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Evaluating fermentation effects on cell growth and crude extract metabolic activity for improved yeast cell-free protein synthesis

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ABSTRACT

Saccharomyces cerevisiae is a promising source organism for the development of a practical, eukaryotic crude extract based cell-free protein synthesis (CFPS) system. Crude extract CFPS systems represent a snapshot of the active metabolism *in vivo*, in response to the growth environment at the time of harvest. Therefore, fermentation plays a central role in determining metabolic activity *in vitro*. Here, we developed a fermentation protocol using chemically defined media to maximize extract performance for *S. cerevisiae*-based CFPS. Using this new protocol, we obtained a 4-fold increase in protein synthesis yields with extracts derived from wild-type S288c as compared to a previously developed protocol that uses complex growth media. The final luciferase yield in our new method was $8.86 \pm 0.28\ \mu g\ mL^{-1}$ in a 4 h batch reaction. For each of the extracts processed under different fermentation conditions, synthesized protein, precursor monomers (amino acids), and energy substrates (nucleotides) were evaluated to analyze the effect of the changes in the growth environment on cell-free metabolism. This study underscores the critical role fermentation plays in preparing crude extract for CFPS reactions and offers a simple strategy to regulate desired metabolic activity for cell-free synthetic biology applications based on crude cell extracts.

1. Introduction

Cell-free protein synthesis (CFPS) is now viewed as a versatile platform for applications in high-throughput protein expression [1,2], synthetic biology [3,4], proteomics and structural genomics [5], and expanding the chemistry of life [6–8]. The reduced process duration for protein expression (hours instead of days), potential for linear scalability, enhanced reaction control in an open environment, and ability to produce toxic and difficult-to-express proteins makes it a powerful technology for developments in biotechnology, proteomics, and synthetic biology [2]. Remarkable progress has been achieved in CFPS over the last two decades [2]. Although the prokaryotic-based *Escherichia coli* CFPS is the most commonly used platform, there remains a need to develop robust, high-yielding eukaryotic CFPS platforms capable of soluble expression of complex eukaryotic proteins with post-translational modifications.

Current eukaryotic CFPS technology includes platforms derived from wheat germ extract [9], insect cell extract [10], rabbit reticulocyte lysate [11], CHO cell extract [12], *Leishmania tarentolae* extract [13], and yeast extract [14,15], among others. Our lab has focused on the development of a robust eukaryotic CFPS system using *S. cerevisiae* extract [14]. Advantages include simple growth, facile genetic manipulation techniques, and utility as a biomanufacturing organism for biotechnology applications [16]. *S. cerevisiae*

Abbreviations: ADP, adenosine diphosphate; AMP, adenosine monophosphate; ANOVA, analysis of variance; ATP, adenosine triphosphate; CFPS, cell-free protein synthesis; CHO, Chinese hamster ovary; E.C., adenylate energy charge; eIF, eukaryotic initiation factor; HPLC, high performance liquid chromatography; IRES, internal ribosome entry site; IVC, integral of cell viability; NTP, nucleoside triphosphate; OD_{600}, optical density at 600 nm; PCR, polymerase chain reaction; RI, refractive index; SC, synthetic complete; SD, synthetic dextrose; YNB, yeast nitrogen base; YPD, yeast extract-peptone-dextrose media.

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has additionally been employed extensively in research and industry and therefore has very well characterized physiology, genetics, biochemistry and fermentation conditions. Furthermore, cell-free translation reactions using extract from *S. cerevisiae* have been a historical resource for studying the function of translation initiation factors [17]. These characteristics make *S. cerevisiae* a promising candidate for the development of an easy-to use, cost–effective, and high yielding eukaryotic CFPS platform. However, the utility of yeast CFPS would be improved with an increase in reaction yield (g protein/L reaction volume) and a better understanding of the underlying metabolism.

During CFPS, crude cellular extract provides the translational machinery that drives the protein synthesis reaction. However, the extract also contains catalysts involved in metabolic pathways, nucleotide recycling enzymes, and protein folding enzymes (*e.g.*, chaperones, foldases, *etc.*), among others. It is now appreciated that the presence of these enzymes leads to non-protein producing metabolic activity in the extract [2]. While some of these reactions can be utilized to support CFPS reactions, such as energy regeneration [18], other active metabolism could be characterized as deleterious to protein synthesis, for example leading to substrate and energy limitations [19,20]. For CFPS reactions, the active metabolism of the crude extract is a function of the active metabolism present in the living cell at the time of harvest. Thus, altering the fermentation prior to extract preparation is a simple way to control the crude extract metabolic activity and potentially increase CFPS productivity.

Previous work has demonstrated that the composition of the fermentation media can be altered to ensure consistent growth of source strains for robust extract preparation [21] and to enhance CFPS performance [22–24]. For example, in the S30 *E. coli* CFPS platform, reaction efficiency and duration was increased by inhibiting deleterious phosphatase activity through supplementing the growth media with 50 mM inorganic phosphate [19] and tryptophan concentration was stabilized by inhibiting tryptophanase activity through growing the cells on glucose [25]. Despite these works, very few examples have yet to systematically quantify the impact of growth media on non-bacterial CFPS systems.

Here, we sought to develop a chemically defined medium to improve yeast CFPS and to better understand the metabolic activity of the extract. The development of a chemically defined media protocol is important because is permits straightforward identification of metabolic transitions during growth, provides flexibility to modify the precise composition of the media, and enables extract batch-to-batch consistency. Understanding the relationship between cell growth and extract metabolic activity is important because various conditions could have a favorable or unfavorable impact on cell-free productivity.

Indeed, it is well known that *S. cerevisiae* has evolved metabolic controls to be very sensitive to the external nutrient environment [26–30]. Changes in nutrient compositions, many of which occur dynamically over the course or batch fermentation, elicit multiple responses regulating major metabolic pathways, including: catabolite repression/derepression, general amino acid control, and stress responses [30–32]. For example, amino acid starvation and glucose limitation have been shown to inhibit translation initiation [32,50]. In another example, glucose catabolite repression in *S. cerevisiae* represses enzymes related to cellular respiration, mitochondrial biosynthesis, and multiple other metabolic processes [33–35]. A shift in central carbon metabolism occurs in *S. cerevisiae* from primarily fermentative to primarily respirative metabolism in response to extracellular glucose levels [30,36]. Previously, this shift in central carbon metabolism has been shown to influence the cellular transcription profile and metabolome [35,37]. These transitions determine the nature of non-protein producing reactions present during CFPS, which affect CFPS yields. Therefore, characterizing and optimizing the fermentation step during extract preparation for *S. cerevisiae* is crucial to ensure optimal biochemical activity during CFPS (Fig. 1A).

With an aim to improve yeast CFPS productivity and better understand the active metabolism present in the reaction, we assessed the impact of different chemically defined media compositions on CFPS yields, quantified metabolic activity in the CFPS reaction in response to media composition, and optimized the ideal growth phase for cell harvest (Fig. 1B). Ultimately this led to the adoption of an improved fermentation protocol using synthetic complete media supplemented with amino acids and a 4-fold increase in productivity compared to cells grown on complex media. This study highlights how the optimization of fermentation conditions can be a simple approach to influence metabolic activity present in cell-free reactions.

2. Materials and methods

2.1. Cell growth and extract preparation

S. cerevisiae S288c was used as the source strain. Cells were cultivated on chemically defined media composed of 6.7 g L^{-1} Yeast Nitrogen Base (YNB) (Sigma–Aldrich, St. Louis, MO), 20 g L^{-1} glucose and 50 mM potassium phosphate buffer, pH 5.5, hereby referred to as Synthetic Dextrose (SD) Media. In addition, Synthetic Complete (SC) Media was used as denoted in the text and is composed of SD media with the addition of Synthetic Complete Drop Out (2.002 g L^{-1}) Supplements (ForMedium™, Norfolk, United Kingdom) [38]. YPD was the complex media used in this study composed of 10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose, and 50 mM phosphate buffer (pH 5.5). An alternate SC media was used as denoted in the text composed of YNB without amino acids and ammonium sulfate (Sigma–Aldrich, St. Louis, MO) and 5.6 g L^{-1} glutamine (Sigma–Aldrich, St. Louis, MO), while the remaining SC components were unaltered.

Fermentation characterization was completed using 1 L BIOSTAT® Q plus bioreactors (Sartorius Stedim Biotech S.A., Aubagne Cedex, France). Batch fermentations were carried out in a 5 L New Brunswick Scientific Fermenter (Eppendorf, Enfield, CT) with a working volume of 5 L, stirred at 400 rpm, and sparged with air at 1 vvm. The pH during fermentation was maintained at 5.5 using 5 N potassium hydroxide. In addition, some extracts were processed from chemostats as denoted in the text. Dilution rates were carried out at 0.25 h^{-1}, 0.35 h^{-1} and 0.45 h^{-1} using the batch SC media as the feed.

When the OD_{600} reached the desired value, the culture was cooled quickly by harvesting through a stainless steel coil immersed in an ice bath. Cells grown on defined media were harvested at 6 OD_{600}, unless otherwise denoted in the text. Cells were pelleted and washed as described previously [14]. Then, extracts were prepared as described previously with high-pressure homogenization for cell lysis and dialysis for buffer exchange [14]. For the extracts prepared from chemostats, the cells were harvested after they had reached steady state at their respective dilution rate. Dry weight, metabolites, dissolved oxygen and carbon dioxide profiles were constant for ~4 residence times before cell collection.

2.2. Cell-free protein synthesis reactions

CFPS reactions were performed as described previously [14], with the exception of using 6.67 μg mL^{-1} Ω-Luc-A_{50} PCR amplified DNA. The Ω-Luc-A_{50} PCR template was constructed as previously described [14] with the exception that a 50-mer poly(A) tail was used at the 3′ end of the coding sequence [39]. Samples were collected from batch CFPS reactions at 0, 0.25, 0.5, 0.75, 1, 1.5,

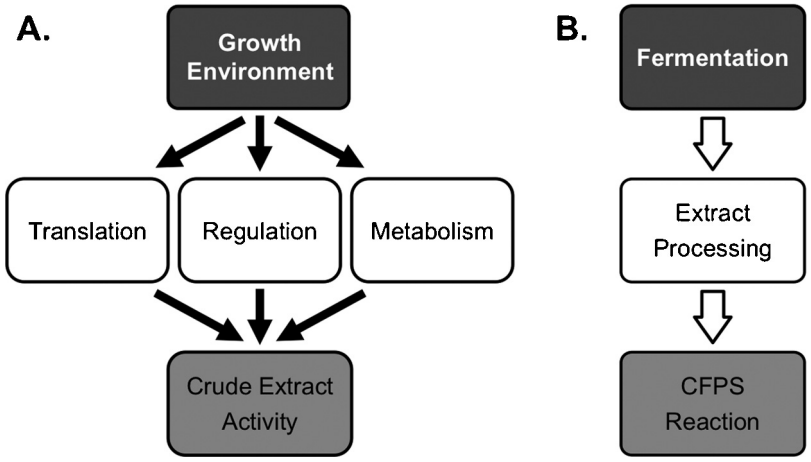

Fig. 1. Flow diagram of fermentation effects on biochemical activity present in CFPS reactions. (A) A logic flow diagram is presented demonstrating how the growth environment during fermentation influences active translation, metabolism, and regulation inside the cell, which is retained by the crude extract used in CFPS reactions. The metabolic activity of the crude extract is a snapshot of intracellular metabolism and regulation, therefore the effects of changes made in the growth environment (Dark Gray Box) is observed in crude extract activity (Light Gray Box). (B) A process flow diagram of the experimental setup. Similarly to the logic flow diagram, the dark gray box represents the processing step where variations in the protocol were made and the light gray box represents the step at which the effect of the change was analyzed.

2, 2.5, 3, and 4 h and assayed for active firefly luciferase. Active firefly luciferase was measured *via* relative luminescence intensity using ONE-Glo™ Luciferase Assay System (Promega, Madison WI) compared to a linear standard curve of recombinant luciferase (Promega).

2.3. Quantitative HPLC analysis of media substrates

High-performance liquid chromatography (HPLC) analysis was used to measure substrates present in the growth media during fermentation. Glucose, ethanol, glycerol, acetate, and pyruvate were measured using an Agilent 1260 series HPLC system (Agilent, Santa Clara, CA). At each appropriate time point, 1 mL of media was sampled and promptly centrifuged at 16,000 × *g* for 1 min at 4 °C to pellet the cells. The supernatant was collected and filtered using a 0.2 μm filter. The filtrate was subsequently analyzed using an Aminex HPX 87-H column (Bio-Rad, Hercules, CA) with an isocratic flow of 0.6 mL min^{-1} of 5 mM sulfuric acid at 55 °C for 30 min. Acetate and pyruvate were measured using UV absorbance at 210 nm. Glucose, ethanol, and glycerol were measured *via* refractive index (RI). Concentrations were determined by comparison to a standard calibration.

2.4. Quantitative HPLC analysis of nucleotides and amino acids in CFPS reactions

Nucleotides and amino acids were measured *via* HPLC as described previously [20].

3. Theory

3.1. Integral of viable cell density approach for mathematical modeling of fermentation

To identify different harvest ODs that might correspond to different metabolic states, we characterized metabolic transitions occurring during batch fermentation by identifying shifts in consumption and production rates of the cellular metabolites using the integral of viable cell (IVC) density approach, which has been previously employed for metabolic characterization of mammalian cell cultures [40,41]. The integral of viable cell density (IVC) approach was used to model the growth rate and substrate utilization/production rates [42].

$$\frac{dx}{dt} = \mu * X;\ \frac{ds}{dt} = -q_s * X;\ \frac{dp}{dt} = q_p * X \quad (1)$$

The IVC approach can be utilized to estimate the values for specific growth rate (μ), specific substrate consumption rate (q_s) and specific product formation rate (q_p), by measuring cell concentration (X), substrate concentration (S), and product concentration (P) in a batch reaction over time (t). According to this approach:

$$\int dX = -\mu * \int Xdt \quad (2)$$

$$\int dS = -q_s * \int Xdt \quad (3)$$

$$\int dP = -q_p * \int Xdt \quad (4)$$

where the term $\int Xdt$ is referred to as the IVC, which represents the time integral of viable cell density. A plot of substrate or product concentration *versus* IVC provides a linear regression used to estimate specific substrate utilization and production rates, respectively. This method has been previously employed for kinetic analysis of experimental data with animal cell cultures [40,41]. Here, our objective varied slightly and we employed the IVC approach to characterize specific product formation and substrate utilization during yeast fermentation to identify the presence of a metabolic shift during cell growth. To employ the IVC model we made the following assumptions:

1. During exponential growth of cells, it was assumed that the cells maintained 100% viability. Therefore, the optical cell density (OD) is an accurate representative of the live cell concentration.
2. The production of ethanol is assumed to be growth dependent, as ethanol is a product of the central carbon fermentative pathway.
3. The loss of ethanol to vaporization was assumed to be negligible and proportional to the aqueous concentration. Using Henry's law equations, we estimated that the maximal ethanol concentration of 7.46 g L^{-1} in the media corresponds to ethanol concentration in the gas phase of approximately 0.0022 g L^{-1}.

For our analysis, we measured biomass, glucose consumption, and ethanol production from three separate fermentations of yeast

grown on SC media. The collective data was consolidated and regression analysis was used to estimate the growth and substrate utilization/production rates before and after the metabolic shift. An example sample calculation is provided in Supplementary Table 1.

4. Results

4.1. Evaluation of chemically defined media with supplements

To evaluate the impact of different growth media on extract performance in yeast CFPS, three common media types were used for yeast fermentation: YPD complex media, Synthetic Dextrose (SD) defined media, and Synthetic Complete (SC) defined media. We prepared yeast extract from cells grown on each media type and compared the CFPS yields (as defined by gram protein per volume reaction) for each extract. In order to ensure the extract processing was consistent, we compared CFPS yields from multiple extracts processed with the three media types and found the percent error in CFPS yields ranged from 4.5 to 13% (Supplementary Fig. 1). The performance of the three lysates was significantly different, as determined by a single-factor ANOVA. Then, we used a pair-wise statistical test to compare each of the matched conditions. Notably, luciferase yields for extracts derived from cells grown on SC media were significantly greater than yields from cells grown on SD or YPAD media ($P < 0.0001$, Student's t-test); whereas extracts derived from cells grown on SD and YPAD media were not significantly different ($P = 0.7814$, Student's t-test).

Time course analysis of CFPS for each extract type was conducted to determine the source of the difference in protein synthesis yields (Fig. 2A). Extracts prepared using complex YPD media and synthetic dextrose media had similar luciferase yields of $2.56 \pm 0.15\ \mu g\ mL^{-1}$ and $2.44 \pm 0.30\ \mu g\ mL^{-1}$, respectively. However, extracts processed using cells grown on SC media saw a 2-fold improvement in active luciferase yields up to $4.97 \pm 0.02\ \mu g\ mL^{-1}$. Interestingly, the majority of the difference in protein synthesis yields between extracts prepared on SD and SC media was due to a difference in linear protein synthesis rate (between 0.25 h and 1 h), which increased from $2.43\ \mu g\ mL^{-1}\ h^{-1}$ for extracts from SD media to $5.03\ \mu g\ mL^{-1}\ h^{-1}$ for extracts from SC media (Fig. 2A). In all CFPS reactions, the reaction duration was similar, with protein synthesis terminating by 2 h (Fig. 2A).

The major difference between SD and SC media is the presence of amino acid supplements. To characterize the difference in amino acid metabolism between the two extracts derived from these media we evaluated the dynamic concentration of 19 amino acids (glutamate was excluded due to a concentration >100 mM) during the course of the CFPS reaction (Fig. 2B–E and Supplementary Fig. 2). Although there was an observable difference in the kinetics of the amino acid metabolic activity over time, there was no obvious amino acid substrate limitation in either reaction.

Because there were no observed amino acid substrate limitations, we then turned to evaluate the difference in reaction energetics between the two extracts derived from SD and SC media (Fig. 3). ATP consumption in non-protein producing metabolic pathways has been shown to cause energy limitations in yeast CFPS reactions [20]. To determine if there was an energy limitation difference between the two extracts, we measured the adenosine nucleoside phosphate pool over the course of the CFPS reaction (Fig. 3). We found the adenosine nucleoside phosphate profiles to be similar (Fig. 3A–C). In addition, adenylate energy charge (E.C.), which has been previously shown to be an important determinant of CFPS reaction lifetime *in vitro* [20,43], was also similar between the two extracts (Fig. 3D).

We also evaluated glutamine as an alternative preferred nitrogen source for fermentation. However the use of glutamine in the media lead to a decrease in protein yields to $3.67 \pm 0.06\ \mu g\ mL^{-1}$ and was not pursued further (Supplementary Fig. 3). Specifically, statistical analysis showed that luciferase yields for extract from cells grown using glutamine is significantly lower than those from cells grown using ammonium sulfate ($P < 0.001$, Student's t-test).

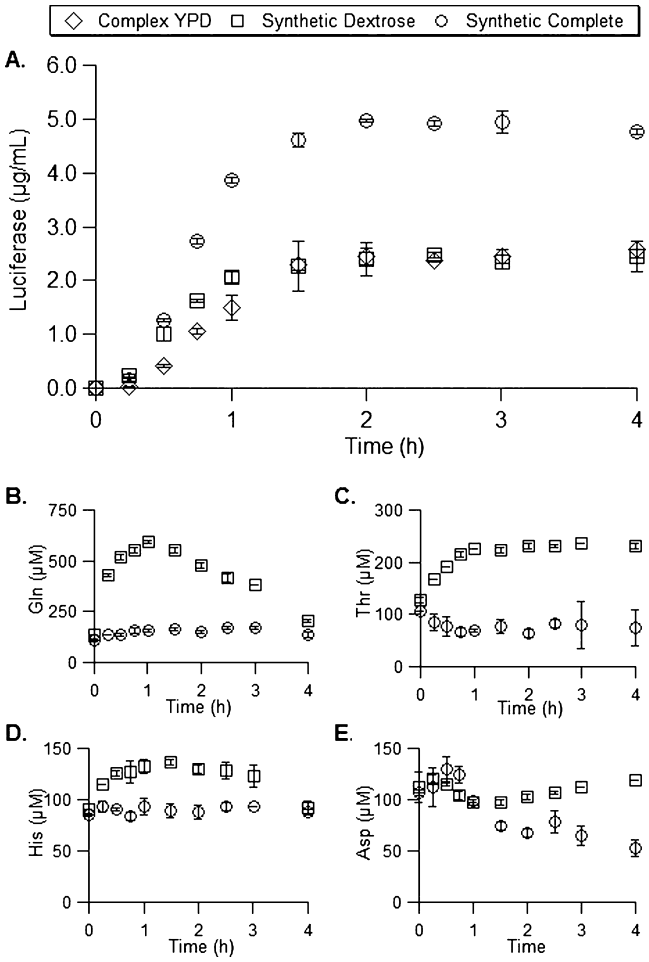

Fig. 2. Time course analysis of protein synthesis and amino acid profiles from CFPS reactions with extracts prepared from various media sources. (A) Active luciferase synthesis is shown over the course of a standard batch reaction. Reactions were carried out using extracts processed from cells grown on complex YPD media (diamonds), Synthetic Dextrose media (squares), and Synthetic Complete media (circles). (B–E) Time course analysis of select amino acids, which have ATP dependent metabolic pathways associated with them, including (B) glutamine (glutamate family), (C) threonine (oxaloacetate/aspartate family), (D) histidine (ribose 5-phosphate family), and (G) aspartic acid (oxaloacetate/aspartate family). All measured amino acids can be found in Supplementary Fig. 2. Values show means with error bars representing the standard deviations of three independent experiments.

4.2. Characterization of fermentation on synthetic complete media

Extracts prepared from cells grown on SC media had the highest CFPS yield from the media evaluated in Fig. 2A. We next sought to further increase protein synthesis yields and quantitatively characterize fermentation of yeast culture grown on SC media by assessing the impact of harvest OD and the dynamic changes in cellular metabolism on S288c extract activity. To do this, we first analyzed the growth rate, substrate consumption, and product formation rates during batch fermentation. A complete carbon analysis was performed on the fermentation broth to measure glucose and its metabolic products, including biomass and ethanol (Fig. 4). The carbon balance is provided in Supplementary Table 2, noting that the balance closed to within 2% of theoretical.

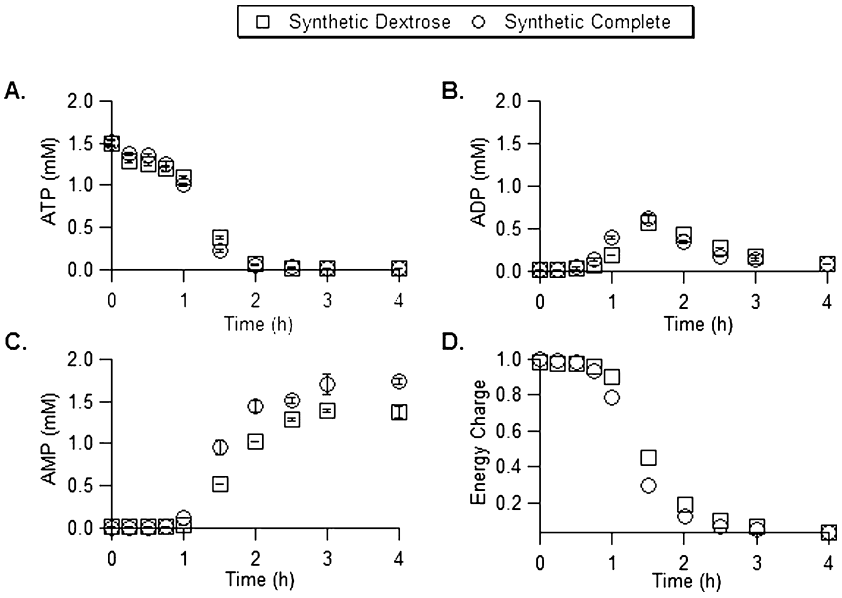

Fig. 3. Time course analysis of adenosine nucleoside phosphates and energy charge from CFPS reactions with extracts prepared from cells grown on various media sources. Batch reactions were sampled for adenosine nucleoside phosphate (AXP) concentrations at appropriate time points and measured using HPLC, including (A) ATP, (B) ADP, and (C) AMP. Reactions were carried out using extracts processed from cells grown on Synthetic Dextrose media (squares) and Synthetic Complete media (circles). Values show means with error bars representing the standard deviations of three independent experiments. (D) Adenylate Energy Charge was calculated using the measured ATP, AMP, and ADP values (A–C) and is displayed over the course of a 4 h CFPS reaction.

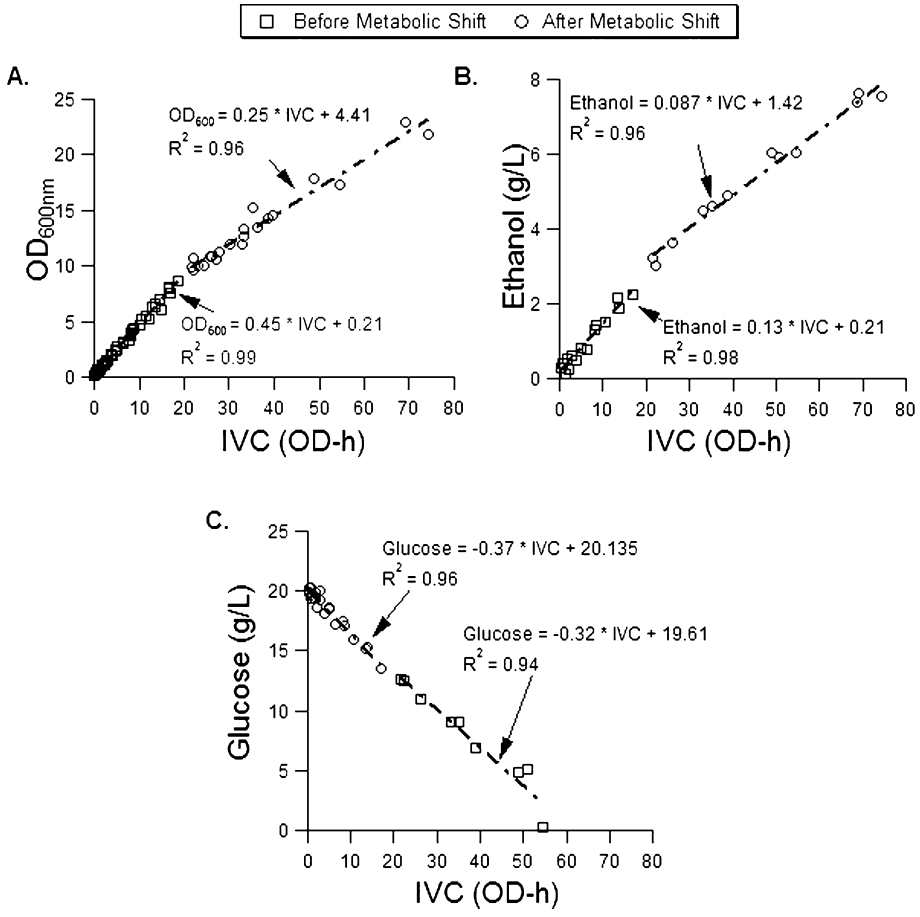

Fig. 4. Characterization of batch fermentation of yeast grown on SC media. One liter bioreactors were used for fermentation of yeast on SC media. (A) Cell density (OD_{600}), (B) ethanol, and (C) glucose were measured at the appropriate time points and pooled together from 3 independent fermentations onto a single plot using the Integral of Viable Cell (IVC) density approach [42].

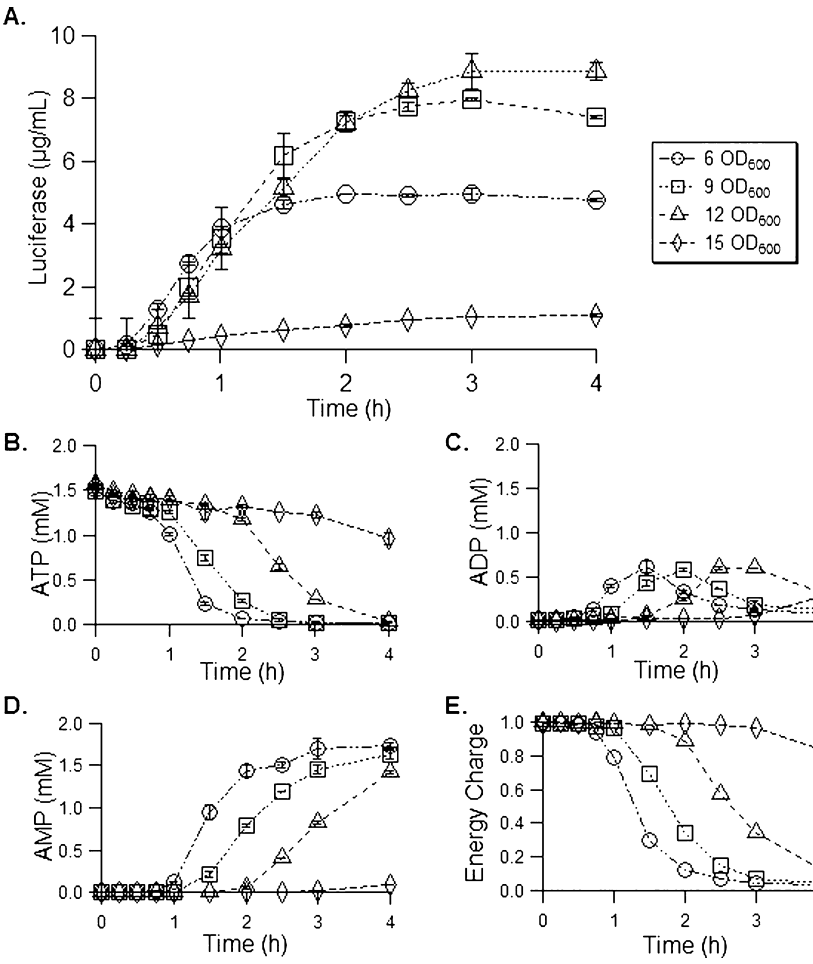

Fig. 5. Time course analysis of protein synthesis, adenosine nucleoside phosphates, and energy charge from CFPS reactions with extract prepared from cells grown on SC media harvested at various OD_{600}. (A) Active luciferase synthesis is shown over the course of a standard batch reaction. Batch reactions were sampled for active luciferase yield at the specified time points and measured using luminescence intensity. Reactions were carries out using extracts processed from cells harvested at 6 OD_{600} (circles), 9 OD_{600} (squares), 12 OD_{600} (triangles) and 15 OD_{600} (diamonds). Reactions were also sampled for adenosine nucleoside phosphate (AXP) concentrations and measured using HPLC, including (B) ATP, (C) ADP, and (D) AMP. Values show means with error bars representing the standard deviations of three independent experiments. (E) Adenylate Energy Charge was calculated using the measured ATP, ADP, and AMP values (A–C) and is displayed over the course of a 4 h CFPS reaction.

We observed a change in the growth rate from 0.45 h^{-1} to 0.25 h^{-1} around 10 OD_{600} during batch fermentation (Fig. 4A) as well as a change in the ethanol production rate from 0.13 to 0.09 g L^{-1} h^{-1} (Fig. 4B). However, the glucose uptake rate remained unchanged (Fig. 4C), suggesting a shift in metabolism downstream of glucose transport.

4.3. Optimization of growth phase for cell harvest

Based on the characterized shift in metabolism at 10 OD_{600}, cells were harvested at 6, 9, 12, and 15 OD_{600}, representing different points in the two exponential growth phases observed. Extracts were processed from each of these cell pellets and protein synthesis was evaluated (Fig. 5A). A 2-fold increase in active firefly luciferase yields was observed from 4.75 ± 0.29 μg mL^{-1} with extract prepared from cells harvested at 6 OD_{600} to 8.86 ± 0.28 μg mL^{-1} with extract prepared from cells harvested at 12 OD_{600}. However, extract prepared from cells harvested at 15 OD_{600} saw a dramatic 8-fold drop in active luciferase yields to 1.11 ± 0.04 μg mL^{-1}. Following the hypothesis that the metabolic phase of harvest influenced the overall cell-free metabolism, we carried out time course analyses of the CFPS reaction to measure concentrations of nucleotides (Fig. 5B–E) and amino acids (Supplementary Fig. 4). We observed that as the harvest OD_{600} increased, the overall rate of ATP depletion decreased (Fig. 5B). Corresponding shifts can also be observed in the metabolism for ADP and AMP (Fig. 5C and D). Finally, the overall duration for which the adenylate energy charge (E.C.) is above the proposed energetically limiting threshold of 0.8 also increases with the harvest OD_{600} (Fig. 5E) [43]. The initial linear protein synthesis rates for the extracts harvested at 6 OD_{600}, 9 OD_{600}, and 12 OD_{600} was similar with an average of 5.02 ± 0.22 μg mL^{-1} h^{-1}, while the duration of protein synthesis increased from 1.5 h for extract harvested at 6 OD_{600} to 2.5 h for extract harvested at 12 OD_{600}. Although protein synthesis for 15 OD_{600} is extended to 3 h and the ATP concentration is mostly stable, the initial linear synthesis rate is greatly reduced to 0.54 μg mL^{-1} h^{-1}.

4.4. Chemostat cell culture

We next evaluated the merits of using a chemostat for cell culture because of the potential bioprocessing advantages it could offer to extract preparation. Moreover, we wanted to assess the impact of growth rate, as well as a nutrient limitation, on CFPS yields. Specifically, we grew the S288c source strain in SC media, which had generated the highest yielding extracts above, using a 1 L jacketed chemostat with a constant working volume of 0.75 L at a dilution rate of 0.25 h^{-1}, 0.35 h^{-1}, and 0.45 h^{-1}. We observed that extracts processed

from cells cultivated using chemostats had low firefly luciferase yields of $0.84 \pm 0.04\ \mu g\ mL^{-1}$ ($D = 0.45\ h^{-1}$), $0.26 \pm 0.01\ \mu g\ mL^{-1}$ ($D = 0.35\ h^{-1}$) and $0.21 \pm 0.01\ \mu g\ mL^{-1}$ ($D = 0.25\ h^{-1}$) (Supplementary Fig. 5). This was 9.50%, 2.97% and 2.44%, respectively, of the total protein synthesized from extract prepared from batch growth harvested at 12 OD_{600}. These results highlight the difficulty of developing fermentation approaches for CFPS systems, which we discuss further below.

5. Discussion

This work has developed a new defined media fermentation protocol for preparation of yeast extract used in CFPS reactions and characterized its impact on cell-free metabolism. Strikingly, by simply altering the growth media, we observed a 4-fold increase in active firefly luciferase yields. As highlighted in Fig. 1A, the fermentation step during extract processing controls the growth environment, which in turn influences metabolism, regulation, and translational activity present *in vivo* and *in vitro*. Not only were yields improved, but a defined chemical medium provides benefits for preparation of more robust and consistent extract batches.

The set of fermentation conditions evaluated were chosen after a thorough review of previous works that have studied the impact of nutrient composition on cell growth, protein synthesis regulation, and metabolic activity [31–33,35,44–47]. Based on these works, we focused our efforts on fermentation conditions that would avoid down-regulating or inhibiting translation and/or metabolic activity (*i.e.*, non-stress conditions). We therefore chose a preferred carbon source of glucose and preferred nitrogen sources of ammonium sulfate and glutamine with established pH, aeration, and agitation rates widely used for *S. cerevisiae* fermentation [32]. We did not vary the fermentation parameters such as pH and agitation because with each fermentation condition investigated, we sought to focus on careful analysis of the protein synthesis kinetics as well as substrate and energy metabolism in the extract. While we expect to examine the effect of other fermentation parameters in future works, our approach allowed us to directly quantify the effects of media components on extract metabolic activity. To our knowledge, such a detailed characterization has never been done before for any eukaryotic CFPS platform.

The variation in amino acid metabolism from extracts prepared using SD media compared to SC media (supplemented with an amino acid mixture) is an illustrative example that showcases how the growth conditions are tied to CFPS metabolism (Fig. 2B–E). Specifically, we observed synthesis of glutamine, threonine, and histidine during the first hour of the reaction in extracts prepared from SD media and not in extracts prepared from SC media (Fig. 2B–D). This is likely a reflection of the activation of amino acid biosynthetic pathways present in yeast cultured on SD media. As an added benefit, previous analysis of *S. cerevisiae* extracts processed using complex YPD media showed instability in aspartic acid during CFPS [20], which we overcome by the usage of chemically defined media reported here (Fig. 2E). Lastly, in CFPS reactions with the best performing extract (cells harvest at 12 OD_{600} using SC media), all amino acids were stable throughout the course of active protein synthesis (Supplementary Fig. 4), suggesting additional considerations such as energy limitations, mRNA degradation, or catalyst instability, could contribute to reaction termination [20].

We also found the growth phase at the time of cell harvest to have a strong influence on cell-free metabolism. *S. cerevisiae* is crabtree-positive organism and at high concentrations of glucose respiratory enzymes are repressed and fermentation is the primary mode for utilizing glucose [47]. Changes in the extracellular glucose concentration can activate a metabolic shift in glucose utilization from fermentation to respiration and leads to a more efficient use of glucose [48]. This is a possible explanation for the metabolic shift observed in the growth and ethanol production rates observed during batch fermentation (Fig. 3A and B).

The effects of dynamic metabolic changes during *in vivo* cell growth of the source strain was observed to impact cell-free metabolism (Fig. 5). For example, we observe a decrease in ATP utilization and stabilization of the E.C. in the CFPS reaction as the harvest OD_{600} increases from 6 up to 12 (Fig. 5B–E). While the exact mechanism remains unknown, a possible explanation could be due to the regulation of intracellular ATP utilizing processes in response to reducing glucose concentrations during cell growth. Previously, it has been shown that an E.C. value greater than 0.8 is an important determinant of reaction lifetime [20,43]. We observed the duration of sustained E.C. is increased from 1 h in CFPS reactions with extracts prepared from cells harvested at 6 OD_{600} to 2 h with extracts prepared from cells harvested at 12 OD_{600} (Fig. 5E) and protein synthesis is prolonged from 1.5 h to 3 h with improved yields (Fig. 5A). Similarly, we observed that dropping below the E.C. threshold not only affects protein synthesis, but also the overall metabolism in the cell-free reaction, as evidenced by shift in amino acid metabolism (Fig. 2B–E and Supplementary Figs. 2 and 4). Furthermore, glutamine was previously reported to increase the intracellular ATP to ADP ratio [49]. Therefore, we evaluated if the utilization of glutamine would lead to an improvement in extract activity for yeast CFPS; however, the extract made with glutamine was only 55% as active as the extract using ammonium sulfate.

Surprisingly, the trend in E.C. duration extension continues with extract prepared from cells harvested at 15 OD_{600}, with E.C. above 0.8 beyond 4 h (Fig. 5E). However, there is a significant reduction in protein synthesis yield from these extracts (Fig. 5A). This reduction in protein synthesis is a result of reduced synthesis rate as opposed to reduced reaction duration. One possible explanation consistent with a reduced protein synthesis rate could be a decreased number of translation initiation events caused by translation initiation down-regulation. In yeast, it has been shown that glucose depletion rapidly inhibits translation initiation [50]. In Fig. 4, we observed that at 15 OD_{600} (corresponding to IVC value of 45–50), the extracellular glucose concentration is reduced from $20\ g\ L^{-1}$ to $<5\ g\ L^{-1}$. Therefore, the reduction in translation rate could be due to translation initiation down-regulation in response to a glucose limitation stress response [44]. A similar phenomenon is seen in extract prepared from SD media compared to extract prepared from SC media (Fig. 2A), which in this case could be a function of amino acid starvation stress response [32,51].

Our results showcase the challenges associated with fermentation development for CFPS systems and how conditions of stress (*e.g.*, glucose starvation or amino acid limitation) can be deleterious to CFPS performance. There are many complex regulatory strategies used by yeast that down-regulate translation activity in times of stress that would prove undesirable for highly active extract preparation. Supplementary Table 3 summarizes some common growth conditions for yeast that would expect to negatively impact CFPS (*e.g.*, ethanol excess, nitrogen limitation, and carbon limitation).

Evidence that stress caused by nutrient limitation might be undesirable for CFPS can be seen from the extracts prepared using a chemostat for cell growth. Of the dilution rates evaluated, all extracts had CFPS yields less than 10% compared to extract prepared from batch growth harvested at 12 OD_{600} (*i.e.*, when substrates were not limiting and the cells were less stressed). The reduced luciferase yields may be attributed to translation inhibition as a response to stress or nutrient limitation. For example, our glucose limited (C-limited) chemostat data (from $D = 0.45\ h^{-1}$) are consistent with the poor protein synthesis performance of the batch reactions harvested at 15 OD_{600} (where glucose was found to be $<5\ g\ L^{-1}$).

While it is clear that the conditions studied here represent only one set of possible cell growth conditions, the methodology

developed by our study could in the future be applied as a template for characterization and optimization of other fermentation conditions. Given that the batch fermentation performed best, this could include the development of high-density fed-batch fermentation with consistent extract metabolic activity, similar to the work completed in the development of the *E. coli* CFPS platform [21,52].

6. Conclusion

In this work, we demonstrated how varying fermentation conditions can be used as an effective method to tune metabolic activity present in CFPS reactions. In addition, we developed a defined media protocol that led to a 4-fold improvement in CFPS yields. Although cell-free systems may offer reduced biological complexity compared to their *in vivo* counterpart, this study re-affirms the presence of vast biochemical complexity and highlights how closely the *in vivo* and *in vitro* platforms are related. In particular, when preparing extract for cell-free reactions the growth conditions play critical role with respect to both promoting the desired activity and suppressing undesired activity. By improving our understanding of how fermentation impacts cell-free metabolism and protein synthesis, we have enhanced our understanding of the overall process and improved CFPS yields. In addition, the general strategy presented here could be used in reverse to optimize growth conditions for fermentation using cell-free extracts as a method to approximate changes in *in vivo* metabolic and translation activity. Furthermore, we expect that methods proposed here would be useful when designing cell-free metabolic engineering platforms derived from crude cell extracts and cell-free protein synthesis platforms from alternative source organisms.

Conflict of interest statement

The authors declare no commercial or financial conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bej.2014.07.014.

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