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Original Research Article

## A cell-free framework for rapid biosynthetic pathway prototyping and enzyme discovery

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## ABSTRACT

Speeding up design-build-test (DBT) cycles is a fundamental challenge facing biochemical engineering. To address this challenge, we report a new cell-free protein synthesis driven metabolic engineering (CFPS-ME) framework for rapid biosynthetic pathway prototyping. In our framework, cell-free cocktails for synthesizing target small molecules are assembled in a mix-and-match fashion from crude cell lysates either containing selectively enriched pathway enzymes from heterologous overexpression or directly producing pathway enzymes in lysates by CFPS. As a model, we apply our approach to *n*-butanol biosynthesis showing that *Escherichia coli* lysates support a highly active 17-step CoA-dependent *n*-butanol pathway *in vitro*. The elevated degree of flexibility in the cell-free environment allows us to manipulate physicochemical conditions, access enzymatic nodes, discover new enzymes, and prototype enzyme sets with linear DNA templates to study pathway performance. We anticipate that CFPS-ME will facilitate efforts to define, manipulate, and understand metabolic pathways for accelerated DBT cycles without the need to reengineer organisms.

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## 1. Introduction

For decades scientists and engineers have turned to engineering biological systems to help meet societal needs in energy, medicine, materials, and more (Bornscheuer et al., 2012; Fritz et al., 2010; Curran and Alper, 2012; Rollié et al., 2012). This has been an attractive, sustainable way to produce small molecules, especially when chemical synthesis is untenable (Erickson et al., 2012; Nielsen et al., 2014). The ability to harness organisms that naturally produce molecules of interest has expanded the available chemical palate (Demain, 2014; Harvey et al., 2015). Often when natural producers are insufficient for production at the optimal titer ( $\text{g l}^{-1}$ ), yield, or volumetric productivity ( $\text{g l}^{-1} \text{h}^{-1}$ ), engineers seek to design biosynthetic pathways and regulatory processes in cells to meet certain manufacturing criteria (Kern et al., 2007; Nielsen, 2001). For example, introducing heterologous pathways into model microorganisms and engineering them to maximize a particular biosynthesis has led to large scale production of 1,3-propanediol, farnesene, and artemisinin with many

more on their way to market (Nielsen et al., 2014; Hodgman and Jewett, 2012). Efforts to make these molecules have resulted in success, but not without a great deal of challenges.

Bringing a biosynthetic molecule to market usually involves countless hours of design-build-test (DBT) cycles (Kwok, 2010). The production of *n*-butanol is a prime example of these challenges. A series of *Clostridia* species are natural producers of *n*-butanol during acetone–butanol–ethanol fermentation, and *Clostridia acetobutylicum* and *Clostridia beijerinckii* are two of which are commonly used in commercial *n*-butanol plants (Green, 2011). However, these species are difficult to engineer because of a biphasic metabolism, unknown regulation patterns, and a limited number of species-specific engineering tools (Lutke-Eversloh and Bahl, 2011). Heterologous expression of *Clostridia* metabolism in model microorganisms like *Escherichia coli* and *Saccharomyces cerevisiae* allows *n*-butanol production to be more easily engineered but can be accompanied by lower titers (Atsumi et al., 2008; Steen et al., 2008). Starting with heterologous expression of the *n*-butanol pathway as a baseline, scientists have been able to increase titers dramatically by knocking out genes from genomes (Atsumi et al., 2008), increasing redox driving forces by introducing pathway-independent enzymes (Shen et al., 2011), and identifying homologous enzymes with better activities (Bond-Watts et al., 2011). Years of iterative metabolic engineering led to

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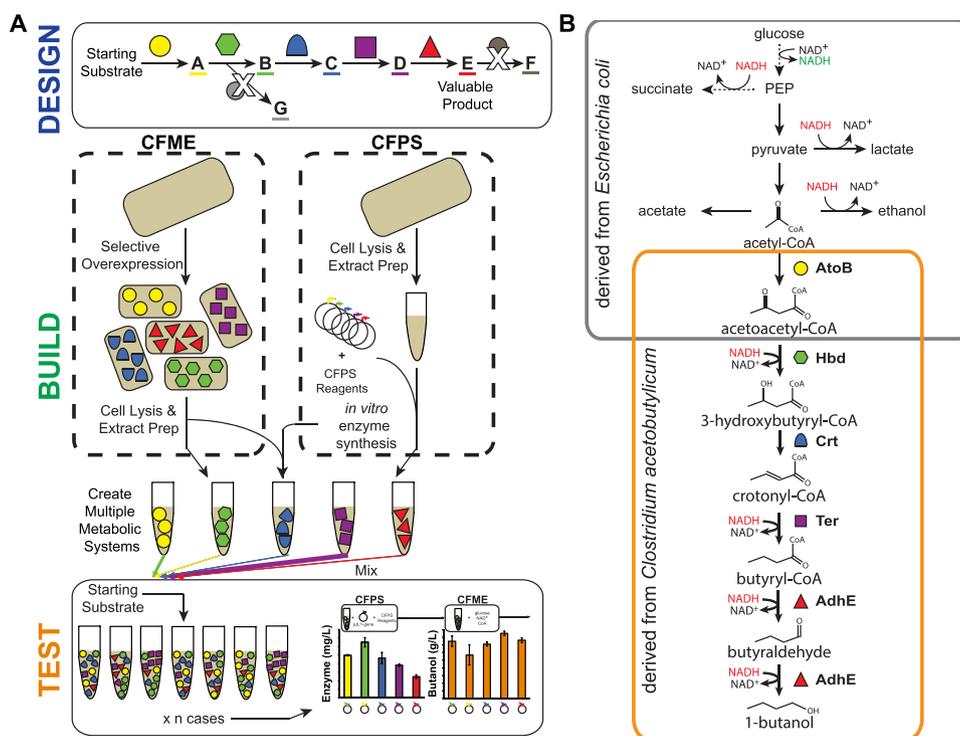
these advances, but titers are still not high enough and scale-up is often too unpredictable to outcompete natural producers for commercial production (Dong et al., 2015). As is the same for many biosynthetic pathways, we cannot quickly enough identify optimal biosynthetic systems and discover the best sets of enzymes that work together as a group. Therefore, metabolic engineering remains costly and time-consuming (Keasling, 2010, 2012).

A key challenge in metabolic engineering is balancing the tug-of-war that exists between the cell's physiological and evolutionary objectives on one side and the engineer's process objectives on the other. Put another way, it is very difficult to balance intracellular fluxes to optimally satisfy a very active synthetic pathway while the machinery of the cell is functioning to maintain reproductive viability. Other challenges include: (i) the need for reliable computational selection and design of enzyme homologs for pathway design, (ii) the limited number of feasible homologs and genetic constructs that can be searched in any one project, and (iii) the unknown effects of optimal pathway enzyme expression on the entire metabolic system (Jensen and Keasling, 2014; Dai and Nielsen, 2015; Lee and Kim, 2015).

Many established and emerging technologies seek to address these challenges. For example, metabolic flux analysis and genome engineering offer generalized capabilities to modify living organisms for improving product titers (Lee et al., 2012; Yadav et al., 2012). In addition, coupling machine-learning algorithms to multiplexed designs can accelerate efforts to rationally engineer cells (Smanski et al., 2014). However, DBT cycle time remains a limitation (Boyle and Silver, 2012). *In vitro* systems offer a complementary, yet underutilized approach to speed up DBT cycles with some potential advantages (Hodgman and Jewett, 2012; Carlson et al., 2012; Sun et al., 2014; Siegal-Gaskins et al., 2014). For example, the open reaction environment allows for the addition of components such as cofactors and intermediates at any time during a cell-free reaction, which can be maintained at

precise concentrations. In addition, cell-free systems have no cell viability constraints. Furthermore, the cell-free format permits DBT iterations without the need to reengineer organisms (Sun et al., 2014), with the potential to reduce DBT cycle time (Siegal-Gaskins et al., 2014). Cell-free metabolic engineering (CFME), or using cell-free techniques to aid metabolic engineering efforts, is emerging as a complementary approach to existing strategies for carrying out biomolecular transformations of interest with *in vitro* ensembles of catalytic proteins, prepared from purified enzymes or crude lysates of cells (Dudley et al., 2015; Zhang, 2015; You and Zhang, 2013; Guterl et al., 2012; Kay and Jewett, 2015; Krutsakorn et al., 2013; Ninh et al., 2015; Welch and Scopes, 1985).

In this work, we develop a cell-free protein synthesis driven metabolic engineering (CFPS-ME) framework to accelerate DBT cycles for optimizing and debugging biosynthetic pathways (Fig. 1A). The foundational principle is that we can construct discrete metabolic pathways through combinatorial and modular assembly of lysates containing enzyme components produced by overexpression in the lysate chassis strain or by cell-free protein synthesis (CFPS). We focus on using CFPS because these systems can help address the growing demand for simple, inexpensive, and efficient protein production technologies for a wide array of applications (Hodgman and Jewett, 2012; Carlson et al., 2012; Swartz, 2012; Dodevski et al., 2015; Henrich et al., 2015; Zemella et al., 2015; Noireaux et al., 2003). In addition, processes that take days or weeks to design, prepare, and execute in cells can be done more rapidly in a cell-free system, because no time-consuming cloning steps are needed (Goshima et al., 2008). Three recent advances enable the use of CFPS for CFME. First, Jewett et al. (2008) demonstrated the ability to stimulate highly active energy and cofactor regeneration pathways in crude cell lysates. Second, Kay and Jewett (2015) showed that crude cell lysate based cell-free systems from *E. coli* could fuel highly active heterologous metabolic transformations. Third, Dudley and Jewett established the



**Fig. 1.** A cell-free framework for pathway prototyping demonstrated with a 17-step *n*-butanol model pathway. (A) Methodology for cell-free metabolic engineering (CFME) and cell-free protein synthesis driven metabolic engineering (CFPS-ME). (B) Schematic (non-stoichiometric) representation of the constructed biosynthetic *n*-butanol pathway. Acetyl-CoA is generated through *E. coli*'s natural glycolysis and funneled into the *C. acetobutylicum*-derived CoA-dependent pathway to produce *n*-butanol. The butyryl-CoA dehydrogenase (Ter) here is from *Treponema denticola*. Four NADH molecules are needed to produce one molecule of *n*-butanol.

ability to build a heterologous biosynthetic pathway by mixing lysates each containing individually overexpressed heterologous enzymes (in preparation). The mix-and-match approach has many advantages including only needing to express one enzyme in each strain, not needing to fine-tune expression, and being able to directly monitor and sample the reaction environment. Here, we extend this approach by demonstrating modular assembly of pathways through the ability to enrich lysates with biosynthetic enzymes using well-defined experimental conditions and CFPS. It is important to note that our goal in this work was not to develop cell-free systems for the highest product titer, an engineered strain for best *in vivo* synthesis of *n*-butanol, or industrial applicability. However, we do show that CFPS-ME offers an even faster approach (hours rather than days) for building pathways directly in lysates for the purpose of enzyme selection and pathway design.

To demonstrate CFPS-ME, we selected the model *n*-butanol biosynthetic pathway derived from *Clostridia* metabolism involving CoA intermediates (Fig. 1B). Endogenous glycolytic enzymes convert glucose to acetyl-CoA, the starting intermediate for *n*-butanol synthesis, another *E. coli* enzyme takes acetyl-CoA to acetoacetyl-CoA, and heterologous enzymes convert acetoacetyl-CoA to *n*-butanol. We first show the ability to mix five crude lysates each with selectively overexpressed enzymes to activate the entire 17-step *n*-butanol production pathway *in vitro* with high yield and productivities. We then establish the CFPS-ME concept by modularly building the *n*-butanol pathway with lysates harboring heterologous pathway enzymes expressed by CFPS or having been overexpressed in the chassis source strain. We apply this framework to rapidly screen enzymes for optimal pathway operation and enzyme discovery. We expect that the CFPS-ME framework will increase the resolution at which we can manipulate biosynthetic pathways by examining enzyme kinetics, measuring metabolic flux, determining catalyst stability, studying redox effects, and prototyping metabolism.

## 2. Material and methods

### 2.1. Bacterial strains and plasmids

*E. coli* NEB Turbo™ (NEB) was used in plasmid cloning transformations and for plasmid preparation. *E. coli* BL21(DE3) (NEB) was used for protein overexpression and for preparation of all extracts (see Supplementary Table 1 for strain details). A modified version of pET-22b (Novagen/EMD Millipore), used in previous studies (Kay and Jewett, 2015), was used for all constructs for *in vivo* over-expression of proteins. For *in vitro* expression of proteins, the pJL1 vector was used. Carbenicillin ( $100 \mu\text{g ml}^{-1}$ ) was used with the pET vector system and kanamycin ( $50 \mu\text{g ml}^{-1}$ ) was used with the pJL1 vector system.

Gibson assembly was used for seamless construction of plasmids (see Supplementary Table 1 for plasmid details). Each gene and vector was amplified *via* PCR using forward and reverse primers designed with NEB's Gibson Assembly Designer (New England Biolabs, Ipswich MA, USA) and purchased from IDT and Phusion® High-Fidelity DNA polymerase (Finnzymes, Thermo Scientific Molecular Biology) (see Supplementary Table 2 for genes and enzymes and Supplementary Table 3 for primer details). Both PCR products were cleaned and mixed with Gibson assembly reactants and incubated at  $50^\circ\text{C}$  for 60 min. Plasmid DNA from the Gibson assembly reactions were immediately transformed into *E. coli* NEB Turbo cells. Propagated constructs were purified using an EZNA Plasmid Mini Kit (Omega Bio-Tek). Completed constructs were used to transform *E. coli* BL21(DE3).

Codon optimized versions of each gene were identified using IDT's codon optimization online tool (Integrated DNA

Technologies®, Coralville, USA) and NCBI's Basic Local Alignment Search Tool (National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda MD, USA). These genes were purchased from Gen9, Inc. (Cambridge MA, USA) (see Supplementary Table 4 for codon-optimized sequences).

### 2.2. Cell extract preparation

*E. coli* BL21(DE3) cells (see Supplementary Table 1 for strains) were grown in  $2 \times$  YTPG media ( $16 \text{ g l}^{-1}$  tryptone,  $10 \text{ g l}^{-1}$  yeast extract,  $5 \text{ g l}^{-1}$  NaCl,  $7 \text{ g l}^{-1}$  potassium phosphate monobasic,  $3 \text{ g l}^{-1}$  potassium phosphate dibasic,  $18 \text{ g l}^{-1}$  glucose). These cells were cultured at the 50 ml scale in 250 ml baffled tunair shake flasks (IBI Scientific, Peosta, IA) in a  $37^\circ\text{C}$  incubator with vigorous shaking at 250 rpm. The cultured cells were monitored by spectrophotometry (Genesys 10S UV-vis, Thermo Fisher Scientific, Waltham, MA). When cells reached  $\text{OD}_{600}=0.6\text{--}0.8$ , the cultures were induced with 0.1 mM IPTG. After induction cultures were grown for 4 h at  $30^\circ\text{C}$ . Antibiotics were not used during cell growth. The cells were harvested by centrifuging at  $8000 \text{ g}$  at  $4^\circ\text{C}$  for 15 min and were washed two times with cold S30 buffer ( $10 \text{ mM}$  Tris-acetate (pH 8.2),  $14 \text{ mM}$  magnesium acetate, and  $60 \text{ mM}$  potassium glutamate). After final wash and centrifugation, the pelleted wet cells were weighed, flash frozen in liquid nitrogen, and stored at  $-80^\circ\text{C}$ . The thawed cells were suspended in 0.8 ml of S30 buffer per 1 g of wet cell mass. In order to lyse cells by sonication, thawed and suspended cells were transferred into 1.5 ml microtube and placed in an ice-water bath to minimize heat damage during sonication. The cells were lysed using a Q125 Sonicator (Qsonica, Newtown, CT) with 3.175 mm diameter probe at frequency of 20 kHz and 50% of amplitude. The input energy (Joules) was monitored and 830 J was used for 1.4 ml of suspended cells. The lysate was then centrifuged twice at  $21,100 \text{ g}$  at  $4^\circ\text{C}$  for 15 min. All of prepared cell extract was flash frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  until use.

### 2.3. Extract protein quantification

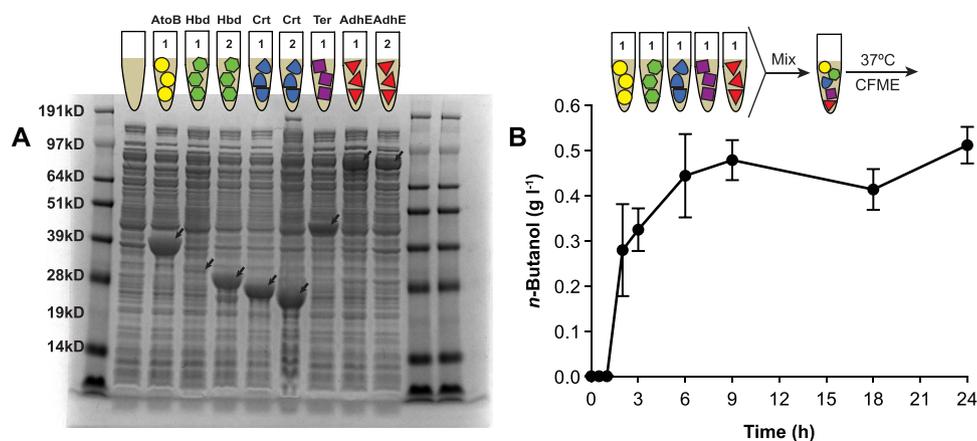
The total protein concentration of the extracts was measured by Quick-Start Bradford protein assay kits (Bio-Rad) with a bovine serum albumin standard. The extracts were subsequently run on a Coomassie-blue stained NuPAGE Bis-Tris 12% SDS-PAGE gel with MOPS buffer (Life Technology, Grand Island, NY). The SeeBlue Plus2 pre-stained ladder (Life Technology, Grand Island, NY) was used and  $\sim 10 \mu\text{g}$  of total protein for each sample was loaded on the gel.

### 2.4. CFME reactions

Reactions were carried out in 1.5 ml Eppendorf tubes at  $37^\circ\text{C}$  in 25  $\mu\text{l}$  volumes. Each reaction consisted of mixing five extracts, containing one enzyme overexpressed each, to complete the biosynthetic *n*-butanol pathway ( $2 \text{ mg ml}^{-1}$ ) along with magnesium glutamate (8 mM), ammonium glutamate (10 mM), potassium glutamate (134 mM), glucose (200 mM), dipotassium phosphate (10 mM, pH 7.2), Bis Tris (100 mM), NAD (1 mM), ATP (1 mM), and CoA (0.5 mM), unless otherwise noted. Reactions were terminated by adding 5% *w/v* trichloroacetic acid in a 1:1 ratio. Precipitated proteins were pelleted by centrifugation at  $15,000\text{g}$  for 10 min. The supernatant was stored at  $-80^\circ\text{C}$  until analysis.

### 2.5. CFPS-ME reactions

CFPS reactions were performed to express enzymes involved in *n*-butanol production prior to starting the CFME portion of the reactions using a modified PANox-SP system (Jewett and Swartz,



**Fig. 2.** Biosynthesis of *n*-butanol achieved via CFME of a coupled *E. coli* and *C. acetobutylicum* metabolic pathway. (A) Via SDS-PAGE, the gel verifies the selective over-expression of pathway enzymes in *E. coli* BL21(DE3) crude cell lysates: AtoB (*Escherichia coli*), Hbd1 (*Clostridia acetobutylicum*, CA), Hbd2 (*Clostridia beijerinckii*, CB), Crt1 (*Clostridium acetobutylicum*, CA), Crt2 (*Pseudomonas putida*, PP), Ter (*Treponema denticola*, TD), AdhE1 (*Clostridium acetobutylicum*, CA), and AdhE2 (*Clostridium pasteurianum*, CP). (B) CFME reactions for *n*-butanol production from glucose were carried out using five crude lysates mixed together (1:1:1:1:1 based on total protein quantification) with glutamate salts ( $Mg^{2+}$ ,  $NH_4^+$ ,  $K^+$ ), phosphate ( $K_2HPO_4$ ), buffer (Bis Tris), and cofactors (ATP, CoA,  $NAD^+$ ). These lysates individually contained AtoB (EC), Hbd1 (CA), Crt1 (CA), Ter1 (TD), and AdhE1 (CA) selectively overexpressed at 37 °C. Error bars represent standard deviations with  $n \geq 3$  independent reactions.

2004). A 25  $\mu$ l CFPS reaction in a 1.5 ml microcentrifuge tube was prepared by mixing the following components: ATP (1.2 mM); GTP, UTP, and CTP (0.85 mM each); folinic acid (34.0  $\mu$ g  $ml^{-1}$ ); *E. coli* tRNA mixture (170.0  $\mu$ g  $ml^{-1}$ ); T7 RNA polymerase (100  $\mu$ g  $ml^{-1}$ ); 20 standard amino acids (2 mM each); nicotinamide adenine dinucleotide (NAD; 0.33 mM); coenzyme-A (0.27 mM); spermidine (1.5 mM); putrescine (1 mM); potassium glutamate (130 mM); ammonium glutamate (10 mM); magnesium glutamate (12 mM); phosphoenolpyruvate (PEP; 33 mM), and cell extract (10 mg  $ml^{-1}$ ). For each reaction plasmid was added at  $\sim 13.3$  or  $\sim 26.6$   $\mu$ g  $ml^{-1}$ . The *n*-butanol production portion of the reaction was initiated by spiking in glucose (200 mM) and additional reagents (NAD, CoA) noted throughout the manuscript.

### 2.6. Quantification of protein produced in vitro

Cell-free protein synthesis reactions were performed as noted above (Section 2.5) with radioactive  $^{14}C$ -Leucine (10  $\mu$ M) supplemented in addition to all 20 standard amino acids. We used trichloroacetic acid (TCA) to precipitate radioactive protein samples. Radioactivity of TCA-precipitated samples was measured by liquid scintillation counting to then quantify the protein produced as previously reported (MicroBeta2; PerkinElmer) (Jewett et al., 2008; Jewett and Swartz, 2004). These reactions were also run on a Coomassie-stained SDS-PAGE gel and exposed by autoradiography. Autoradiographs were imaged with a Typhoon 7000 (GE Healthcare Life Sciences, Pittsburgh, PA). Multiple proteins produced *in vitro* were further quantified by gel image intensity comparisons using ImageJ (NIH).

### 2.7. *n*-butanol quantification

High-performance liquid chromatography (HPLC) was used to analyze the components in the reactions. *n*-Butanol was measured with an Agilent 1260 series HPLC system (Agilent, Santa Clara, CA) via a refractive index (RI) detector. Analytes were separated using the Aminex HPX-87H anion exchange column (Bio-Rad Laboratories) with a 5 mM sulfuric acid mobile phase at 55 °C and a flow rate of 0.6  $ml\ min^{-1}$ . Commercial standard of *n*-butanol was used for quantification of experimental samples by linear interpolation of external standard curves. An example chromatogram for *n*-butanol is given in Supplementary Fig. 1.

## 3. Results

In developing a framework for biosynthetic pathway prototyping, we constructed a 17-step pathway for the production of *n*-butanol. *n*-butanol synthesis was selected as a model because of its importance as a potential biofuel, it is easily quantified by HPLC, and it has multiple heterologous steps. We sought to combine *E. coli*'s endogenous 11-step glycolytic pathway from glucose to acetyl-CoA (AcCoA) with the *Clostridia*-derived six-step *n*-butanol pathway from AcCoA (Fig. 1B). The idea that natural energy and cofactor regeneration would be harnessed in the lysate to fuel *n*-butanol production is a distinct break from typical *in vitro* approaches, which use purified enzymes (Dudley et al., 2015). Complementary to those systems, our approach allows for studying pathway performance in a setting that better mimics the *in vivo* operation (e.g., from glucose rather than AcCoA). The crude lysate system also allows us to focus on expressing only the necessary heterologous enzymes to complete the entire pathway. These enzymes include a thiolase to merge two AcCoAs followed by a number of dehydrogenases to perform a series of reductions through CoA intermediates to obtain *n*-butanol (See Supplementary Table 2 for Genes and Enzymes).

### 3.1. Cell-free metabolic engineering for *n*-butanol production

To enable cell-free biosynthesis of *n*-butanol, we first introduced genes encoding the five enzymes needed to convert AcCoA to *n*-butanol individually into our extract source strains, in this case BL21(DE3) (See Supplementary Table 1 for Strains and Plasmids and Supplementary Table 3 for Primers). We selected two homologs each for hydroxybutyryl-CoA dehydrogenase (Hbd), crotonase (Crt), and bifunctional aldehyde/alcohol dehydrogenase functionalities. For the thiolase (AtoB) and butyryl-CoA dehydrogenase (Ter) we chose *E. coli*'s endogenous enzyme and a widely used enzyme from *Treponema denticola*, respectively. Next, we selectively overexpressed each heterologous enzyme in separate strains using a tightly controlled T7 promoter and strong ribosome binding site. As expected, we observed that the heterologous proteins were overexpressed as the dominant bands, with the exception of Hbd1, on an SDS-PAGE gel (Fig. 2A). The low expression of Hbd1 is likely due to RBS used for expression.

After lysis and extract preparation, we then reconstituted the 17-step pathway from glucose to *n*-butanol by mixing equal total protein concentrations of five separate extracts containing each

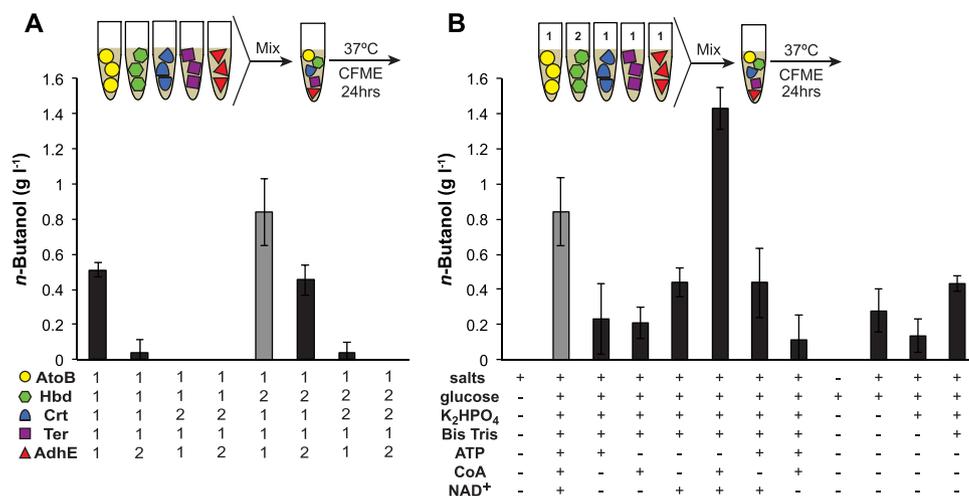
enzyme. Specifically, we started with the following enzyme set: *E. coli*'s AtoB, *C. acetobutylicum*'s Hbd, Crt, and AdhE2, as well as Ter from *T. denticola*. This set was chosen to include most of *C. acetobutylicum*'s enzyme set, one of the most widely used sets for *n*-butanol production, along with previously identified best enzymes for thiolase and butyryl-CoA dehydrogenase functions (Shen et al., 2011; Dong et al., 2015). Upon incubation with essential substrates, salts, and cofactors (e.g., magnesium, potassium, and ammonium salts, glucose, phosphate, buffer, NAD, CoA, ATP), we assessed *n*-butanol synthesis in 25  $\mu$ l CFME batch reactions carried out for 24 h at 37 °C via high performance liquid chromatography (HPLC). We observed production of  $0.51 \pm 0.04$  g l<sup>-1</sup> *n*-butanol (~0.05 mol *n*-butanol/mol glucose) over the course of a 24 h reaction (Fig. 2B), without any optimization to improve titers. As expected, we also observed lactate, acetate, and ethanol as byproducts seen in previous reports of *n*-butanol production, which could be addressed through genome modifications (e.g., deletion of *ldh* gene in the source strain) (Dong et al., 2015). Butanol production shows that both the heterologous pathway and endogenous glycolysis is activated with cofactors being regenerated. However, *n*-butanol production stops after ~9 h. In our previous work, substrate depletion was shown to be the most typical cause for reaction termination (Kay and Jewett, 2015). One way to avoid this limitation is to run reactions in fed-batch or continuous reactor set-ups or use substrates that are metabolized slower (e.g. polymeric sugars). Except in few instances (Welch and Scopes, 1985; Korman et al., 2014), limited cofactor regeneration has historically plagued *in vitro* synthetic enzymatic pathway conversions (Dudley et al., 2015; You and Zhang, 2013; Guterl et al., 2012). Here, however, native glycolytic enzymes in the lysate provide a simple route to fuel highly active heterologous metabolic conversions. For example, to produce ~7 mM *n*-butanol we would need ~56 NADH turnover events, exceeding typical turnover numbers of ~5 to 20 for purified *in vitro* systems (Dudley et al., 2015).

Following demonstration of activating *n*-butanol synthesis, we next aimed to modularly build *n*-butanol synthesis pathways with different enzyme homologs to improve pathway performance. We cycled through multiple distinct ensembles of enzymes by mixing and matching lysates containing different versions of enzymes necessary to complete the biosynthetic *n*-butanol pathway. Trying out different homologs in this manner allowed us to quickly identify a better set of enzymes producing *n*-butanol at

$0.84 \pm 0.19$  g l<sup>-1</sup> (0.09 mol *n*-butanol / mol glucose) (Fig. 3A). Specifically, we showed that Hbd2 from *C. beijerinckii* enabled a 65% increase in *n*-butanol synthesis titers over Hbd1 from *C. acetobutylicum*. A follow-up experiment doubling the Hbd1 enzyme did not alter the amount of *n*-butanol produced, suggesting that this increase was not due to discrepancies in enzyme concentrations in the lysate (Supplementary Fig. 2). However, further studies of these enzymes would elucidate whether the observed *n*-butanol production was a result of using BL21(DE3) extract without heterologous genes expressed (used for normalization), which may have more active glycolytic and byproduct pathways that could divert flux away from *n*-butanol.

While the selection of enzymes is crucial to improving *n*-butanol production, the value of each physiochemical parameter of the cell-free system also affects *n*-butanol production and becomes key in further optimization and debugging of the pathway. To demonstrate the facile nature of combinatorial optimizations in our cell-free framework, we explored changes in the ionic composition because the composition of salts added to *in vitro* systems affects the systems' performance (Jewett et al., 2008; Jewett and Swartz, 2004; Record et al., 1998; Jewett et al., 2013). Specifically, we tested the effect of using glutamate, acetate, and chloride salts on *n*-butanol production and found that glutamate salts perform more than 15% better than the other salt compositions (Supplementary Fig. 3). Our results are consistent with previous works, which have shown that glutamate salts better mimic the intracellular cytoplasmic conditions of *E. coli* to co-activate authentic biological processes such as the *in vitro* co-activation of central metabolism, oxidative phosphorylation, and protein synthesis (Jewett et al., 2008).

Beyond studying pathway performance by altering the ionic composition, the states of critical cofactors (organic molecules necessary for enzyme catalysis) can also be studied. The balance of cofactors, such as oxidized and reduced NAD, is critical to energy regeneration within the lysate by also the heterologous pathway under investigation. In our cell-free framework, the lack of a cell wall enables direct sample acquisition, reaction monitoring, and control. We used this flexibility to study the impact of the ratio of initial cofactors in the reaction to see the ratio's effect on *n*-butanol production. We found that the ratio of NAD(H) at the start of the reaction (e.g., NAD:NADH: 1:0, 1:0.5, 1:1, 0.5:1, 0:1), keeping the total cofactor concentration at 0.5 mM, plays a minimal role in



**Fig. 3.** Enzyme and physiochemical optimizations lead to increased yields of CFME *n*-butanol production. (A) Reactions for *n*-butanol production from glucose were performed using different sets of five crude lysates mixed together to obtain unique combinations of selectively overexpressed enzymes with AtoB, Hbd, Crt, Ter, and AdhE activities. Lysate mixes were combined with glutamate salts (Mg<sup>+</sup>, NH<sub>4</sub><sup>+</sup>, K<sup>+</sup>), phosphate (K<sub>2</sub>HPO<sub>4</sub>), buffer (Bis Tris), and cofactors (ATP, CoA, NAD<sup>+</sup>) and incubated for 24 h at 37 °C. (B) To enhance yields and optimize pathway performance, a physiochemical optimization was performed with or without glutamate salts (Mg<sup>+</sup>, NH<sub>4</sub><sup>+</sup>, K<sup>+</sup>), phosphate (K<sub>2</sub>HPO<sub>4</sub>), buffer (Bis Tris), and cofactors (ATP, CoA, NAD<sup>+</sup>) of cell-free reactions producing *n*-butanol. Reactions incubated for 24 h at 37 °C. The gray bars represent the same recipe in (A) and in (B). All error bars represent standard deviations with  $n \geq 3$  independent reactions.

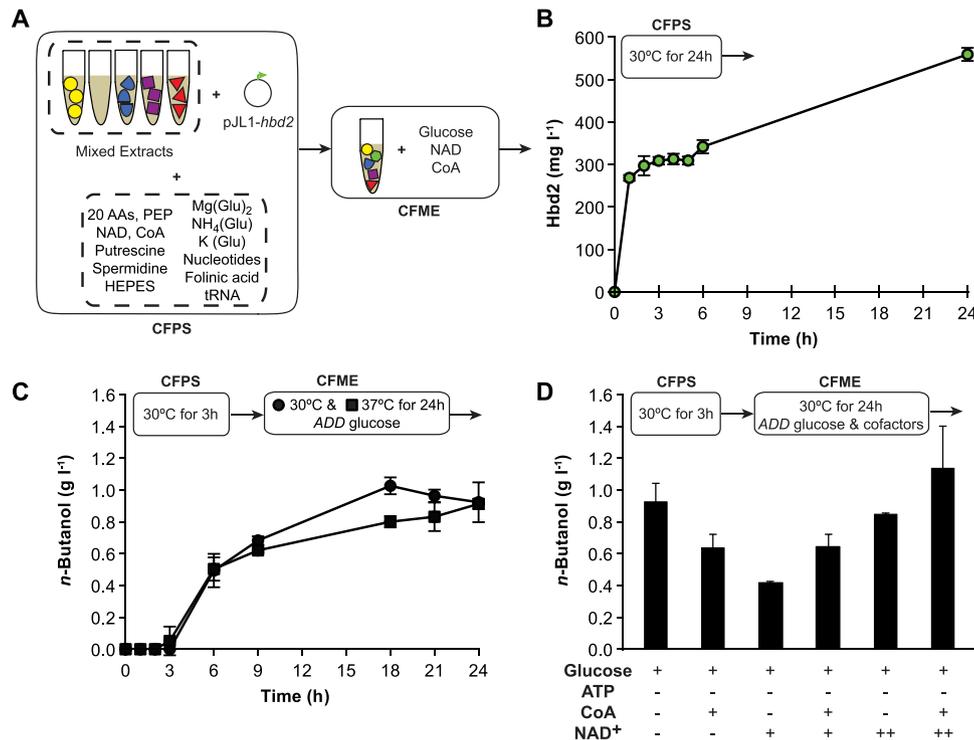
how much *n*-butanol can be produced (Supplementary Fig. 4). This suggests that metabolism in the lysate may control the overall levels of reduced and oxidized cofactor, which is consistent with data from Kay and Jewett (Kay and Jewett, 2015).

Understanding that some components play more of a role in pathway performance than others, we next performed a number of reactions to identify which added components are necessary for *n*-butanol production with a particular interest in the three added cofactors (ATP, NAD, and CoA). The supplementation of cofactors to cell-free reactions would be costly and hinder industrial practicality of this technology if it were proposed as a biomanufacturing platform. In our study of cell-free systems as a prototyping framework, we surprisingly found that omitting ATP boosts *n*-butanol production by greater than 180% from  $0.84 \pm 0.19 \text{ g l}^{-1}$  to  $1.43 \pm 0.12 \text{ g l}^{-1}$  ( $0.11 \text{ mol } n\text{-butanol/mol glucose}$ ) (Fig. 3B). More unexpectedly, by just adding salts to mimic the cytoplasm and glucose as a starting substrate we are able to produce *n*-butanol at  $0.28 \pm 0.12 \text{ g l}^{-1}$ . In other words, if lysates are prepared without dialysis, as we have done, cofactors remaining in the lysate are sufficient for the cell-free transformation and do not need to be added. Collectively, our results here show that the cell-free framework offers a strategy to explore how enzyme variants, substrates, cofactors, ionic composition, etc. can be varied in unique combinations to influence pathway performance. While CFME (i.e., selective enriching or functionalizing the lysate with pathway enzymes prior to extract generation) provides us with a rather quick way to screen enzyme ensembles and reaction conditions, this approach is limited by the cell's ability to produce the enzymes individually *in vivo*, a limitation that we address below.

### 3.2. Cell-free protein synthesis driven metabolic engineering

We next aimed to combine CFPS and CFME to modularly build the *n*-butanol pathway for forward engineering. This is dissimilar from previous works in which synthetic *in vitro* pathways have been built by purified enzymes or using lysates selectively enriched by heterologously expressed enzymes. Integration of CFPS enables one to speed up DBT cycle time for prospecting biosynthetic pathways. Indeed, using CFPS to express enzymes can reduce the time to build pathways to hours rather than days. As a proof-of-concept of this approach, we tested making Hbd2 (the non-native entry enzyme to the pathway) by CFPS to activate *n*-butanol production (Fig. 4A). The key idea of the experiment was that the pathway would remain inactive (as downstream enzymes will not have their substrates) until active Hbd2 was synthesized. We chose to validate CFPS-ME in a three-step process. First, we quantified our ability to express Hbd2 in a CFPS reaction comprised of a mixture of lysates harboring selectively enriched pathway enzymes lacking Hbd2. This was important because typical CFPS systems use lysates from cells harvested in mid-late exponential phase, where as our lysates were collected 4 h post-induction of pathway enzymes. Second, we studied the ability to activate the entire pathway by combining CFPS and CFME. Third, we carried out a series of optimizations to try to increase yields.

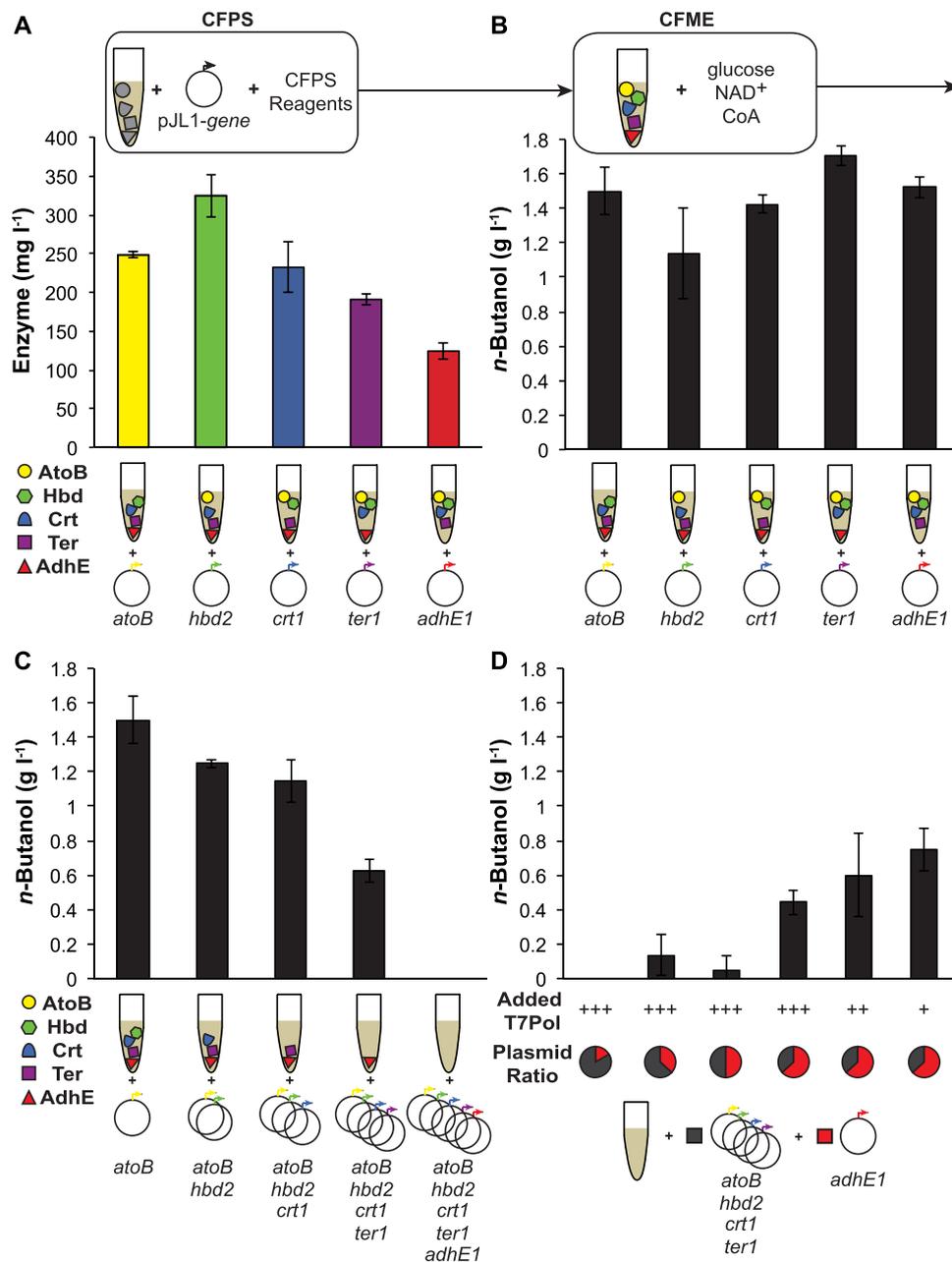
For CFPS, we used the tunable and well characterized PANOx-SP CFPS system developed by Jewett and Swartz (Jewett and Swartz, 2004) to quantitatively test the synthesis of Hbd2. CFPS reactions at 30 °C were allowed to run for 24 h in batch operation and the yields of cell-free synthesized Hbd2 was quantified by monitoring  $^{14}\text{C}$ -leucine incorporation. We based the system on a mixture of lysates used above, except the lysate with Hbd2 was not



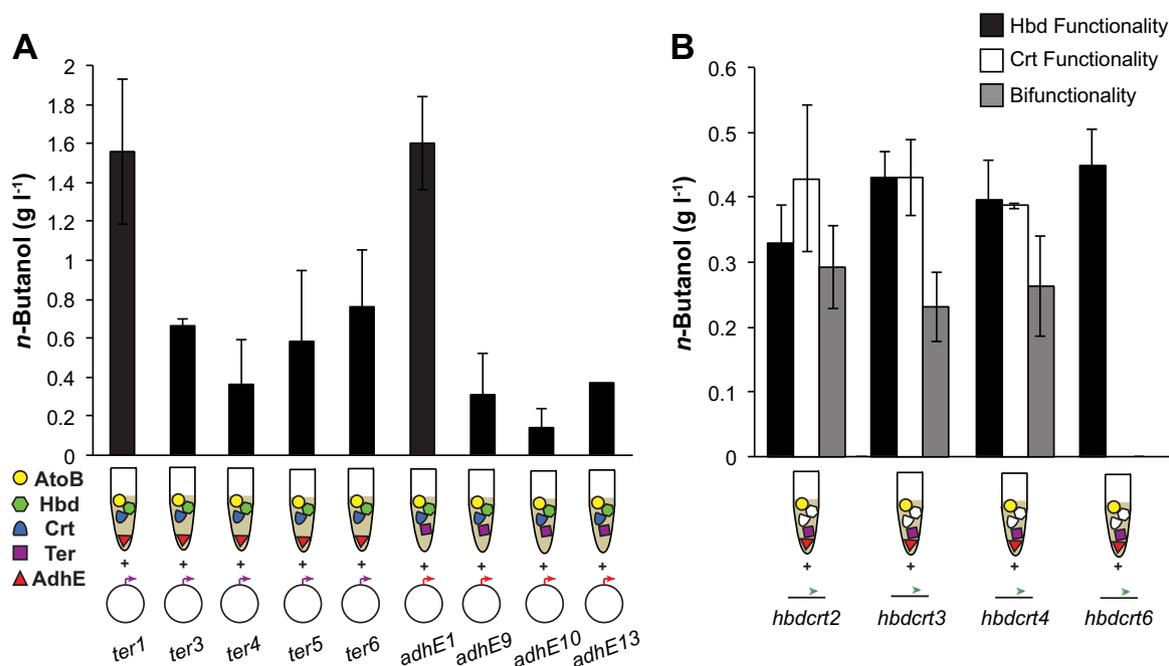
**Fig. 4.** Cell-free protein synthesis of entry enzyme activates *n*-butanol production *in vitro* by CFPS-ME approach. (A) Diagram describing the CFPS-ME experimental design. (B) Cell-free protein synthesis titers of Hbd2 from pJL1-*hbd2* in a crude lysate mixture containing AtoB (EC), Crt1 (CA), Ter1 (TD), and AdhE1 (CA) overexpressed as determined by radioactive  $^{14}\text{C}$ -leucine incorporation. CFPS reactions incubated over a 24-h period at 30 °C. (C) *n*-butanol production in the same mixed lysate system activated by cell-free synthesis of Hbd2 run at 30 °C for 3 h. Glucose was added to activate the *n*-butanol pathway and CFME reactions were incubated over a 24-h period at both 30 °C and 37 °C. (D) Cofactor (ATP, CoA, NAD<sup>+</sup>) optimization of downstream (ME portion of the CFPS-ME approach) cell-free reactions producing *n*-butanol were performed. Minus (-) signs represent no cofactor added, plus (+) signs represent mM amounts of cofactor to match conditions in CFME-alone experiments, and plus-plus (++) reactions represent double the amount of that cofactor. Reactions incubated for 24 h at 30 °C. All error bars represent standard deviations with  $n \geq 3$  independent reactions.

included. Endogenous protein synthesis machinery should act to synthesize and fold desired protein products upon incubation with essential substrates (e.g., amino acids, nucleotides, DNA or mRNA, energy substrates, cofactors, and salts). In this case, we showed that when the DNA for the Hbd2 enzyme on a pJL1 vector was added, the mixed extract could produce  $559 \pm 15 \text{ mg l}^{-1}$  of Hbd2 over a 24-h period (Fig. 4B). Based on this result and the fact that this reaction was over 50% complete by three hours, we chose to run all subsequent CFPS reactions for three hours, which should provide sufficient protein quantities for prototyping.

We next investigated the ability of the cell-free synthesized Hbd2 to activate the full *n*-butanol pathway. After three hours of CFPS, we initiated *n*-butanol metabolism by adding 200 mM glucose to the reactions. We showed that CFPS of Hbd2 could activate *n*-butanol metabolism reaching a titer of  $0.92 \pm 0.13 \text{ g l}^{-1}$  (Fig. 4C). Negative control reactions without synthesis of the Hbd2 did not produce *n*-butanol. Notably, the CFME portion resulted in the same *n*-butanol yields when carried out at either 30 or 37 °C, so for ease we selected 30 °C for all future experiments to have the CFPS and CFME portions performed at the same temperature. As in the CFME system alone, we found that small molecules, cofactors, etc.



**Fig. 5.** Using cell-free protein synthesis to activate metabolism from any node in the biosynthetic pathway. (a) Cell-free protein synthesis titers of AtoB (EC), Hbd2 (CB), Crt1 (CA), Ter1 (CA), and AdhE1 (CA) off pJL1 constructs in separate reaction mixtures as determined by radioactive <sup>14</sup>C-leucine incorporation. Each reaction mixture contained crude lysates with all pathway enzymes except the one made by CFPS. CFPS reactions were incubated for 3 h at 30 °C. (b) *n*-butanol production in the same mixed lysate system activated by CFPS of each enzyme run at 30 °C for 3 h. Glucose, CoA, and NAD<sup>+</sup> were added to activate the *n*-butanol pathway and CFME reactions were incubated for 24 h at 30 °C. (c) *n*-butanol production activated by CFPS of enzymes in combinations: (1) AtoB (EC); (2) AtoB (EC) and Hbd2 (CB); (3) AtoB (EC), Hbd2 (CB), and Crt1 (CA); (4) AtoB (EC), Hbd2 (CB), Crt1 (CA), and Ter1 (TD); and (5) AtoB (EC), Hbd2 (CB), Crt1 (CA), Ter1 (TD), and AdhE1 (CA). The CFPS reactions were run at 30 °C for 3 h. Glucose, CoA, and NAD<sup>+</sup> were added to activate the *n*-butanol pathway and reactions were incubated for 24 h at 30 °C. (d) A plasmid ratio optimization of pJL1-*adhE1* vs. all other pJL1 constructs along with a test of three concentrations of T7 polymerase. For each, CFPS was run at 30 °C for 3 h. Glucose, CoA, and NAD<sup>+</sup> were added to activate the *n*-butanol pathway and reactions were incubated for 24 h at 30 °C. All error bars represent standard deviations with  $n \geq 3$  independent reactions.



**Fig. 6.** Using CFPS-ME to rapidly screen pathway enzymes. (A) *n*-butanol production activated by CFPS of unique Ter homologs and AdhE homologs from pJL1 constructs: Ter3 (*Fibrobacter succinogenes*, FS), Ter4 (*Flavobacterium johnsoniae*, FJ), Ter5 (*Spirochaeta bajacaliforniensis*, SB), Ter6 (*Cytophaga hutchinsonii*, CH), AdhE9 (*Thermosynechococcus* sp. NK55a, TN), AdhE10 (*Providencia burhodogranariae*, PB), and AdhE13 (*Serratia marcescens*, SM). Ter homologs were expressed in crude lysate mixtures containing AtoB (EC), Hbd2 (CB), Crt1 (CA), and AdhE1 (CA) overexpressed, and AdhE homologs were expressed in lysates containing AtoB (EC), Hbd2 (CB), Crt1 (CA), and Ter1 (TD) overexpressed. (B) *n*-Butanol production activated by CFPS putative bifunctional enzymes for Hbd and Crt activity: Hbdcr2 (*Aeropyrum camini*, AC), Hbdcr3 (*Pyrobaculum aerophilum*, PA), Hbdcr4 (*Sulfolobus islandicus*, SI), and Hbdcr6 (*Sulfolobus acidocaldarius*, SA). CFPS reactions were performed from linear DNA in crude lysate mixtures containing: (1) AtoB (EC), Ter1 (TD), and AdhE1 (CA) overexpressed to test bifunctionality, (2) AtoB (EC), Crt1 (CA), Ter1 (TD), and AdhE1 (CA) overexpressed to test Hbd functionality alone, and (3) AtoB (EC), Hbd2 (CB), Ter1 (TD), and AdhE1 (CA) overexpressed to test Crt functionality alone. For each, CFPS was run at 30 °C for 3 h. Glucose, CoA, and NAD<sup>+</sup> were added to activate the *n*-butanol pathway and reactions were incubated for 24 h at 30 °C. All error bars represent standard deviations with  $n \geq 3$  independent reactions.

can modulate pathway performance. For example, we found that adding both NAD and CoA with glucose to initiate *n*-butanol metabolism after CFPS gave us  $1.22 \pm 0.22 \text{ g l}^{-1}$  *n*-butanol (Fig. 4D). Collectively, our results prove for the first time to our knowledge the ability to combine CFPS and CFME to support a highly active biosynthetic pathway.

We further extended this proof-of-concept to activate *n*-butanol production using CFPS at any pathway node by producing each *n*-butanol pathway enzyme. Using mixed extracts with all but one necessary enzyme, we performed CFPS of the ‘missing enzyme’ and saw that each enzyme could be produced individually at more than  $100 \text{ mg l}^{-1}$  without optimization (Fig. 5A). We then proved that full product of each protein is made exclusively in each reaction by an autoradiogram (Supplementary Fig. 5). After validating expression of each enzyme, we then performed CFPS-ME reactions. We carried out three-hour CFPS reactions and then initiated the *n*-butanol pathway by adding glucose, NAD, and CoA, because supplementation of CFPS-ME reactions with both NAD and CoA increased *n*-butanol titers for Hbd2 (Fig. 4D). Strikingly, CFPS-ME could be used for each of the pathway enzymes to produce *n*-butanol at levels as high as  $1.71 \pm 0.06 \text{ g l}^{-1}$  (Fig. 5B).

We next set out to demonstrate we could build the entire pathway by CFPS of the pathway enzymes in our extracts. To this end, we extended the number of enzymes made *in vitro* one by one, by adding equal amounts of DNA of each, and saw that when we made one, two, three, and four of the five enzymes necessary *in vitro* we could produce *n*-butanol at levels between  $\sim 0.6$  and  $\sim 1.4 \text{ g l}^{-1}$  (Fig. 5C). Again, full-length product of each protein is made in each reaction as shown by autoradiogram (Supplementary Fig. 6). However, as we increase the number of enzymes produced by CFPS, the amount of *n*-butanol synthesized decreases. In fact, when we tried to produce all five enzymes *in vitro* we were

initially unable to make any *n*-butanol. We attribute this drop in *n*-butanol production to there not being enough of the last enzyme in the pathway, AdhE, seen by quantification of the enzymes produced by CFPS (Supplementary Fig. 7). However, we were able to make all enzymes *in vitro* at sufficient levels necessary to make *n*-butanol at  $0.75 \pm 0.12 \text{ g l}^{-1}$  by increasing the plasmid DNA encoding AdhE to more than 50% of the total DNA added, (Fig. 5D; Supplementary Fig. 8). Reduced T7 polymerase added shows improvements in *n*-butanol production. Typical CFPS systems supplement T7 polymerase stored in glycerol, and increasing glycerol concentrations can be deleterious to the CFPS system. The extract used in this study contains T7 polymerase expressed *in vivo* prior to extract preparation, so T7 polymerase in the extract is expected to be sufficient without supplementation. Based on our result that added ATP was deleterious to *n*-butanol production by CFME (Fig. 3B), the ATP used in CFPS might be expected to inhibit CFPS-ME *n*-butanol titers if ATP is long-lived. We have previously shown that ATP concentrations are stable around  $200 \mu\text{M}$  over a  $\sim 6$  to 8 h batch CFPS reaction (Jewett and Swartz, 2004). Though, a negative effect from ATP is expected, it is difficult to use the CFME optimization conditions for CFPS-ME, given the added complexity of protein synthesis. Our results importantly showed that we could build a five-step heterologous pathway to make *n*-butanol *in vitro* in three hours.

### 3.3. Rapid prototyping and enzyme discovery with CFPS-ME

The ability to use CFPS-ME to produce enzymes for *n*-butanol biosynthesis allows us to test pathway enzymes without expressing enzymes in the host cell. As a model case study, we decided to test for improved pathway performance (increased *n*-butanol production) by swapping out some of our initial Ter and AdhE

enzymes for a variety of homologs. In less than a day, we studied 4 Ter and 3 AdhE homologs in a combined CFPS-ME reaction. In all cases, we observed synthesis of *n*-butanol, though lower than our previous best-performing enzymes (Fig. 6A). Five of these variants come from species never tested before.

Having demonstrated the ability to explore enzyme homologs using CFPS-ME, we then set out to demonstrate the potential for using linear DNA templates instead of plasmids. Using linear DNA molecules, *i.e.* PCR products, would expedite the process since the entire process could be done without cells and we could avoid laborious cloning steps. As a model system, we first repeated the experiments presented in Fig. 6 with linear templates and observed that the linear DNA templates can successfully be expressed to complete the *n*-butanol biosynthesis pathway (Supplementary Fig. 9). Next, we chose to screen multifunctional enzymes that to our knowledge have never before used for *n*-butanol production. We selected four enzymes with proposed Hbd and Crt functionalities that were identified by NCBI-BLAST searches. By preparing reactions with three different enzyme mixtures (mixed extracts with overexpressed enzymes prior to lysis) (1) without Hbd, (2) without Crt, and (3) without Hbd and Crt, we could characterize each enzyme variant by their ability to perform each enzymatic function. We discovered that each of these enzymes could activate *n*-butanol synthesis, and the proposed Hbdcrt6 from *Sulfolobus acidocaldarius* only had Hbd functionality (Fig. 6B). The ability to use linear DNA templates for CFPS-ME makes possible the ability to rapidly screen individual and sets of enzymes completely *in vitro*. Here, we used this approach to parse out individual functionalities of multi-functional enzymes.

#### 4. Discussion

In this study, we developed a new cell-free framework for prototyping biosynthetic pathways and screening enzymes. In one scenario, we overexpress individual pathway components in cells, lyse these cells, and mix and match lysates in cell-free cocktails to study biochemical pathway performance. In a distinct thrust from typical *in vitro* systems, our approach allows us to study heterologous pathways in the context of native metabolism. In another scenario, we bypass *in vivo* expression altogether by using CFPS to enrich lysates with different enzymes for combinatorial assembly of different pathways. The combination of CFPS to express homologs of individual biosynthetic enzymes for studying pathway performance is also a distinction of our workflow. In addition, the use of linear PCR templates, which could be improved by DNA stability techniques (*e.g.*, the addition of purified GamS protein) (Sun et al., 2014), allows us to avoid *in vivo* cloning steps altogether. Our CFPS-ME approach should therefore be faster than conventional approaches to select enzymes and pathway designs in cells (hours instead of days/weeks), and enables parallelized pathway construction of combinatorial designs to accelerate DBT cycles.

A key conceptual innovation of our work is that the DBT unit can be cell-free lysates rather than genetic constructs. Engineering large biosynthetic systems composed of many genes in microbes remains challenging (Smanski et al., 2014). One of the many obstacles is simply how many different genetic designs with beneficial chances are feasible to make. Cell-free systems have already been shown to screen genetic designs to improve enzyme performance at a rapid rate (Daugherty et al., 2013). Our CFPS-ME framework should allow researchers to study more designs than previously possible by rapidly prototyping enzyme performance *in vitro* before putting designs into a host. As an example, a six-step biosynthetic pathway testing 5 homologs for each enzymatic step would require testing of 15,625 pathway combinations. While

this set of combinations exceeds typical pipelines pursued in cells today, our CFPS-ME system could leverage robotic or automated liquid-handling systems to access such design space.

The goal of this manuscript was to provide a new approach to building biosynthetic pathways in a modular fashion *in vitro*. Now achieved, we plan to optimize a large-scale fermentation process with the CFPS-ME approach in the future. Towards this goal, we additionally carried out experiments to show that protein expression in the cell-free system translates to the *in vivo* system. Specifically, we took all Ter homologs screened *in vitro* by CFPS-ME (Fig. 6) and expressed them in whole cells *in vivo*. All but one of the Ter homolog proteins can be expressed in cells on a first pass (as determined by SDS-page expression, Supplementary Fig. 10). These data show that protein expression in the cell-free system can translate to the *in vivo* system. Thus our approach holds promise for identifying good enzymes that can be expressed in cells, following a body of work that uses *in vitro* enzyme assays to identify enzymes with the best-performing biochemical characteristics for desired metabolic transformations prior to putting them into a host. For example, Liao and colleagues showed that *in vitro* reconstitution could be used to construct the non-oxidative glycolytic pathway prior to *in vivo* expression (Bogorad et al., 2013), and Zhu et al. (2014) reconstituted the mevalonate pathway *in vitro* to study pathway kinetics before using the pathway *in vivo* for the production of farnesene.

Our cell-free approach mimics the intracellular environment of *E. coli*, where endogenous glycolytic enzymes from the cell extract convert glucose to AcCoA. Thus, our platform enables many different biosynthetic pathways to be studied in the context of central metabolism with enhanced control inherent to *in vitro* systems. Here, we were able to increase *n*-butanol production by ~200% of our initial starting conditions (up to ~1.5 g l<sup>-1</sup>) by simply testing the performance of different enzymes sets and adjusting the physicochemical environment. While it is difficult to compare in a normalized fashion the *in vitro* process to the *in vivo* process, our results (given as final measured concentration) are higher than some published reports of *n*-butanol production in comparable genomically unmodified hosts (Supplementary Fig. 11) (Krutsakorn et al., 2013; Gulevich et al., 2012; Nielsen et al., 2009; Inui et al., 2008). However, Bond-Watts et al. (2011) notably reported titers of 4.6 g l<sup>-1</sup> in a genomically unmodified host by selecting a particular set of synergistic enzymes and taking advantage of their chemistries. Given the reasonable yields, we were curious as to how the CFPS-ME reactions would perform at increased scale. We thus performed additional experiments of increasing size reactions to give confidence in our quantitative yields. Specifically, the reaction volume of CFPS-ME reactions was scaled from 25 to 250 μL, an order of magnitude increase (Supplementary Fig. 12). Our data shows that these reactions are scalable and are consistent with several previous works showing the ability of cell-free systems to scale (Kay and Jewett, 2015; Yin et al., 2012; Zawada et al., 2011; Voloshin and Swartz, 2005; Hong et al., 2015).

Looking forward, specialty chemicals, natural products, and materials offer an extremely diverse set of compounds with a seemingly infinite set of structures and bioactivities. Our CFPS-ME approach offers a new discovery pipeline to leverage advances in DNA sequencing and DNA synthesis to optimize biosynthetic pathways, discover new enzymes, and test new hypotheses. Because it is an open system, cell-free reactors can be readily interrogated for intermediate product formation, such as by the on-line, high speed LC/MS approaches used by Panke and colleagues for optimization of glycolysis in cell-free extracts (Bujara et al., 2011). Cell-free systems in tandem with high-end metabolomics could offer a high degree of flexibility to model the kinetics and stability of individual enzymes, measure metabolite fluxes in

multistep pathways, and experimentally isolate many other parameters confounded in living organisms. This has potential to speed up metabolic engineering DBT cycles.

### Author contributions

A.S.K. and M.C.J. conceived and designed the experiments. A.S.K. performed all of the experimental work. A.S.K. and M.C.J. wrote the manuscript.

### Competing financial interests

The authors declare no competing financial interests.

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### Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ymben.2016.03.002>.

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