Biochemical Engineering Journal 91 (2014) 140–148

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

Regular Article

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

Alaksh Choudhury[a,b,c,1], C. Eric Hodgman[b,c,1], Mark J. Anderson[b,c], Michael C. Jewett[a,b,c,d,e,*]

[a] Masters in Biotechnology Program, Northwestern University, 2145 Sheridan Rd Technological Institute E136, Evanston, IL 60208-3120, USA
[b] Department of Chemical and Biological Engineering, Northwestern University, 2145 Sheridan Rd Technological Institute E136, Evanston, IL 60208-3120, USA
[c] Chemistry of Life Processes Institute, Northwestern University, 2170 Campus Drive, Evanston, IL 60208-3120, USA
[d] Member, Robert H. Lurie Comprehensive Cancer Center, Northwestern University, 676 N. St Clair St, Suite 1200, Chicago, IL 60611-3068, USA
[e] Institute for BioNanotechnology in Medicine, Northwestern University, 303 E. Superior St, Suite 11-131, Chicago, IL 60611-2875, USA

ARTICLE INFO

Article history:
Received 10 March 2014
Received in revised form 1 July 2014
Accepted 29 July 2014
Available online 4 August 2014

Keywords:
Cell-free protein synthesis
Defined media
Enzyme biocatalysis
Fermentation
Growth kinetics
Modelling Yeast

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.

* Corresponding author at: Department of Chemical and Biological Engineering, Northwestern University, 2145 Sheridan Road, Technological Institute E136, Evanston, IL 60208-3120, USA. Tel.: +1 847 467 5007.
E-mail address: m-jewett@northwestern.edu (M.C. Jewett).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bej.2014.07.014
1369-703X/

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,