Cell-free Protein Synthesis for High-throughput Biosynthetic Pathway Prototyping

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Running Head: CFPS for Biosynthetic Pathway Prototyping

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Abstract

Biological systems provide a sustainable and complimentary approach to synthesizing useful chemical products. Metabolic engineers seeking to establish economically viable biosynthesis platforms strive to increase product titers, rates, and yields. Despite continued advances in genetic tools and metabolic engineering techniques, cellular workflows remain limited in throughput. It may take months to test dozens of unique pathway designs even in a robust model organism, such as *Escherichia coli*. In contrast, cell-free protein synthesis enables the rapid generation of enzyme libraries that can be combined to reconstitute metabolic pathways *in vitro* for biochemical synthesis in days rather than weeks. Cell-free reactions thereby enable comparison of hundreds to thousands of unique combinations of enzyme homologs and concentrations, which can quickly identify the most productive pathway variants to test *in vivo* or further characterize *in vitro*. This cell-free pathway prototyping strategy provides a complementary approach to accelerate cellular metabolic engineering efforts toward highly productive strains for metabolite production.

Key Words

Cell-free, metabolic engineering, TX-TL, high-throughput screening, *in vitro*, enzyme assay, biosynthetic pathways

1. Introduction

Metabolic engineering seeks to establish efficient biological platforms for chemical production by increasing flux through desired pathways through optimizing expression of heterologous enzyme variants and combinations while downregulating competing native pathways [1]. This process is slowed by the constant need to balance product formation with cell growth and viability, as well as the large time and labor investment required for genetic manipulations [2]. Engineering cellular metabolism with these constraints limits the ability to thoroughly screen pathway variants

comprising different enzyme homologs, combinations, and expression levels. Cell-free systems, on the other hand, enable biosynthesis in the absence of genetic regulation or biomass generation, providing the opportunity for direct modulation of the reaction environment outside normal physiological conditions or viability constraints [3-6]. These cell-free systems are also amenable to immobilization, compartmentalization, and high-throughput assembly through automated liquid handling [7, 8]. Purified enzymatic reactions are the most common example of cell-free biochemical synthesis (**Figure 1A**). This approach enables pathway design from the ground up with precise control of enzyme concentrations and has led to a wide range of products, from simple acids [9, 10] and alcohols [11, 12] to more complex terpenes [13], bioplastics [14], and cannabinoids [15]. However, the time and cost associated with purifying enzymes often limit the number of homologs tested at each step in the pathway, and the fully synthetic environments of purified reactions lack native metabolism, including cofactor regeneration. These factors limit the ability to effectively screen purified enzyme libraries and reduce applicability to *in vivo* systems that contain other pathways competing for substrate and cofactor pools [3-5].

Extract-based cell-free metabolic engineering (CFME) provides a cheaper and faster route to test heterologous enzymes in the context of native metabolism, although these platforms do sacrifice some of the extensive control afforded by purified systems [3, 16]. Crude cell extracts have been generated from a diversity of organisms by growing cells to a desired density, lysing them through physical or chemical disruption, and removing insoluble components by centrifugation [17]. Among these extract-based cell-free platforms, *Escherichia coli* extracts have been extensively optimized and modified for an array of applications, including metabolite synthesis [18]. One key benefit of these cell-free extracts is that native *E. coli* glycolysis can convert the carbon substrate (most commonly glucose) to the appropriate central carbon metabolite for the investigated biosynthetic pathway while recycling the cofactors ATP and NADH [3]. Studies have demonstrated that *E. coli* extract generated from cells expressing the pathway for 2,3-butanediol biosynthesis result in high-yielding CFME reactions that are more tolerant to

toxic substrates than cells [19, 20]. While this approach may be useful for biomanufacturing, it does not increase the ability to screen enzymes for cellular biosynthesis. Greater control over CFME reactions can be attained by generating extracts from several strains that each overexpress one enzyme homolog from the desired pathway, providing the ability to mix-and-match enzymes at different relative concentrations (**Figure 1A**). The extract mixing approach has successfully produced mevalonate [21, 22], terpenes [23, 24], and butanol [25, 26]. However, the scope of this technique is limited by the need to grow separate strains for expression of each target enzyme, and the concentration of heterologous enzymes must be estimated relative to native enzymes. A recent advance combined cell-free protein synthesis (CFPS) and CFME for pathway prototyping using extract from a wildtype strain of *E. coli* to generate enzymes for butanol biosynthesis in a single reaction, to which glucose and cofactors were added to activate the pathway [27]. This provides greater flexibility for enzyme expression by simply exchanging plasmids, without any additional growth steps. Although relative enzyme levels can be modulated by adjusting plasmid DNA concentrations, inevitable resource competition reduces the ability to precisely tune the pathway composition [28].

Here we describe a 2-pot approach to cell-free protein synthesis-driven metabolic engineering (CFPS-ME) that enables high-throughput expression and testing of enzyme variants, combining the benefits of endogenous metabolism in cell extract for cofactor regeneration with the quantitative control over enzyme concentrations normally afforded by purified systems (**Figure 1B**). First, cell extract is generated from a highly productive *E. coli* strain for CFPS [29]. Second, proteins are expressed *in vitro* from plasmid DNA or linear expression templates [30]. ^{14}C-leucine incorporation enables quantification of only the heterologous protein expressed during CFPS, which increases the precision of this CFME technique compared to other extract-based approaches. Third, enzyme-enriched extracts are combined with the desired substrate and catalytic concentrations of cofactors to activate native *E. coli* catabolism and the heterologous anabolic pathway. Biosynthesis can then be quantified via chromatography, chemical assays, or

biosensors to indicate the most active enzyme combinations and reaction conditions. This method provides a high-throughput platform for individual and combinatorial enzyme screening with unique flexibility, as illustrated by the rapidly produced acetone biosynthesis dataset below (**Figure 2**). While enzyme libraries can be generated through CFPS to screen pathways of interest, other cell-free approaches may be used in tandem to add well-characterized accessory enzymes (*e.g.,* purified enzymes [31] and overexpression extracts [23]). Applications of CFPS-ME to date include the biosynthesis of 3-hydroxybutyrate [32], butanol [33], styrene [34], valinomycin [35], and indole alkaloids [36] as well as the combinatorial assessment of glycosylation pathways [31]. Coupling the high-throughput workflows of two-pot CFPS-ME with machine learning algorithms can reduce the experimental test space of combinatorial pathway prototyping to quickly identify effective enzyme combinations and ratios [33, 37]. The ability to use linear expression templates additionally carries the potential for direct screening of enzyme libraries without the need for cellular transformations [38] and production of enzyme homologues from inexpensive gene blocks within 24 h [39].

Pairing cell-free protein synthesis with the combinatorial assembly of metabolic pathways *in vitro* compounds recent developments in cell-free synthetic biology to provide a powerful tool for high-throughput pathway prototyping [28]. The modular nature of this approach allows for the design of tailored strategies for each optimization problem. For example, extract preparation methods may be altered based on available equipment for lysis, and utilizing knockout strains can prime cell-free metabolism for different pathways through rerouted carbon flux. Additionally, linear expression templates may be used for CFPS to avoid cellular transformation bottlenecks, and reaction composition can be altered to improve protein and/or metabolite yields. Overall, the CFPS-ME framework is poised to facilitate multiplexed study of biosynthetic pathways at an unprecedented speed and throughput in order to inform cellular engineering efforts [33] and eventually large-scale cell-free biosynthesis platforms [40] with implications for producing commodity chemicals [3], biomaterials [41], and natural products [42].

2. Materials

2.1. Cell Extract Preparation

1. LB broth and LB agar plates: Combine 10.0 g of tryptone, 5.0 g of yeast extract, 5.0 g of NaCl with deionized water to 1 L and dissolve completely. If making agar plates, add 15 g of agar. Autoclave or filter to sterilize.
2. 2xYTPG media: Combine 16.0 g of tryptone, 10.0 g of yeast extract, 5.0 g of NaCl, 7.0 g of K_2HPO_4, and 3.0 g of KH_2PO_4 with deionized water to 750 mL. Dissolve completely and adjust pH to 7.2 using 5 N NaOH or KOH. Dissolve 18.0 g of glucose in 250 mL of deionized water. Autoclave separately to avoid Maillard reaction and combine prior to growth. Alternatively, combine all reagents in water to 1 L and sterile filter after adjusting pH.
3. 1.0 M Isopropyl β-D-1-thiogalactopyranoside (IPTG)
4. S30 buffer: Prepare 1.0 M Tris base (adjusted to pH 8.2 with glacial acetic acid), 1.4 M magnesium acetate, and 6.0 M potassium acetate. Sterile filter stock solutions and combine 10 mL of each solution with 970 mL of water for final concentrations of 10 mM Tris base, 14 mM magnesium acetate, and 60 mM potassium acetate. Add 2 mL of 1.0 M dithiothreitol (DTT) per L of buffer immediately prior to use.
5. BL21 Star™ (DE3) or desired knockout strain
6. pJL1-derived plasmids or linear templates (Addgene plasmid # 102634) containing genes of interest for biosynthetic pathways
7. 250-mL baffled flask
8. 2.5-L Tunair flask
9. High-speed centrifuge with 1-L centrifuge bottles
10. Tabletop centrifuge with 50-mL conical tubes
11. Lysing instrument: Sonicator or homogenizer

12. 10-30 mL Luer lock syringes
13. Liquid nitrogen
14. Vortex

2.2. Cell-free Reactions

1. 15x salt solution: 120 mM magnesium glutamate, 150 mM ammonium glutamate, 2.01 M potassium glutamate.
2. 15x nucleotide master mix: 18 mM adenosine triphosphate (ATP), 12.75 mM guanosine triphosphate (GTP), 12.75 mM cytidine triphosphate (CTP), 12.75 mM uridine triphosphate (UTP), 0.51 mg/mL folinic acid, 2.559 mg/mL *E. coli* tRNAs.
3. 1 M phosphoenolpyruvate (PEP)
4. 50 mM amino acid solution (all 20 canonical amino acids at 50 mM)
5. 100 mM nicotinamide adenine dinucleotide (NAD)
6. 50 mM coenzyme A (CoA)
7. 250 mM putrescine
8. 250 mM spermidine
9. 1.0 M oxalic acid
10. 1.0 M HEPES buffer (pH 7.2)
11. 2.2 M glucose
12. 1.0 M Bis-Tris buffer
13. 100 mM ATP
14. 50 mg/mL kanamycin
15. 5% and 10% (w/v) trichloroacetic acid (TCA) or another quenching reagent (see Note 15)
16. Echo 550 liquid handling robot (Labcyte Inc., CA, USA)

2.3. Protein Quantification via Radioactivity

1. 0.32 mM ^{14}C-Leucine
2. 0.5 N KOH
3. 5% (w/v) TCA
4. Styrofoam blocks with the center cut out wrapped in aluminum foil
5. 0.5 mm diameter metal pins with glass heads
6. Microbeta scintillation counter or other means of protein quantification
7. Filtermat A (Perkin Elmer)
8. Meltilex A scintillation wax sheets (Perkin Elmer)
9. Adjustable heating block
10. Geiger counter

3. Methods

3.1. Cell Extract Preparation

3.1.1. Cell Growth and Harvest

1. Streak glycerol stock of BL21 Star™ (DE3) (or desired strain) on LB-agar plate, including antibiotic if necessary. Incubate plate overnight at 37 °C.
2. Inoculate 30 mL of LB broth with a single colony from step 1. Incubate at 37 °C with shaking at 220 rpm for 16-18 h.
3. Ensure that centrifuge rotors, S30 buffer, and centrifuge bottles are cooling to 4 °C.
4. Use the overnight culture to inoculate 1 L of 2xYTPG with an initial OD_{600} of 0.05-0.1 in a 2.5-L Tunair flask. Incubate at 37 °C with shaking at 220 rpm.
5. For BL21 Star™ (DE3), induce T7 expression with 0.5 mL of 1 M IPTG at OD_{600} of 0.5-0.6.
6. Continue monitoring cell density, diluting culture as necessary to be within the linear range of your spectrophotometer.

7. Harvest the cells in mid-exponential phase (OD_{600} 3-3.5) by transferring the culture to a 1-L centrifuge bottle or dividing the culture into 2 bottles. Centrifuge for 5 minutes at 8,000 g and 4 °C.
8. Carefully discard the supernatant and immediately place the centrifuge bottle(s) on ice.
9. Transfer the pellet to two 50-mL conical tubes per liter of cell culture using a spatula. Keep the tubes on ice.
10. Wash the cell pellets with 25 mL of S30 buffer (with DTT added to a final concentration of 2 mM) using a Vortex to resuspend the cells. Ensure that the slurry remains cold by alternating with 15 seconds on the vortex and 15 seconds on ice.
11. When the pellets are completely resuspended and no cell clumps are visible, centrifuge at 4 °C for 2 minutes at 10,000 g.
12. Carefully discard the supernatant.
13. Repeat steps 10-12 twice more for a total of 3 washes.
14. Dry the interior and exterior of the conical tubes with Kimwipes, taking care not to disturb the pellet.
15. Measure and record the cell mass in each tube.
16. Flash freeze the cell pellets in liquid nitrogen and store at -80 °C until lysis. Alternatively, omit flash freezing and immediately begin lysis and clarification steps.

3.1.2. Lysis and Clarification

1. If cell pellets were frozen, thaw for 1 h on ice. Otherwise, proceed directly to step 2.
2. Add 1 mL of S30 buffer per gram of cell pellet.
3. Resuspend the cell pellet using a vortex at max speed, alternating with 15 s intervals on the vortex and on ice to keep cells cold and metabolism slow. Continue until the cell pellet is fully resuspended and no clumps remain.

4. A layer of foam may form atop the cell suspension, particularly if the cells were frozen. Allow the cells to rest on ice for 15 minutes so that foam can dissipate.
5. Lyse the cells via sonication or high-pressure homogenization.
    1. Sonication:
        1. Transfer 1 mL aliquots of cell suspension to 1.5-mL microcentrifuge tubes.
        2. Lyse each aliquot using a QSonica Q125 Sonicator with 3.175 mm diameter probe at 20 kHz and 50% amplitude, keeping the tube in an ice water bath. Input 680 J for 1 mL aliquots, alternating 15 s on and 15 s off to reduce overheating.
        3. Immediately place tubes of cell lysate on ice.
    2. Homogenization:
        1. Transfer resuspended cells without foam to a syringe using an 18-guage needle to break up any remaining cell clumps.
        2. Lyse the cells using an Avestin EmulsiFlex-B15 homogenizer with a single pass at a pressure of 20,000-25,000 psi. Maintain a controlled flow rate for consistent pressure to maximize the percentage of cells lysed.
        3. Immediately transfer cell lysate to a clean conical tube on ice.
        4. Aliquot lysate into 1.5-mL microcentrifuge tubes on ice.
6. Optional: Add 3mM DTT (3 μL of 1 M DTT per 1 mL lysate) to the lysate and mix by inverting the tube (see Note 9).
7. Centrifuge tubes of lysate for 10 minutes at 12,000 g and 4 °C.
8. Carefully remove the clarified supernatant (now referred to as cell extract) without disturbing the pellet of insoluble cell debris and transfer to fresh 1.5-mL microcentrifuge tubes on ice.
9. Centrifuge tubes of extract again for 10 minutes at 12,000 g and 4 °C to remove any residual insoluble debris. This will produce a small pellet, if any.

10. Transfer final extract to a conical tube and mix to ensure homogeneity in the batch.
11. Aliquot cell extract in desired volume in either 200-µL PCR tubes or 1.5-mL microcentrifuge tubes on ice.
12. Flash freeze aliquots in liquid nitrogen and store at -80 °C until use.
13. Determine the bulk protein content of your cell extract using a Bradford assay or similar protein quantification method, expecting ~50-60 mg/mL.

3.2. Cell-free Protein Synthesis (CFPS)

1. Prepare pJL1 expression templates for proteins of interest using either plasmids or linear templates.
    a. Purify plasmids using high-quality midi- or maxi-prep kits. Ethanol precipitation may increase CFPS yields by removing residual salts.
    b. Amplify linear expression templates using a high-fidelity polymerase, such as Q5.
        i. Forward primer: 5'-ctgagatacctacagcgtgagc-3'
        ii. Reverse primer: 5'-cgtcactcatggtgatttctcacttg-3'
2. Assemble a CFPS master mix from stock solutions in nuclease-free water according to **Table 1**. If using an extract strain without chromosomally induced T7 polymerase, add purified T7 polymerase to a final concentration of 0.1 mg/mL.
3. Add 1 µL of purified expression template to 2-mL tubes to reach final concentrations of 5-10 nM. Use 2 µL of linear expression template when using unpurified PCR products to avoid transcriptional limitation. Ensure that the droplet containing expression template is at the bottom of the tube.
4. Aliquot 14 µL of CFPS master mix to each tube with expression template and gently mix at the bottom of the tube.
5. Incubate CFPS reactions at 30 °C for 6-20 h.

3.3. Protein Quantification

1. Set up CFPS reactions in triplicate as described above with the addition of 10 μM ^{14}C-leucine and incubate for desired time. Include a blank reaction (using water instead of expression template) to determine background radioactivity, and express green fluorescent protein as a positive control. For alternatives to radiolabeling, see Note 12.
2. Prepare PCR strips with 5 μL of 0.5 N KOH to dissolve total and soluble fractions of each CFPS reaction.
3. Add 5 μL from your reaction to a PCR tube and pipette to mix with base. This is the total fraction. Incubate samples at 37 °C for 20 min.
4. During the incubation, centrifuge remaining CFPS volume for 10 min at 12,000 g and 4 °C to isolate the soluble fraction.
5. Add 5 μL of supernatant to PCR tubes and pipette to mix with base. Take care not to disturb the pellet, which will be wide in a 2-mL tube. Incubate samples from soluble fraction at 37 °C for 20 min
6. Prepare 2 Filtermats. One will be washed, and the other will not. Pin them to the foil-wrapped Styrofoam blocks with the center cut out to ensure your samples remain elevated and do not touch the surface. This reduces error substantially.
7. After incubation, place 4 μL from each sample onto corresponding wells of the washed and unwashed Filtermats. Avoid air bubbles to ensure the same volume of each sample is placed on each Filtermat.
8. Allow Filtermats to dry under the heat lamp for 20 min.
9. Take the Filtermat designated for washing and place in a plastic tray. Cover with cold 5% TCA, shake gently, and place at 4 °C for 15 min. Carefully pour liquid into the appropriate radioactive waste container, and repeat this step for a total of 3 washes.

10. Cover the washed Filtermat with 100% ethanol, shake gently, and incubate at room temperature for 15 min. Carefully pour liquid into the appropriate radioactive waste container.
11. Place the Filtermat back on the Styrofoam block and allow to dry under the heat lamp for 20 min.
12. Turn on the heating block to 80-90 °C and apply a clean transparency sheet on top to contain melted wax.
13. Carefully place dry Filtermat on the transparency and melt scintillation wax to align with the edges of the sample wells. Use metal forceps in both hands to keep the mat flat for even melting. Once the wax has soaked into the Filtermat, lift it up with forceps on both sides and gently blow on it until the wax becomes opaque again.
14. Place the waxed Filtermat in a plastic plate holder, taking care to align the holes with wells on the Filtermat.
15. Load your washed and unwashed mats into the MicroBeta and run desired program for scintillation counting.
16. Determine the amount of protein produced in each reaction:
    a. Calculate the percent of leucine incorporated into proteins by dividing counts from the washed sample by counts from the corresponding unwashed sample.
    b. Subtract the background incorporation percent (quantified in the blank reaction) from all samples.
    c. Divide the corrected incorporation percent by the number of leucine residues in the expressed protein and multiply by the total concentration of leucine in the reaction.
    d. Divide this value by the molecular weight for each protein expressed to obtain micromolar concentrations.

[((Washed count - Background count) / Unwashed count) * [Leu] μM * (MW Protein μg/μmol)] / [(Leu residues in protein) * (1000 mL/L)] = μg/mL.

3.4. Cell-free Metabolic Engineering (CFME)

3.4.1 CFME assembly by hand

1. Reconstitute biosynthetic pathways *in vitro* by combining desired μM concentrations of enzymes produced by CFPS. Correct for different amounts of CFPS added by adding spent CFPS reaction without plasmid template or with sfGFP template to normalize the concentrations of reagents and native enzymes between samples.
2. Combine enzyme-enriched CFPS reactions with reagents listed in **Table 2** in nuclease-free water to 20 μL in 1.5-mL microcentrifuge tubes (see note 14).
3. Incubate CFME reactions at 30 °C for 6-24 h.

3.4.2 Assembly using an Echo 550 automated liquid-handling robot

1. Set up CFPS as described previously calculating in the dead volume needed for the Echo source plate (20 μL for PP^+ 384 plates and 2 μL for LDV plates).
2. Combine CFPS reactions in 96- or 384-well plate using 384PP/LDV_AQ_CP as a fluid type on an Echo 550 liquid handling robot (Labcyte Inc., CA, USA). Normalize CFPS volume added to each combination using spent CFPS reaction without plasmid template or with sfGFP template.
3. Start reactions by adding the same amount of a master mix containing the above listed components dissolved in nuclease free water to each well using 384PP_AQ_BP as a fluid type on the Echo or an Integra VIAFLO 96/384 multichannel pipette.
4. Seal plate, incubate at 30 °C, and optimize concentrations as stated in 3.4.1.

4. Notes

The modularity and adaptability of CFPS-ME to fit the specific needs of the pathways under investigation is a major advantage of the system. It does, however, make it difficult to write a generalizable protocol that can be used for any case. Here we offer some of the adaptations to the above protocol that might benefit or enable certain experiments, and we included comments about common pitfalls and sensitive steps of the outlined protocol above.

1. Endogenous enzymes in the BL21 Star™ (DE3) strain can affect product yields by diverting the starting substrate or interacting with other metabolic pathways. Extracts from strains containing knockouts of these enzymes can be made for CFPS-ME experiments, and this workflow can even be used to accelerate the characterization of a gene knockout's impact on a metabolic pathway. However, these knockouts may impact the growth of cells for high-yielding CFPS extract preparation. For this reason we suggest optimization of the harvest OD_{600} (3.1.1 Step 7) by comparing timepoints in early, mid, and late exponential growth.
2. As an alternative to knockout strains, recent studies have shown that undesired enzymes can be removed or reduced post-lysis by adding protease sites [43] or affinity tags [44] (adding an additional processing step). This reduces the detrimental effect on cell growth caused by some gene knockouts and could enable highly productive CFPS-ME extracts even when essential genes are targeted for removal.
3. The growth medium (and especially the carbon source) can directly influence metabolism of the extract. The addition of glucose to 2xYTP does not influence CFPS yields in our experience, but it increases glycolytic activity in cell extracts. Alternative carbon sources for growth and/or cell-free biosynthesis might benefit CFPS-ME for pathways relying on different parts of central carbon metabolism.

4. Induction of T7 polymerase is not possible in all strains, and purified T7 polymerase can be added to those extracts during CFPS (3.2 Step 2). Alterations to this method are required for robust expression from native *E. coli* promoters [45].
5. Lysis through sonication or homogenization requires specialized equipment, such as the QSonica Q125 Sonicator with 3.75 mm tip or Avestin EmulsiFlex-B15 Homogenizer. Less expensive methods (including bead-beating, French press, and chemical lysis) have successfully produced functional cell extracts as well [17, 46].
6. A run-off reaction after lysis and initial centrifugation (after 3.1.2 Step 7), which consists of a 60-80 minute incubation at 37°C, can improve CFPS yields for some strains, including K strains of *E. coli* [29].
7. Centrifugation speed during extract preparation (3.1.2 Step 7) influences diameter, size distribution, and concentration of inverted membrane vesicles in the extract. This may influence oxidative phosphorylation (and therefore ATP regeneration) in both CFPS and CFME reactions [47, 48]. This dependence on respiration makes the reactions sensitive to oxygen availability, so surface area to volume ratios can impact efficiency.
8. Organic salts used in S30 buffer (3.1.1 Step 3), in the CFPS reaction mix (3.2 Step 2), and during CFME reactions (3.4.1 Step 2) can directly influence yields of the CFME reaction depending on the pathway under investigation. For example, acetate has been shown to enter cell-free metabolism during synthesis of hydroxymethylglutaryl-CoA and mevalonate [21]. Glutamate undergoes deamination and enters the TCA cycle at α-ketoglutarate, which eventually leads to a build-up of succinate. Acetate or glutamate can be used interchangeably in all three steps depending on pathway demands.
9. Addition of 3 mM DTT to lysed cells prior to centrifugation is included in some published protocols, but empirically this has shown little difference in CFPS yields. DTT added to S30 buffer should provide a sufficiently reducing environment.

10. Addition of oxalic acid to CFPS master mix increases protein production, but it can reduce titers of some target metabolites [33].
11. Linear expression templates are stable in extracts derived from BL21 Star™ (DE3), but stability varies between extracts of different strains based on exonuclease activity. GamS nuclease inhibitor can stabilize linear templates when added in the CFPS mix at 30 μg/mL (3.2 Step 2) [30].
12. Alternative methods for quantifying expression levels from CFPS include reporter protein domains (*e.g.*, split-GFP [49], cleavable N-terminal GFP fusion [50], or NanoLuc [51]) and chemical labeling kits (*e.g.,* FluoroTect™ or Transcend™ from Promega [52]).
13. Reaction pH and buffer choice may be optimized to balance pH optima of desired catabolic and anabolic enzymes [26, 34].
14. CFME reactions require optimization of substrate, cofactor, and enzyme concentrations to maximize product titers and/or reaction rates. Additional cofactors, such as NADP(H) may be beneficial for some pathways. Optimize incubation time and temperature for investigated pathway.
15. Use an appropriate quenching method for the downstream analytic method to precipitate proteins in the CFME reaction or otherwise stop metabolic activity. For liquid chromatography, add a common quenching reagent such as 5-10% acid (trifluoroacetic, trichloroacetic, or formic), 50% acetonitrile, or 50% methanol. Centrifuge samples at 10,000xg for 10 min and transfer supernatants to appropriate vials or plates for your instrument. For gas chromatography, extract samples with an appropriate solvent (*e.g.*, ethylacetate, hexane, dodecane), adding 6.25% H_2SO_4 and 7.5% NaCl to precipitate proteins if desired. After extraction, further derivatization may be appropriate for target molecule detection.

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Table 1. CFPS master mix composition.

Reagent	Stock Concentration	Final Concentration
Salt solution	15x	1x
Nucleotide master mix	15x	1x
PEP	1 M	33.33 mM
Canonical amino acid mix	50 mM	2 mM
NAD	100 mM	0.4 mM
CoA	50 mM	0.27 mM
Putrescine	250 mM	1 mM
Spermidine	250 mM	1.5 mM
Oxalic acid* (see Note 10)	1 M	4 mM
HEPES (pH 7.2)	1 M	57 mM

Table 2. CFME reaction mix composition.

Reagent	Stock Concentration	Final Concentration
Salt solution	15x	1x
Bis-Tris Buffer	1 M	100 mM
Glucose	2.2 M	50-200 mM
ATP	100 mM	0-10 mM

NAD	100 mM	0-10 mM
CoA	50 mM	0-10 mM
Kanamycin	50 mg/mL	1.25 mg/mL
Fresh *E. coli* extract	~50 mg/mL	8 mg/mL

Figure Legends

Figure 1. Methods for cell-free metabolite synthesis. (A) Enzymes expressed *in vivo* can be purified for precise control of concentration or obtained in a crude cell extract to retain native metabolism prior to mixing *in vitro*. (B) Combining cell-free protein synthesis with *in vitro* chemical synthesis results in a high-throughput method that includes quantitative determination of protein expression and native *E. coli* catabolism and cofactor regeneration. Cell extract is generated from *E. coli* through physical lysis and centrifugation. Enzymes are expressed individually using plasmid DNA of linear expression templates, and expression is quantified by radioactive leucine incorporation. Enzymes are then combined at defined concentrations with substrate and cofactors to recapitulate metabolic pathways *in vitro*, followed by biochemical analysis.

Figure 2. Sample data from CFPS-ME workflow. (A) Pathway diagram for conversion of glucose to acetone, utilizing native enzymes from *E. coli* cell extract and homologs of 4 heterologous enzymes. (B) CFPS with radioactive incorporation from 44 linear expression templates provides accurate protein yields in 2 days. Oxalic acid was omitted to enhance acetone titers in downstream reactions (see Note 10), resulting in ~60% of the sfGFP reporter yields (construct 43) seen in previous CFPS optimizations. (C) Combinatorial assembly of 81 pathway combinations (varying homologs and enzyme concentrations of 0.05, 0.1, and 0.5 μM) produces *in vitro* acetone titers ranging from the limit of detection to 67 mM in a matter of days, with HPLC analysis as the limiting step.