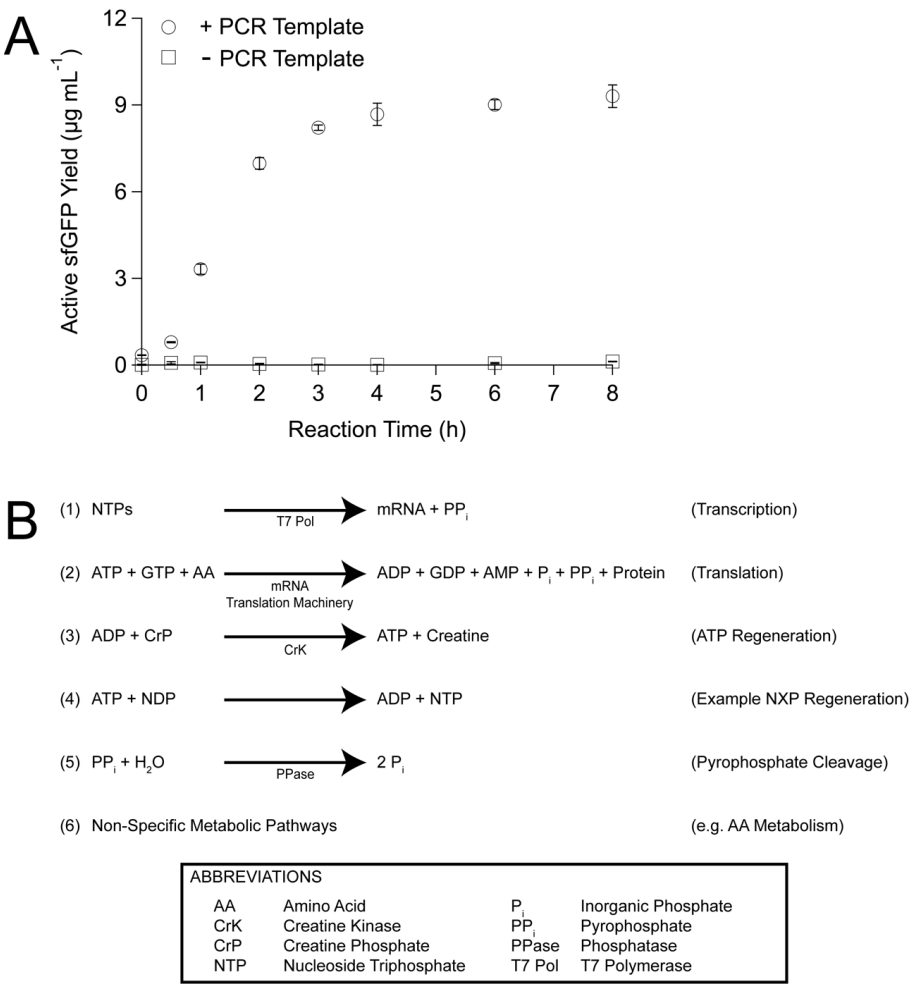


Figure 1. Time course analysis of protein yield for a batch yeast CFPS reaction. (**A**) Active sfGFP synthesis is shown over the course of a standard batch reaction incubated at 21°C. Fifteen μL batch reactions were prepared in separate 1.5 mL tubes and sampled for active sfGFP yield at the appropriate time points and measured using fluorescence intensity. Reactions were supplied with (circles) or without (squares) sfGFP-encoding PCR template. Values show means with error bars representing the standard deviations of three independent experiments. (**B**) Outline of metabolic activity within a yeast cell-free protein synthesis reaction.

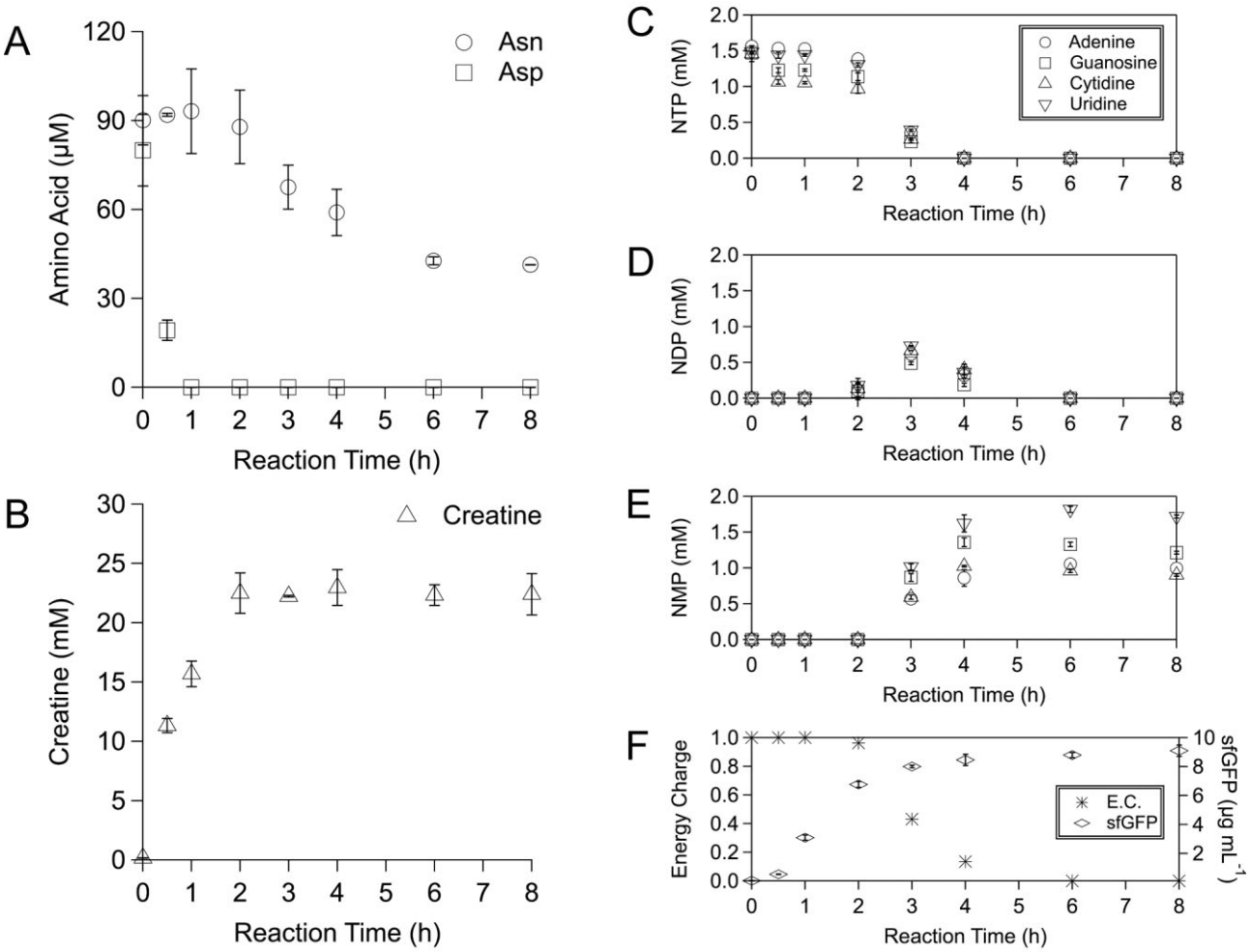


Figure 2. Time course analysis of amino acid, creatine, and nucleotide concentrations with energy charge during a batch yeast CFPS reaction. Fifteen μL batch reactions were prepared in separate 1.5 mL tubes and sampled for active sfGFP yield at the appropriate time points. (**A**) Concentrations of select amino acid species were measured using HPLC. (**B**) Creatine was measured using a chemical assay. (**C**) Nucleoside triphosphate, (**D**) diphosphate, and (**E**) monophosphate concentrations were measured by HPLC. Values show means with error bars representing the standard deviations of data pooled from three independent experiments. (**F**) Energy charge (E.C.; left axis) and sfGFP (right axis) are plotted against the reaction time. sfGFP yields were measured using fluorescence intensity.

In addition to measuring active sfGFP, each sample was assayed for concentration of amino acids, nucleotides, creatine, phosphate, and mRNA in order to characterize the reaction environment during the course of the cell-free reaction. A metabolism diagram that summarizes the major substrate flows within the yeast CFPS reaction is shown in Fig. 1B. HPLC analysis of 19 amino acids (Supporting information, Fig. S1; glutamate was excluded from the analysis due a concentration of >100 mM), show that aspartic acid and asparagine fall below the exogenously supplied amount (80 μM) within 3 h (Fig. 2A). In fact, no detectable aspartic acid is measured after 1 h reaction time, suggesting a possible amino acid limitation (Fig. 2A).

We next measured consumption of creatine phosphate, the secondary energy substrate. Because creatine is not part of a natural biosynthetic pathway in yeast, we are able to measure consumption of creatine phosphate by measuring the synthesis of creatine (Fig. 2B). A plateau in creatine synthesis occurs after 2 h, which implies the creatine phosphate supply has been exhausted. Once creatine phosphate has been depleted, it is reasonable to assume that ATP can no longer be regenerated.

Quantitative HPLC analysis of the nucleotide pool also shows fluctuation of each species over time. Nucleoside triphosphate (NTP) concentrations stay relatively stable around 1.5 mM each for the first 2 h of the reaction (Fig. 2C). This timing corresponds with the exhaustion of the creatine phosphate supply. As NTPs are consumed, nucleoside diphosphates (NDPs) and nucleoside monophosphates (NMPs) are accumulated sequentially (Fig. 2D–E). NDPs do not accumulate above 0.75 mM each

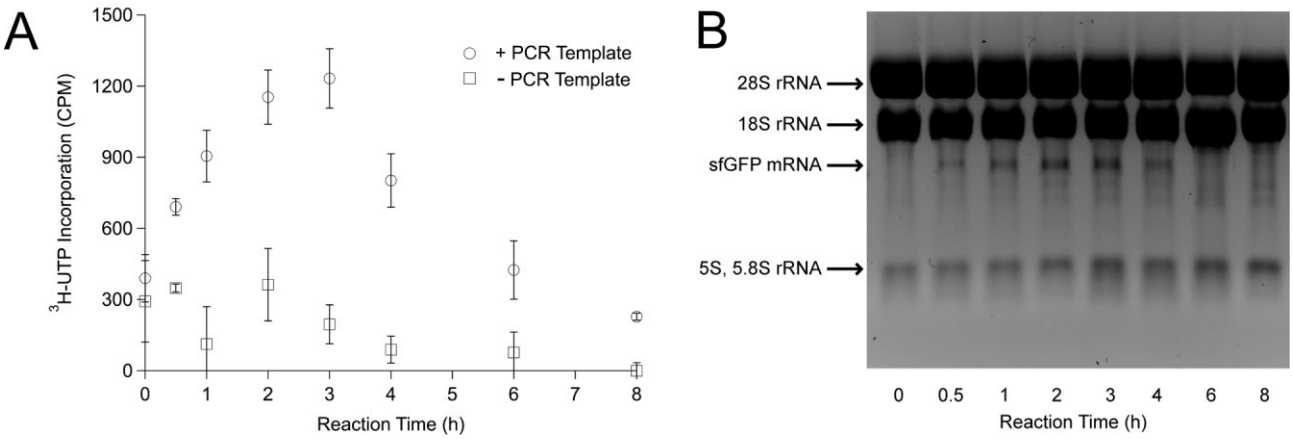


Figure 3. Time course analysis of mRNA synthesis during a batch yeast CFPS reaction. (**A**) Synthesized mRNA is shown using tritiated UTP incorporation over the course of a standard batch CFPS reaction. Fifteen µL batch reactions were prepared in separate 1.5 mL tubes supplied with 0.5 µL ^{3}H-UTP and sampled at the appropriate time points. The reaction was supplied with and without sfGFP-encoding PCR template. Synthesized mRNA was quantified via scintillation intensity. Values show means with error bars representing the standard deviations of four independent experiments. (**B**) mRNA was measured via denaturing gel electrophoresis over the course of a standard batch CFPS reaction. Twenty micrograms of purified RNA was added to each lane. A gel image without DNA added to the reaction is supplied as Supporting information, Fig. 2. This experiment was replicated twice with similar results.

and are quickly converted to their monophosphate form, with the concentration of NMPs stabilizing shortly after 4 h. Evaluating the Energy Charge (E.C.), which is defined by Atkinson as the overall status of energy availability in the system (Eq. 1), allows us to hypothesize when energy in the system becomes limiting [14].

$$\text{E.C.} = \frac{[\text{ATP}] + \frac{1}{2}[\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]} \quad (1)$$

It has been reported that in vivo E.C. measurements below 0.8 indicate an energy limitation [15]. This E.C. threshold is reached in our CFPS system between 2 and 3 h (Fig. 2F).

Lastly, we investigated the synthesis and degradation of mRNA encoding for sfGFP during the course of the CFPS reaction. Figure 3 shows that sfGFP mRNA is synthesized between 0 and 2 h, maintains a relatively constant concentration from 2 to 3 h, and is degraded after 4 h. These results agree with the exhaustion of NTP supply, which would limit transcription, between 2 and 4 h of the reaction. Because protein synthesis terminates and slows prior to 4 h, the timing of sfGFP mRNA degradation suggests that the mRNA template is not a limiting substrate during batch CFPS reactions.

3.2 Fed-batch experiments

From the information gained while characterizing the batch reaction, we designed fed-batch experiments to feed the substrates that were determined to be limiting over the course of the reaction: aspartic acid and creatine phosphate. The feed times of 0.5, 1.0, and 1.5 h were chosen based on the time of depletion of aspartic acid (Fig. 2A) and the consumption of creatine phosphate (Fig. 2B). Aspartic acid and creatine phosphate additions were performed individually at 0.5×, 1.0×, and 1.5× the initial concentrations (Fig. 4A and B, respectively) and combined at 1.0× initial concentration (Fig. 4C). The intent was to feed the potentially limiting substrates prior to their depletion, so that protein synthesis could be sustained, as opposed to restarted. As can be seen in Fig. 4A–C, neither feed time nor feed supplement affected active sfGFP yield.

The lack of any positive effects on protein synthesis from the fed-batch reactions indicates that substrate depletion is not the only cause of reaction termination. Inorganic phosphate has been previously characterized as a toxic molecule byproduct during CFPS reactions because it sequesters magnesium [7]. Furthermore, we would expect phosphate accumulation during CFPS with creatine phosphate used as a secondary energy source. Here, a phosphate is donated from creatine phosphate to ADP to regenerate ATP. However, once the phosphate bond is cleaved from an ATP-dependent reaction, the phosphate is unable to be recycled and thus accumulates in the system.

To evaluate the effect of phosphate on the system, potassium phosphate was added to the system at the start of the reaction in known concentrations (Fig. 4D). With as little as 30 mM additional phosphate added to the system, sfGFP production is reduced by 50%. Similarly we see the accumulation of additional 30 mM phosphate within 3 h of the reaction (Fig. 4E). To offset inorganic phosphate accumulation, one established strategy is to include magnesium in a fed-batch reaction [7]. However,

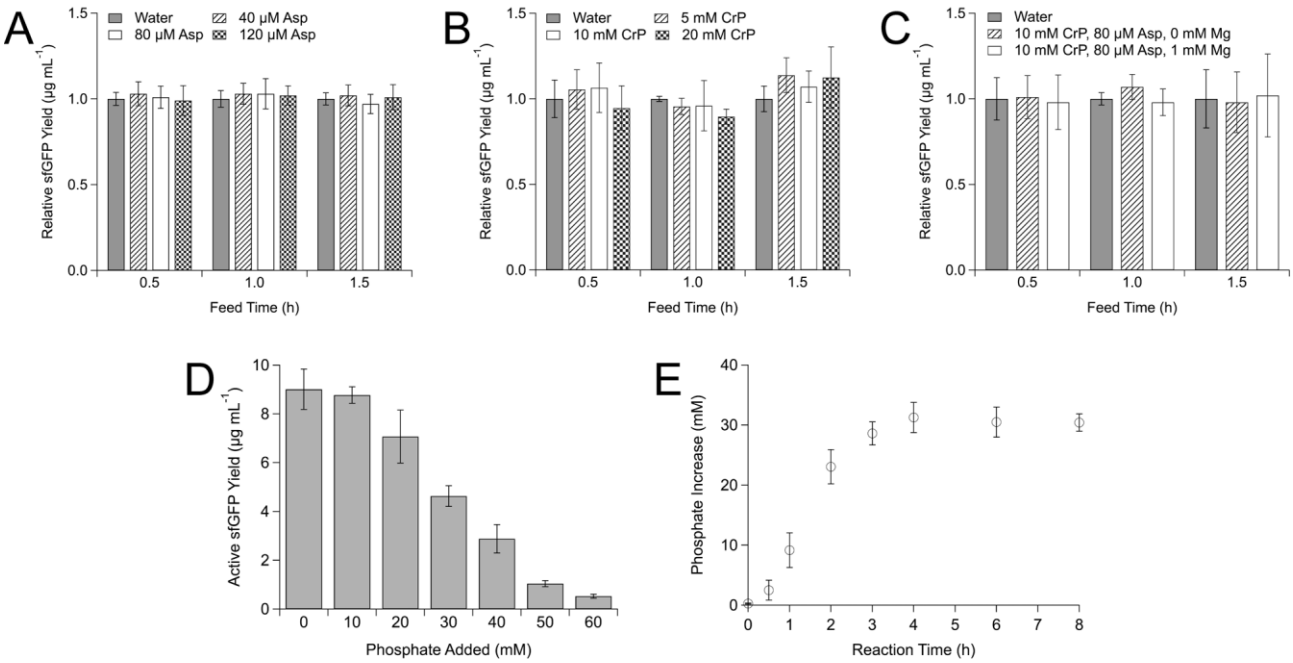


Figure 4. Effects of feeding limiting substrates and accumulating toxic byproducts during a cell-free protein synthesis reaction. Fifteen μL fed-batch reactions were prepared in separate 1.5 mL tubes. After 0.5, 1.0, or 1.5 h of incubation, 1 μL of feeding solution was added to each of the reactions, in order to feed (**A**) aspartic acid, (**B**) creatine phosphate, or (**C**) both with additional magnesium glutamate supplied at the specified concentration. Active sfGFP was quantified via fluorescence intensity after 8 h of incubation. (**D**) Additional phosphate was added to 15 μL batch reactions at time 0 h in the form of potassium phosphate (pH 7.4), ranging from 0 to 60 mM. The additional potassium was offset by a reduction in potassium glutamate added to the reaction. (**E**) Phosphate concentration was measured using a enzymatic assay and analyzed over the course of a standard batch CFPS reaction. Values show means with error bars representing the standard deviations of data pooled from three independent experiments.

this too showed no benefit in overall yield as can be seen in Fig. 4C. Despite the addition of magnesium and limiting substrates, it is possible that inorganic phosphate accumulation remains a factor preventing the anticipated increase with respect to protein production in fed-batch reactions.

3.3 Semi-continuous exchange experiments

To allow for limiting substrate replenishment and byproduct removal, we employed a semi-continuous exchange method, seen in Fig. 5A. The 1 kDa molecular weight cut off (MWCO) semipermeable membrane allows for passive diffusion of small molecules between the reaction mixture inside the membrane tube and the dialysate, while keeping the enzymes and translation machinery contained within the reaction. While these experiments were performed for 100 μL reaction volumes, the increase in reaction volume has no effect on sfGFP yield (Supporting information, Fig. S3). When samples of the batch and semi-continuous reactions at time points of 0, 0.5, 1, 2, 3, 4, 6, 8, and 10 h were assayed for active sfGFP (Fig. 5B), the semi-continuous method led to a 70% improvement in protein synthesis yields over the batch reaction, producing 17.0 ± 3.8 μg mL^{-1}. In addition, the NTP measurements clearly show that, compared to the batch reaction where NTPs are completely depleted by 4 h, the semi-continuous method is able to sustain the NTP pool above 1 mM up to 10 h (Fig. 5C). Similarly, Fig. 5D shows that the semi-continuous reaction maintains reduced concentrations of both creatine and phosphate compared to the batch reaction. Finally, Fig. 5E also shows that the aspartic acid is no longer limiting in the semi-continuous reaction, despite its depletion in the batch reaction. In addition, Supporting information, Fig. S4 shows that the other amino acids – excluding arginine, which is synthesized in the semi-continuous reaction – are at the same concentration in the semi-continuous reaction and the dialysate, indicating good diffusion across the membrane. These results demonstrate that the exchange of small molecules, including limiting substrates and toxic byproducts, does indeed prolong the reaction length, leading to a higher protein yield.

4 Discussion

Eukaryotic CFPS platforms are known to experience limited batch synthesis yields, in part due to short protein synthesis timeframes. By characterizing the cell-free

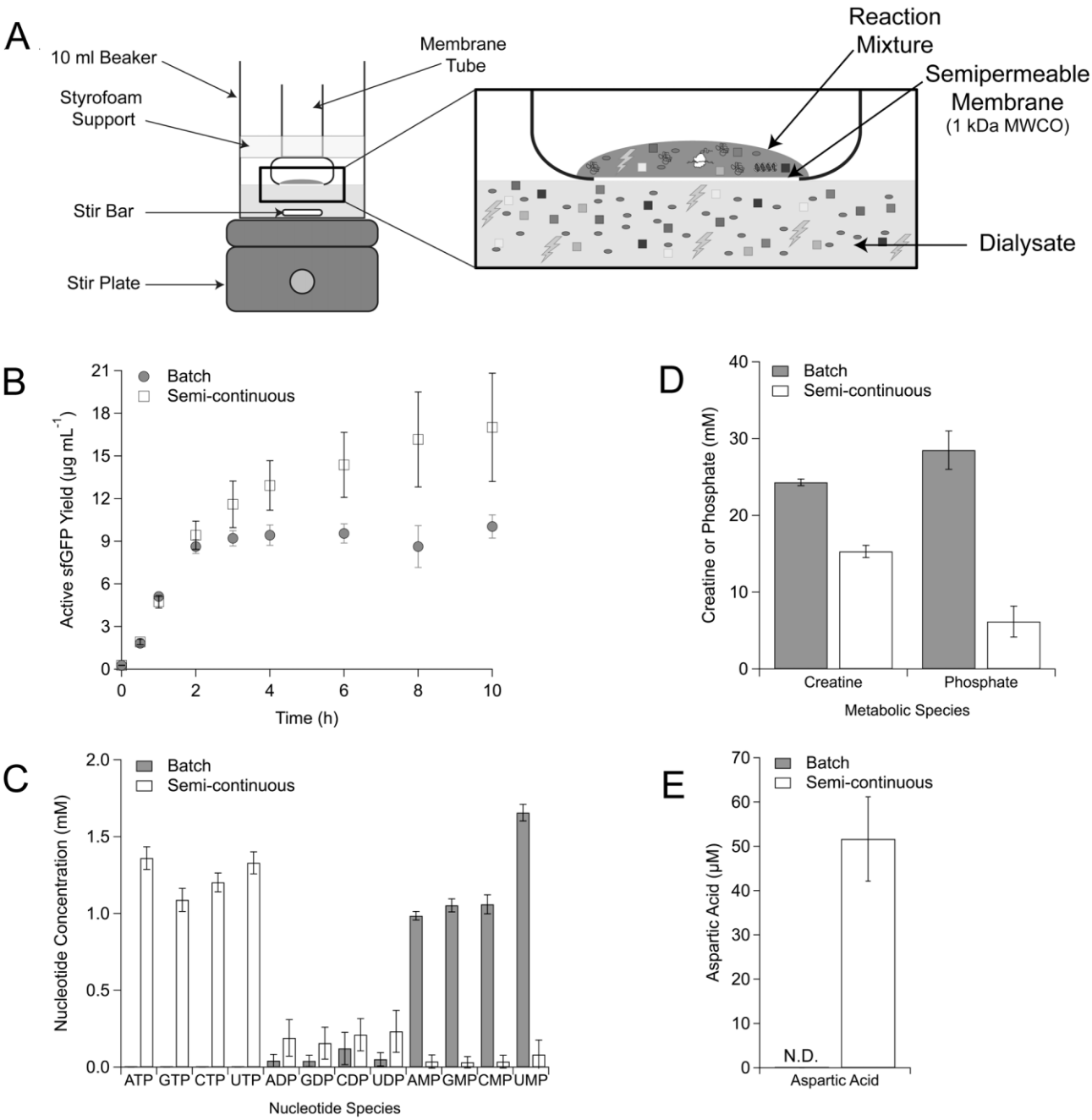


Figure 5. Semi-continuous exchange reaction. (**A**) A schematic of the experimental set-up for the semi-continuous exchange reactions. (**B**) sfGFP synthesis is shown over the course of a semi-continuous CFPS reaction. Reactions were sampled at the appropriate time points to measure active sfGFP yield using fluorescence intensity. (**C**) Endpoint nucleotide measurements after a 10 h semi-continuous reaction using HPLC. (**D**) Endpoint creatine and phosphate measurements after a 10 h semi-continuous reaction using chemical assays. (**E**) Endpoint aspartic acid measurements after a 10 h semi-continuous reaction using HPLC. N.D., none detected. Values show means with error bars representing the standard deviations of data pooled from at least three independent experiments.

reaction environment using a systematic approach, we have uncovered substrate limitations (specifically ATP) that limit protein synthesis during a yeast CFPS batch reaction, as well as outlined a path for characterizing other cell-free platforms. The study began by running a time course analysis of batch CFPS reactions characterizing synthesized protein, small molecules of interest, and mRNA. The time course analysis for protein synthesis revealed that sfGFP synthesis slows dramatically between 2–4 h of reaction time (Fig. 1A).

Energy limitations were identified as the key factor contributing to reaction termination. We found that protein synthesis cessation correlates closely with both ATP depletion and E.C. drop (Figs. 1A and 2C–F). This is similar to previous work in *E. coli*-based CFPS systems [7, 16]. Additionally, we observed that creatine concentration plateaus around the 2 h reaction mark (Fig. 2B), suggesting an exhaustion of the creatine phosphate secondary energy supply at the same time. Not surprisingly, NTP species are stable for the first 2 h of reaction (≈1.5 mM) and then are noticeably consumed between 2 and 3 h (≈0.3 mM each at 3 h) after the exhaustion of creatine phosphate. Concurrently, the E.C. of the system drops below the energy-limiting value of 0.8 from 1.0 to 0.4. The cascading result continues with the synthesis and consumption of NDPs as well as synthesis of NMPs between 2 and 4 h. Further, mRNA synthesis slows and stops between 2 and 3 h and begins degrading thereafter, coinciding with the depletion of NTPs (Fig. 3). Because mRNA concentration remains significantly non-zero up to 4 h of reaction time, while protein synthesis stops closer to 3 h, it seems unlikely that mRNA is a limiting substrate in yeast CFPS batch reactions. The most likely explanation for reaction termination, therefore, is the depletion of the primary energy source (ATP; fully consumed around 3 h), which is strongly correlated to the depletion of the secondary energy source (creatine phosphate; fully consumed by 2 h).

While energy substrates are clearly limiting, amino acid supply does not appear to be an issue. Amino acid limitations have been previously determined to be important factors in limiting CFPS reactions in *E. coli*-based CFPS [8, 9]. Interestingly, our results show that only aspartic acid and asparagine concentrations are significantly non-productively consumed. However, it is a curious result that aspartic acid concentration drops below the detection limit of our HPLC method after 1 h reaction time while protein synthesis continues for at least an additional 2 h (sfGFP contains 17 aspartic acid residues). Further investigation is necessary to explain this result.

If energy substrate limitations are indeed the cause of CFPS batch reaction termination, obvious solutions would be to adopt fed-batch or semi-continuous reaction formats to replenish creatine phosphate supply. Interestingly, we found that expectations were only met when adopting the semi-continuous approach (increasing active sfGFP yield 70%; Fig. 5) and not for fed-batch reactions (no change in sfGFP yield; Fig. 4A–C). This is counter to previous work in *E. coli*-based systems [7, 17]. This result suggests the reaction is more complicated than initially thought. One potential reason why the fed-batch approach for feeding additional creatine phosphate was not beneficial is due to the synthesis of the inhibitory toxic byproduct, inorganic phosphate (Fig. 4D–E), which has been previously shown to inhibit other CFPS reactions as well [7]. In contrast, the semi-continuous reaction had clear diffusion of small molecules between the reaction mixture and dialysate that allowed (i) the reaction to retain high concentrations of NTPs, which were demonstrated to be correlated with reaction termination, (ii) higher concentration of aspartic acid, and (iii) reduced concentrations of inorganic phosphate (Fig. 5C–E), compared to the batch reaction.

Ultimately, we are concerned with maximizing protein synthesis in a batch reaction, which is the most economical and simple high-throughput reaction format [1]. By designing a study to characterize the small molecules in a batch reaction, we have determined that reaction termination is correlated with a drop in NTP supply and E.C.. However, reaction termination is unlikely attributed to a single cause. Beyond the scope of this study there are other possibilities that could be associated with CFPS reaction termination. For example, phosphorylation of the α-subunit of eIF2 has been shown to limit extended eukaryotic CFPS reactions by blocking translation initiation in hybridoma- [18], HeLa- [19], and mouse embryo fibroblast- [20] based extracts. This notion could also partially explain the lack of additional synthesized proteins in a fed-batch reaction as well as the reduced protein synthesis rate seen in the semi-continuous reaction after 3 h.

With batch yields on the order of 10 $\mu g\ mL^{-1}$, yeast CFPS is on par with other eukaryotic cell-free platforms under development. For example, the commonly used wheat germ extract and rabbit reticulocyte lysate CFPS kits available from Promega (Madison, WI) each yield 1–10 $\mu g\ mL^{-1}$ in combined Tx/Tl batch reactions. Additionally, insect and CHO cell extracts recently published by the Kubick lab have reported batch Tx/Tl yields of 14 and 49 $\mu g\ mL^{-1}$, respectively [21]. However, major advantages of our platform include technically simple and inexpensive extract preparation methods [5] as well as low batch-to-batch variability with a variance of 1.21 $\mu g^2\ mL^{-2}$ among three individually prepared extracts.

To continue to improve yeast CFPS yields, several strategies will likely need to be employed. One such strategy might involve genome engineering, which was successful for the improvement of *E. coli*-based CFPS. The idea here is to engineer the source strain for improved extract performance. For example, successful gene deletions for *E. coli*-based extracts have been implemented to stabilize amino acid degradation pathways [8, 9], inhibit protein degradation [22, 23], and promote disulfide bond formation [24]. Amino acid biosynthetic pathways are clearly active in yeast CFPS reactions (Supporting information, Fig. S1) and could be potential genome engineering targets. One advantage of starting with *S. cerevisiae* as our source for eukaryotic-based extract is the wealth of information associated with this organism, including a fully sequenced genome.

A second strategy would involve template engineering. Although the tobacco mosaic virus Ω sequence has been shown to be effective at promoting translation initi-

ation in CFPS [25], it is unclear if there are other alternatives that might offer improved CFPS yields. For example, the Ω sequence is thought to recruit the translation initiation complex eukaryotic initiation factor 4F, which is related mechanistically to cap-dependent translation [26]. Alternatively, several eukaryotic CFPS platforms employ the use of internal ribosome entry sites (IRES) to initiate cap-independent translation including those derived from the picornavirus *Dicistroviriadae* family of intergenic region (IGR) IRES [27–29]. Furthermore, the use of IGR IRES sequences bypass eukaryotic initiation factor 2α phosphorylation regulation [28]. An additional alternative for CFPS 5′ untranslated region of the DNA template would be the use of species-independent translational sequences [30].

A final strategy used to improve *E. coli* CFPS has involved activating alternative energy pathways that do not depend on supplementing the reaction with high-energy phosphate compounds. This strategy can be used to both extend CFPS reaction lifetime and prevent phosphate accumulation. Examples of this include fueling the reaction with dextrins and polymeric carbohydrates [31, 32] or a hybrid system that uses both creatine phosphate and glucose [33]. Adopting one of these techniques could limit phosphate production during yeast CFPS reactions, thus extending the reaction lifetime. An added benefit would be the removal of dependence on the costly secondary energy source, thus improving the economics associated with yeast CFPS.

In summary, we designed a systematic approach intended to characterize the yeast-based CFPS reaction previously developed. Although our focus was on characterizing yeast-based CFPS reactions, this study could easily be adapted to studying any CFPS platform of interest. By evaluating biochemical reactions, we determined that the source of early reaction termination results from a cascade of events resulting from loss of available energy sources. We went on to show that a semi-continuous reaction format could alleviate these limitations to realize a ≈70% increase in active protein synthesis and information gained can now be leveraged to improve batch reaction performance. Moving forward, several strategies have been identified that we expect to continue to improve the emerging yeast CFPS platform.

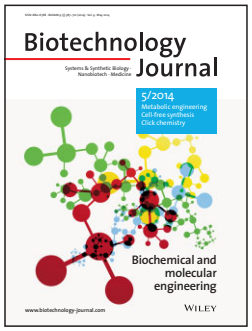
The authors would like to thank Dr. Brad Bundy and Dr. James R. Swartz for supplying the pY71sfGFP plasmid used in this study. We acknowledge Northwestern University and the DARPA Biomedicines on Demand program (N66001-13-C-4024) for support. J.A.S. was supported by the National Science Foundation Graduate Research Fellowship, grant number DGE-1324585.

The authors declare no financial or commercial conflict of interest.

5 References

[1] Zawada, J. F., Yin, G., Steiner, A. R., Yang, J. et al., Microscale to manufacturing scale-up of cell-free cytokine production – a new approach for shortening protein production development timelines. *Biotechnol. Bioeng.* 2011, *108*, 1570–1578.
[2] Carlson, E. D., Gan, R., Hodgman, C. E., Jewett, M. C., Cell-free protein synthesis: Applications come of age. *Biotechnol. Adv.* 2012, *30*, 1185–1194.
[3] Yin, J., Li, G., Ren, X., Herrler, G., Select what you need: A comparative evaluation of the advantages and limitations of frequently used expression systems for foreign genes. *J. Biotechnol.* 2007, *127*, 335–347.
[4] Goshima, N., Kawamura, Y., Fukumoto, A., Miura, A. et al., Human protein factory for converting the transcriptome into an in vitro-expressed proteome. *Nat. Methods* 2008, *5*, 1011–1017.
[5] Hodgman, C. E., Jewett, M. C., Optimized extract preparation methods and reaction conditions for improved yeast cell-free protein synthesis. *Biotechnol. Bioeng.* 2013, *110*, 2643–2654.
[6] Swartz, J., Developing cell-free biology for industrial applications. *J. Ind. Microbiol. Biotechnol.* 2006, *33*, 476–485.
[7] Kim, D.-M., Swartz, J. R., Prolonging cell-free protein synthesis by selective reagent additions. *Biotechnol. Prog.* 2000, *16*, 385–390.
[8] Michel-Reydellet, N., Calhoun, K., Swartz, J., Amino acid stabilization for cell-free protein synthesis by modification of the *Escherichia coli* genome. *Metab. Eng.* 2004, *6*, 197–203.
[9] Calhoun, K. A., Swartz, J. R., Total amino acid stabilization during cell-free protein synthesis reactions. *J. Biotechnol.* 2006, *123*, 193–203.
[10] Bundy, B. C., Swartz, J. R., Site-specific incorporation of *p*-propargyloxyphenylalanine in a cell-free environment for direct protein–protein click conjugation. *Bioconjug. Chem.* 2010, *21*, 255–263.
[11] Iizuka, N., Najita, L., Franzusoff, A., Sarnow, P., Cap-dependent and cap-independent translation by internal initiation of mRNAs in cell extracts prepared from *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 1994, *14*, 7322–7330.
[12] Swartz, J. R., Jewett, M. C., Woodrow, K. A., Cell-free protein synthesis with prokaryotic combined transcription-translation. *Methods Mol. Biol.* 2004, *267*, 169–182.
[13] Jewett, M. C., Swartz, J. R., Rapid expression and purification of 100 nmol quantities of active protein using cell-free protein synthesis. *Biotechnol. Prog.* 2004, *20*, 102–109.
[14] Atkinson, D. E., Energy charge of the adenylate pool as a regulatory parameter. Interaction with feedback modifiers. *Biochemistry* 1968, *7*, 4030–4034.
[15] Chapman, A. G., Fall, L., Atkinson, D. E., Adenylate energy charge in *Escherichia coli* during growth and starvation. *J. Bacteriol.* 1971, *108*, 1072–1086.
[16] Catherine, C., Lee, K.-H., Oh, S.-J., Kim, D.-M., Cell-free platforms for flexible expression and screening of enzymes. *Biotechnol. Adv.* 2013, *31*, 797–803.
[17] Jewett, M. C., Swartz, J. R., Substrate replenishment extends protein synthesis with an in vitro translation system designed to mimic the cytoplasm. *Biotechnol. Bioeng.* 2004, *87*, 465–471.
[18] Mikami, S., Kobayashi, T., Yokoyama, S., Imataka, H., A hybridoma-based in vitro translation system that efficiently synthesizes glycoproteins. *J. Biotechnol.* 2006, *127*, 65–78.
[19] Mikami, S., Masutani, M., Sonenberg, N., Yokoyama, S., Imataka, H., An efficient mammalian cell-free translation system supplemented with translation factors. *Protein Expr. Purif.* 2006, *46*, 348–357.
[20] Zeenko, V. V., Wang, C., Majumder, M., Komar, A. A. et al., An efficient in vitro translation system from mammalian cells lacking the translational inhibition caused by eIF2 phosphorylation. *RNA* 2008, *14*, 593–602.

[21] Brödel, A. K., Sonnabend, A., Kubick, S., Cell-free protein expression based on extracts from CHO cells. *Biotechnol. Bioeng.* 2014, *111*, 25–36.
[22] Goerke, A. R., Loening, A. M., Gambhir, S. S., Swartz, J. R., Cell-free metabolic engineering promotes high-level production of bioactive *Gaussia princeps* luciferase. *Metab. Eng.* 2008, *10*, 187–200.
[23] Jiang, X., Oohira, K., Iwasaki, Y., Nakano, H. et al., Reduction of protein degradation by use of protease-deficient mutants in cell-free protein synthesis system of *Escherichia coli*. *J. Biosci. Bioeng.* 2002, *93*, 151–156.
[24] Knapp, K. G., Swartz, J. R., Evidence for an additional disulfide reduction pathway in *Escherichia coli*. *J. Biosci. Bioeng.* 2007, *103*, 373–376.
[25] Takai, K., Sawasaki, T., Endo, Y., Development of key technologies for high-throughput cell-free protein production with the extract from wheat embryos, in: Andrzej, J. (Ed.), *Advances in Protein Chemistry and Structural Biology*, Academic Press 2008, pp. 53–84.
[26] Gallie, D. R., The 5′-leader of tobacco mosaic virus promotes translation through enhanced recruitment of eIF4F. *Nucleic Acids Res.* 2002, *30*, 3401–3411.
[27] Kobayashi, T., Yukigai, J., Ueda, K., Machida, K. et al., Purification and visualization of encephalomyocarditisvirus synthesized by an in vitro protein expression system derived from mammalian cell extract. *Biotechnol. Lett.* 2013, *35*, 309–314.
[28] Stech, M., Brödel, A. K., Quast, R. B., Sachse, R., Kubick, S., *Cell-Free Systems: Functional Modules for Synthetic and Chemical Biology*, Springer, Berlin/Heidelberg 2013, pp. 1–36.
[29] Bergamini, G., Preiss, T., Hentze, M. W., Picornavirus IRESes and the poly(A) tail jointly promote cap-independent translation in a mammalian cell-free system. *RNA* 2000, *6*, 1781–1790.
[30] Mureev, S., Kovtun, O., Nguyen, U. T. T., Alexandrov, K., Species-independent translational leaders facilitate cell-free expression. *Nat. Biotechnol.* 2009, *27*, 747–752.
[31] Kim, H.-C., Kim, T.-W., Kim, D.-M., Prolonged production of proteins in a cell-free protein synthesis system using polymeric carbohydrates as an energy source. *Process Biochem.* 2011, *46*, 1366–1369.
[32] Wang, Y., Zhang, Y.-H. P., Cell-free protein synthesis energized by slowly-metabolized maltodextrin. *BMC Biotechnol.* 2009, *9*, 58.
[33] Kim, T.-W., Oh, I.-S., Keum, J.-W., Kwon, Y.-C. et al., Prolonged cell-free protein synthesis using dual energy sources: Combined use of creatine phosphate and glucose for the efficient supply of ATP and retarded accumulation of phosphate. *Biotechnol. Bioeng.* 2007, *97*, 1510–1515.



Special issue: Biochemical and molecular engineering, edited by George Chen and Brian Pfleger.
Cover image displaying an abstract colorful DNA molecule design from Fotolia.com © burak çakmak

Biotechnology Journal – list of articles published in the May 2014 issue.

Regular Articles