Perspective

Transforming Biochemical Engineering with Cell-Free Biology

James R. Swartz
Dept. of Chemical Engineering and Dept. of Bioengineering, Stanford University, Stanford, CA 94305

DOI 10.1002/aic.13701
Published online December 1, 2011 in Wiley Online Library (wileyonlinelibrary.com).

Keywords: cell-free biology, cell-free protein synthesis, cell-free metabolic engineering, synthetic enzymatic pathways, biocatalysis, in vitro protein synthesis, metabolic engineering

Introduction

This perspective introduces modern cell-free biology as a rapidly developing technology offering disruptive, game-changing capabilities to chemical engineers. The foundational goal is to conduct complex biomolecular transformations using well characterized homogeneous catalysis. Consequently, the field becomes accessible to the fruits of decades of chemical engineering progress toward producing low-unit value products at high volumes. Although historically used only for research, crude-cell lysates can now be engineered for the rapid and effective production of a wide variety of complex biochemical products… and this is just the beginning. The general concepts will first be introduced followed by a brief history, a few illustrative examples, and a look toward future applications.

Overview

What is Cell-Free Biology? It is simply the activation of complex biological processes without using living cells. Instead, the cells are opened up (lysed) and unpurified portions are used (see Figure 1). The use of purified enzymes also provides important technologies (Hodgman and Jewett, 2011). However, crude cell lysates offer significantly lower catalyst costs and much greater system capabilities, and this latter approach is the primary focus here.

Cell-free biology has been practiced for decades, mostly to study biological phenomena, and, in fact, was used to first decipher the genetic code 50 years ago (Matthaei and Nirenberg, 1961). For decades, practitioners were intimidated by the complexity of crude cell extracts containing hundreds of active biological catalysts. More recently, the "black box" has been illuminated by multiple examples showing that the reaction networks can be understood, altered, and controlled. Notably, even membrane dependent energy metabolism has been activated (Jewett et al., 2008). Table 1 lists some of the many advantages gained with direct access to the reaction network and, in addition, by avoiding the many mechanisms that have evolved for maintaining cell viability and preserving the species. These will be discussed further.

While early approaches were designed to answer important scientific questions, more recent cell-free production technologies produce complex biological products and are efficient and scalable. For example, cell-free pharmaceutical protein production has been demonstrated at the 100-L scale with nearly identical performance to that observed in 20 μL experiments, an expansion factor of 5×10^6 (Zawada et al., 2011). Even proteins as complex as [FeFe] hydrogenases with six iron sulfur centers (see Figure 2) can be produced in such cell-free reactions (Boyer et al., 2008). More recently, cell lysates are being optimized for producing natural and modified metabolites (Bujara et al., 2011). Such advances open a wide range of new opportunities for biochemical engineers.

Cell-free Biochemical Engineering. Cell-free technologies simplify and harness cellular biology and also utilize the entire reactor volume instead of the 5 to 20% volume fraction (the intracellular volume) that is used in a typical fermentation process. Most importantly, cell-free approaches provide direct access to the complex set of chemical reactions that take place in a living cell. The process then takes on the form and function of chemical processes employing homogeneous catalysis; processes that have been practiced and improved by chemical engineers for decades.

Figure 1 depicts the basic concepts and the process sequence for cell-free technologies. The source cells are first grown, harvested, and lysed. The lysate can then be used directly or centrifuged to remove suspended solids and further processed, if needed. Also illustrated is the degree to which the intracellular contents become diluted (often 10- to 20-fold) and the direct access that is gained. The direct

Correspondence concerning this article should be addressed to J. R. Swartz at jswartz@stanford.edu.

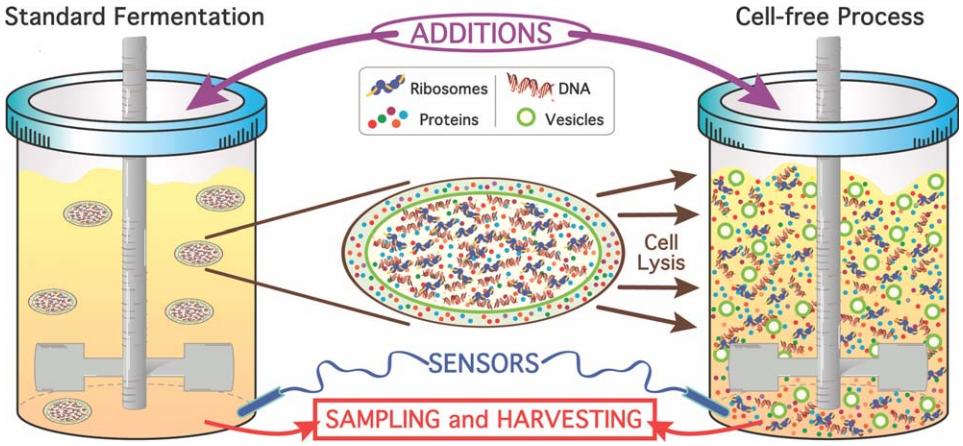


Figure 1. Industrial cell-free biology.

The schematic illustrates the fundamental concepts of cell-free technologies. In the general approach, cells are grown in a standard fermentor harvested and analyzed by high-pressure homogenization. The cell-free reaction is typically conducted in a batch or feed-batch mode. The biological reaction networks fill the entire working volume of the reactor and are 20-fold more dilute than the extremely crowded intracellular environment. Yet virtually all of the cellular catalysts are provided. This includes catalysts that are membrane associated since the inner membrane is fragmented during cell lysis, and the fragments round up to from functional membrane vesicles. The net result is that complex biotransformations are affected by homogeneous catalysis.

access allows precise sensing, sampling, and control of the reaction environment. After lysis and reaction initiation, hundreds of different enzymes are distributed uniformly throughout the reactor.

To gain a full appreciation of the potential, it is useful to draw parallels to the early development of chemical engineering. One of the major objectives was the efficient and selective conversion of one molecule into another. Early

Table 1. Advantages of Cell-Free Systems

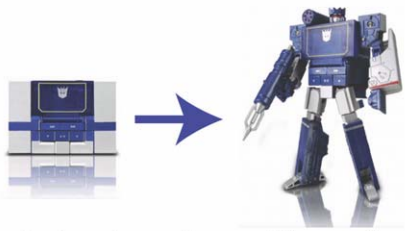
Directly Influence Complex Reaction Networks
- Efficiently supply energy
- Control reducing equivalent flux
- Use PCR products for protein expression
- Add or remove catalysts
- Add or remove reagents
  - Use complex enzymatic substrates
  - Adjust DNA template concentrations
  - Augment cofactor concentrations
  - Add "non-natural" reagents

Directly Monitor Complex Reaction Networks
- Easily acquire representative samples
- Amenable to on-line monitoring

Maintain a Stable Catalytic System
- Determined by cell state when harvested
- No new protein synthesis (unless desired)
- Limited protein hydrolysis

Reduced Effects of Toxicity
- No need for cell viability
- Must maintain only desired metabolism

Less Crowded Environment
- Improved protein folding
- Reduced rates of undesired reactions
- Improved diffusion rates



Like the transformer toys, cell-free methods convert a living cell into a catalytic system with dramatically enhanced capabilities

No Barriers to Product Access
- No need for transporters
- Facile protein evolution
  (no cell lysis or product purification)
- Simplified on-line product harvesting
- Easily monitor product levels

Ability to Focus Metabolism
- Produce only the desired product
- Adjust cofactors, enzymes, pH, redox
- Radically shift metabolism after cell lysis
- Combines intracellular compartments
  - Lysis can activate desired pathways
  - Lysis can inactivate competing enzymes
    (See Figure 6)

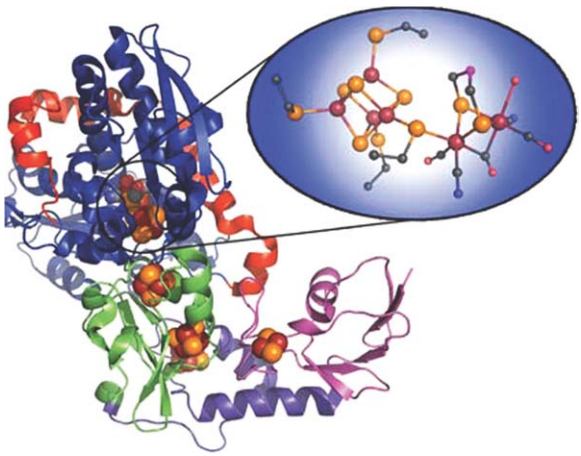


Figure 2. Structure of an (FeFe) hydrogenase showing the multiple iron sulfur centers and an expanded view of the active site cofactor composed of two iron sulfur centers, two cyanide ligands, three carbon monoxide ligands, and a bridging molecule.

Such complex proteins can now be produced with cell-free technologies to enable convenient enzyme evolution.

chemical engineers often did not fully understand the chemical reactions or the mass transfer and hydrodynamic phenomena taking place in their reactors. Nonetheless, extensive progress was achieved as experimentation produced useful correlations, the use of dimensionless parameters provided insights, and improved online monitoring and process control technologies enabled further advances. In nearly all cases, progress was dependent on rapid and highly informative monitoring and sample acquisition. As the field developed, catalysts were used to accelerate desired reactions and, although they often lost activity over time, the activities usually did not dramatically change. Online monitoring coupled with the ability to add reagents directly to the reaction volume again enabled effective real-time process control for further process improvement.

This scenario contrasts sharply with biochemical engineering processes using living organisms. The reactions of interest take place behind a highly selective barrier, the cell wall. Not only are the influential reactions sequestered, reactants are actively added and expelled by membrane associated transporters according to an evolved agenda that frequently contravenes process objectives. Furthermore, the concentrations of hundred of catalysts are actively controlled and often change dramatically during the course of a batch process. The script dictating these changes has evolved to optimize survival of the individual and the species, objectives that are diametrically opposed to the overproduction and release of a single product. While recombinant DNA technologies and metabolic engineering approaches have delivered impressive progress, it is still virtually impossible to control the overall suite of reactions within the cells. It is also very difficult to acquire representative samples from this crucially important reaction chamber. Cell-free technologies totally change this scenario. The two most notable applications, cell-free protein synthesis and cell-free metabolic engineering, illustrate this game-changing epiphany.

Cell-free Protein Synthesis (CFPS). The initial and more developed cell-free technology is CFPS. *E.*coli bacterial cells (Swartz, 2006), red blood cells (Findeis and Whitesides, 1987), and wheat kernels (Sawasaki et al., 2002)) have all been used as the source of the protein synthesis catalysts. In all cases, the cells are lysed and the contents diluted. The dilution relieves the crowding that exists in the cells, and this has several effects. Most noticeably, for *E.coli* CFPS, there is essentially no background protein synthesis directed by the chromosomal DNA. More than likely, the loosely associated sigma factors fall off the native RNA polymerase so that it is no longer functional to produce messenger RNA from DNA carried over in the cell extract. For CFPS, however, monomolecular RNA polymerases such as those from the T7 and SP6 bacteriophages are added and function effectively with their respective promoters to produce only the desired mRNAs (and proteins).

The dilution also appears to be beneficial for protein folding. Translational protein elongation is slowed to about 10% of its normal rate because of the lower concentrations of the required elongation factors. This allows more time for newly synthesized protein domains to fold before the following domains emerge from the ribosome. Also, since only one type of protein is being produced into the diluted solution, there is less chance for nascent, unfolded proteins to become tangled with each other. While the dilution may also reduce the effectiveness of chaperones, these can be overexpressed in the source cell before lysis, if required. Notably, the catalytic system will also be stabilized as the dilution also reduces the concentrations of proteases and nucleases.

Amino acids are typically added to the lysate to serve as a major class of substrates, but protein synthesis is also one of the most energy demanding processes in typical cells. The entropic cost of assembling such precise macromolecules is high. This is another reason that expressing a single protein during CFPS is beneficial as chemical energy is not dissipated toward the synthesis of undesired proteins. Most CFPS systems use a high-energy phosphate bond donor such as phosphoenolpyruvate (PEP), acetyl phosphate, or creatine phosphate, but newer systems activate normal aerobic catabolism to use glucose or glutamate as the energy source. The discovery that such complex metabolism could be activated and controlled in cell lysates has now sparked interest in using cell-free biology to produce important smaller biomolecules as well as proteins.

Cell-free Metabolic Engineering (CFME). CFME is an emerging area that seeks to take full advantage of the reaction accessibility offered by the cell-free approach. As will be discussed later, by totally separating the growth (catalyst production) and the production (catalyst utilization) phases, additional efficiencies can be gained. For a full appreciation of these advantages, it is useful to review basic concepts in metabolism. These are illustrated in Figure 3 in the context of cell-free metabolism.

Metabolism is roughly divided into two broad categories: anabolism and catabolism (although some reactions support both). Catabolism is typically the breaking down of larger molecules into smaller ones to create molecular precursors, to capture reducing equivalents (electrons), and to convert incoming chemical energy into a form that can be generally used to power the cell (typically ATP and GTP). In contrast,

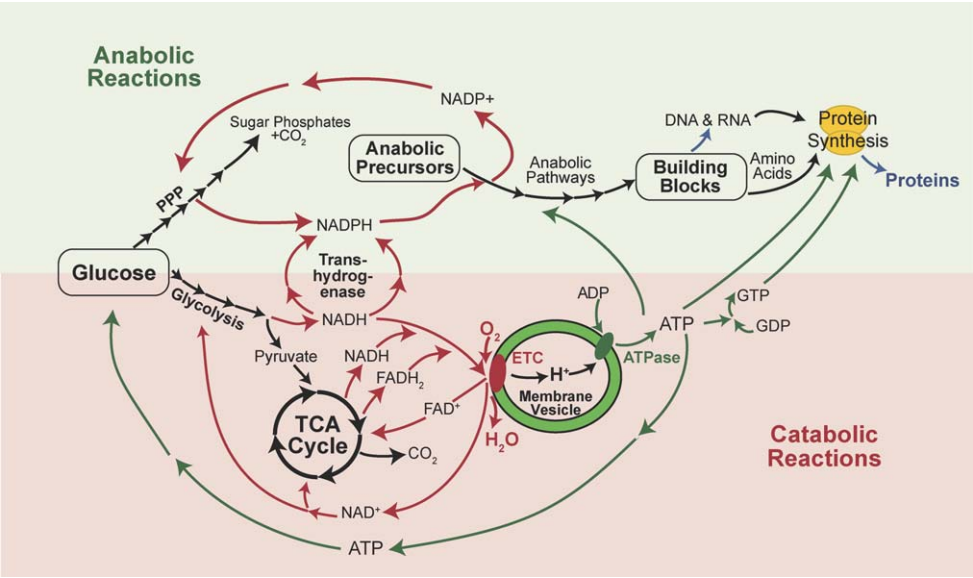


Figure 3. Conceptual diagram of metabolic networks showing reducing equivalent fluxes (red), and the flow of the high-energy compounds (green) required to fuel metabolism.

Cell-free biology allows these fluxes to be adjusted for efficient product formation independent of the needs of a living cell. Note that the oxidized reducing equivalent carriers must be recycled for metabolism to proceed. Not shown, but also crucial is there cycling of the energy carriers. The relatively low concentrations of the carriers require rapid recycling flux rates to support active metabolism.

Abbreviations: pentose phosphate pathway (PPP); tricarboxylic acid (TCA); electron transport chain (ETC); nicotinamide adenine dinucleotide (NADH), reduced form, and NAD^+, oxidized form; nicotinamide adenine dinucleotide phosphate (NADPH), reduced form, and $NADP^+$, oxidized form; flavin adenine dinucleotide (FADH2), reduced form, and FAD^+, oxidized form; adeno-sine diphosphate (ADP) and triphosphate (ATP); guano sine diphosphate (GDP) and triphosphate (GTP.

anabolism is the process of converting smaller molecules, the molecular precursors, into the larger molecules required to fill various needs for the cell. The top portion of Figure 3 shows a very coarse grained sense of anabolism, while the bottom depicts catabolism. The flow of atoms (mostly carbon, oxygen, hydrogen, and nitrogen) in the form of biomolecules is depicted in black, the flow of reducing equivalents in red, and directly usable chemical energy in green. Even though this diagram has been highly simplified, the complex and interconnected nature of metabolism is obvious. To enable the cell to separately control reducing equivalent flux in anabolism and in catabolism, different carriers are used; NADPH for anabolism and NADH for catabolism. Yet, just to keep options open, organisms also provide transhydrogenase activities to regulate electron flow between the two. In this diagram, aerobic metabolism is shown. The luxury of delivering reducing equivalents to oxygen provides a plentiful source of usable chemical energy. In contrast, anaerobic metabolism captures only about 7% of the usable energy from glucose. However, if the objective is to retain most of the chemical energy in the biomolecular product (as, for example, for fuel ethanol), this can be advantageous.

In seeking to reengineer this complex reaction network for the production of a targeted metabolite, the first step is simply to provide higher catalytic capability for the desired anabolic reactions. Typically, several enzymes are required, and these can be accumulated to supernatural levels to support higher productivities in the cell lysates. Clearly the anabolic precursors will also be required at comparable rates. These can be supplied by a variety of central pathways, and these precursor supply reactions may also need higher catalytic capacity. However, now comes the more difficult part. The precursor supply and anabolic reactions for each product will vary significantly as to whether they require or produce reducing equivalents as well as whether they require or produce usable chemical energy. The magnitude of the consumption or production also varies widely depending on the anabolic pathway, but in almost all cases, the required recycling rates for the electron and energy carriers are significantly faster than the central molecular fluxes. In a living cell, all of this must be balanced both to produce large quantities of the desired product and to maintain the electron and energy supplies required to keep the cell alive and in good health. In contrast, in cell-free metabolism, all metabolic fluxes can be controlled and directed solely to benefit product formation. The challenge is to monitor and control the supporting fluxes for the most efficient production of the desired metabolite. In fact, production may well be limited by these factors and not by the central pathway enzymes. Cell-free metabolic engineering approaches offer unprecedented opportunities to monitor and control the entire metabolic system for maximal production rates and conversion efficiencies.

These same basic metabolic principles are conserved throughout nature, and this means that not only can enzymes from different organisms be combined into a single pathway, but entire pathways from different organisms can be combined. For example, the synthetic pathway for a complex secondary metabolite can be supported by the reducing equivalent and energy supply infrastructure from another unrelated organism. While the feasibility for this mix and match concept has been amply demonstrated *in vivo* (e.g., Pfeifer et al., 2001), it is likely to be much more successful when implemented using CFME. Taken further, the open nature of the system allows entirely new and non-natural enzymatic substrates to be used as metabolic precursors. Fully

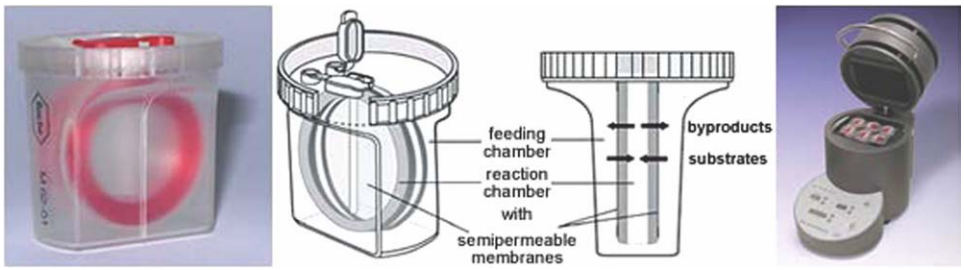


Figure 4. Commercialized ultra-filtration device to support CFPS reactions.

As indicated, substrates can diffuse into the reaction chamber and potentially toxic byproducts can diffuse out. A 10 mL feeding chamber supports a 1-mL reaction volume.

developed, the concept would enable the enzymatic synthesis of complex biomolecules composed of moieties derived both from traditional chemical synthesis and from enzymatic synthesis (see below). This hybrid approach promises the potential for more economical production of currently used biomolecules such as antibiotics. It may also allow the efficient production of new and more effective biomedicines that are very difficult if not impossible to make by either chemical synthesis or by natural pathways.

A Brief History

In 1897, Eduard Buchner was helping his brother, a physician, by looking for a way to preserve extracts from yeast cells. Thinking about jams and jellies, he tried adding sugar. To his surprise the mixture began bubbling as glycolysis converted the sugar to alcohol with the release of CO_2. This was the first realization that biological reactions could occur outside of a living cell (contradicting Louis Pasteur's beliefs), and it earned Buchner the 1907 Nobel Prize in Chemistry. This basic discovery helped establish the new science of biochemistry and is probably the first known example of cell-free biology.

The subsequent pursuit of biochemical knowledge profited mostly from reductionist approaches, but more complicated biological processes require multiple catalysts. Early experimentation with cell-free protein synthesis began in the 1950's (Borsook, 1950; Winnick, 1950) with the observation that radioactively labeled amino acids could be incorporated into proteins using crude cell extracts. These studies progressed, and, in 1961, Nirenberg and Matthaei reported the use of CFPS to decipher the genetic code, earning the 1968 Nobel Prize in Physiology or Medicine. Such methods were improved empirically with notable advances coming from the Zubay laboratory at Columbia University. For example, in 1973, he and his coworkers reported the cell-free synthesis of rat growth hormone (Bancroft et al., 1973).

A substantial advance then came in 1988 when Alexander Spirin reported the use of ultrafiltration membranes to stabilize the small molecule environment (Spirin et al., 1988) by continually providing substrates and removing inhibitors. Protein synthesis catalyzed by either *E.coli* or wheat germ extracts continued at constant rates for more than 40 h and accumulated 100-fold more product than a batch reaction. This remarkable approach was commercialized with the device shown in Figure 4. While these results suggested significant potential for commercial cell-free biology, the large volume of the feeding solution made the technique relatively expensive.

Several subsequent advances then came from the Swartz laboratory. Many years of rDNA protein research at Genentech had left a strong impression that living cells have their own agenda, and it does not include making foreign protein. Cell-free approaches offered a way to bypass that agenda. With a focus on commercial scale protein production, the first step was to conduct a process cost and commercialization feasibility analysis. It became apparent that the energy source and nucleotide costs dominated and were unacceptable, that simple scale-up methods were required, and that protein folding was inadequate, particularly for proteins requiring disulfide bonds.

The costs were initially addressed by developing the PANOx (PEP, amino acids, NAD^+, oxalic acid) system (Kim and Swartz, 2001). Early observations had suggested some degree of energy metabolism in the cell extracts. It was reasoned that the secondary pathway shown in Figure 5 could be activated by adding NAD^+ and coenzyme A to the reaction mixture. This proved to be correct, and yields were further improved by increasing amino acid concentrations and by adding oxalic acid to inhibit an energy wasting futile cycle. The net result was a 4- to 5-fold increase in product

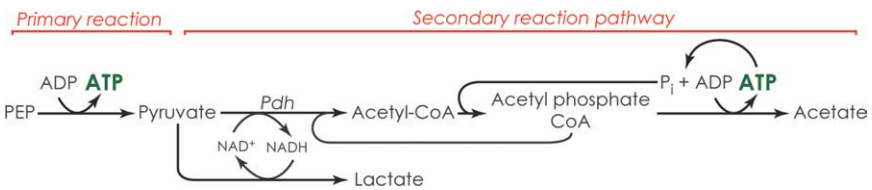


Figure 5. The reactions activated in the PANOx system by addition of NAD+ and CoenzymeA (CoA).

Because one pyruvate must be used to regenerate the NAD^+ for each pyruvate going to acetyl-CoA, the combined pathway generates 50% more ATP than the primary pathway alone.

accumulation. The PANOx system was licensed for use with the devices shown in Figure 4 providing similar yield increases.

Encouraged by these results and realizing that the use of PEP was still too expensive, activation of oxidative phosphorylation was explored (the ATP generation process diagrammed in Figure 3). Initial tests showed no activity in the standard reaction mixture, and it was reasoned that a more natural chemical environment might foster full natural functionality. Michael Jewett accepted this challenge, consulted the literature relative to *E.coli*'s internal chemical environment, and developed the Cytomim (cytoplasmic mimic) system with radical changes in the reaction mixture (Jewett et al., 2008). Changing the chemical environment did indeed activate oxidative phosphorylation, and high-protein yields were obtained using inexpensive energy substrates such as pyruvate, glutamic acid, and glucose. Active and prolonged energy metabolism also allowed the use of less expensive nucleotides, and overall raw material costs were reduced by approximately 30-fold (Calhoun and Swartz, 2005) to levels attractive for pharmaceutical protein production.

Protein folding was then improved by doing the opposite, by inactivating endogenous metabolism. Iodoacetamide was used to inactivate the cytoplasmic enzymes that normally reduce disulfide bonds. Only then could the addition of a thiol/disulfide redox buffer stabilize the relevant redox potential so that disulfide bonds could be formed and, if necessary, shuffled to facilitate the proper folding of human pharmaceutical proteins. Augmentation with *E.coli*'s disulfide isomerase stimulated the latter process. Each of the changes was necessary, but not sufficient, and, when combined, provided efficient folding for proteins with multiple disulfide bonds (Kim and Swartz, 2004).

Finally, the scale-up challenges had been intensified by the need to provide oxygen for ATP production. Early tests with gas sparging produced the expected horrendous foaming. Amazingly, addition of standard fermentation antifoams controlled the foaming without inhibiting energy metabolism, protein production, or protein folding. By activating natural energy metabolism, inactivating disulfide bond reducing metabolism, and using standard scale-up technology; each of the major barriers to commercialization had been solved. Sutro Biopharma (initially FAB) was then established and has now reported effective production of granulocyte macrophage colony stimulating factor (GMCSF) at the 100-L scale (Zawada et al., 2011). Purification to pharmaceutical quality product was achieved with two chromatography steps, and additional data suggest that the fidelity of amino acid incorporation is significantly better from the cell-free reaction than from *in vivo* production. Sutro is now establishing the first cell-free GMP capability for pharmaceutical protein production.

In parallel, Yaeta Endo's group at Ehime University in Japan discovered that early wheat germ extracts contained a protein synthesis inhibitor. This could be removed by extensive washing to dramatically increase protein expression yields (Madin et al., 2000). The resultant system has now been extensively developed for the production of multiple proteins in parallel (Sawasaki et al., 2002; Goshima et al., 2008). Professor Takuya Ueda at the University of Tokyo extended the concept even further by demonstrating protein synthesis using only purified components; i.e., the PURE system (Shimizu et al., 2001). This system includes 36 purified enzymes, 46 tRNAs, and purified ribosomes in addition to a number of small molecular weight reactants. Even though this approach is prohibitively expensive for most commercial applications, its well-defined nature makes it ideal for studying protein expression and folding. Probably its most important value is to fully "demystify" the complex process of protein synthesis.

As mentioned, cell-free metabolic engineering for smaller biomolecules is still in a nascent form. Notable work has emerged from the laboratory of Sven Panke at ETH in Zurich, and a new company, GreenLight Biosciences, has been established to pursue the significant potential advantages. More about this is given later.

A Few Illustrative Examples

Perspectives are not intended as full topical reviews, but the power of this technology can best be appreciated by examining a few examples of complex cell-free biology. These have been categorized as indicated later, and examples of synthetic enzymatic pathways (SEP; Zhang et al., 2007; Hodgman and Jewett, 2011) are given first by way of comparison.

Synthetic enzymatic pathway examples

In 2000, Jonathan Woodward and colleagues extended work begun by Eli Greenbaum to demonstrate highly efficient hydrogen production from glucose 6-phosphate (G6P) (Woodward et al., 2000). In this work, the pentose phosphate pathway was reconstituted with purified enzymes to supply reducing equivalents in the form of NADPH. These were then used by a thermophilic hydrogenase to produce hydrogen at 97% of the maximal theoretical yield. This is in sharp contrast to the maximal fermentation yield of about 30% obtained with living organisms. Percival Zhang and colleagues have since demonstrated the harvesting of reducing equivalents from cellobiose (a key breakdown product from renewable biomass) with a yield of 95% using a 12 enzyme system (Wang et al., 2011). To activate even more complex pathways, the Williamson laboratory at Scripps assembled pathways composed of 28 and 18 enzymes for producing purines (Schultheisz, 2008) and pyrimidines (Schultheisz, 2011). This included enzymes to supply chemical energy (ATP) and consume excess reducing equivalents. Jewett and Hodgman (2011) and Zhang et al. (2011) have provided recent reviews of this rapidly growing field.

Cell-free protein synthesis examples

As this field developed, CFPS revealed many of the expected advantages, but also provided unexpectedly efficient protein folding, as explained earlier. One striking example is a small, but very active luciferase isolated from the copepod *Gaussia princeps*. The ability to control the disulfide/sulfhydral redox potential, the uncrowded folding environment, and the addition of a disulfide isomerase produced approximately 100 times the active enzyme concentration in the cell-free reaction relative to *in vivo* yields (Goerke et al., 2008). Simple virus-like particles (VLPs) can also be efficiently produced when the coat protein is

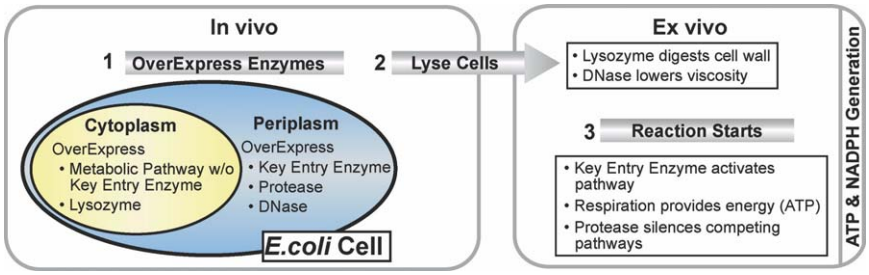


Figure 6. GreenLight's compartmentalization concept for producing more active extracts for cell-free metabolic engineering.

In step 1 the cells are grown and induced to overproduce the desired metabolic pathway as well as enzymes that will mature the cell extract. Enzymes that would otherwise hamper cell growth are accumulated in a compartment where they will not have access to their substrates. Upon cell lysis (step 2), the enzymes act upon their substrates (step 3) to improve extract properties and to activate the desired metabolic pathway. Active membrane vesicles and direct access to the reaction chamber then allow control of the energy and reducing equivalent fluxes.

expressed by CFPS (Bundy et al., 2008). Since the accessibility of CPFS enables effective site-specific incorporation of uniquely reactive non-natural amino acids (Goerke and Swartz, 2009), these VLPs could then be surface-modified by attaching proteins and DNA onto the VLP outer surface to produce potential vaccines and imaging agents (Patel and Swartz, 2011).

Cell-free protein evolution

CFPS is very well suited for screening large libraries of protein variants to identify improved traits. The ability to produce proteins directly from linear DNA (PCR products) and to directly evaluate the bioactivity of the product without cell lysis or product purification enables convenient high throughput protein evolution. Yamane, Nakano, and colleagues demonstrated this in 2003 with a system they called SIMPLEX (single-molecule-PCR-linked *in vitro* expression) (Koga et al., 2003). The library of mutated genes was diluted to the point that a single molecule of DNA was, on average, placed in each microtiter well. This was amplified by PCR to provide the template for CFPS. Using this approach they isolated lipase mutants with inverted enantioselectivity. In parallel, Griffiths and Tawfik (2003) demonstrated even higher throughput by using emulsions in which each aqueous droplet was a nanoscale CFPS bioreactor. Their approach allows the screening of millions of candidates to find very rare functional improvements. In this landmark report, they described how they increased the catalytic activity of an already very efficient enzyme by 63-fold. Further development of emulsion approaches coupled with FACS (fluorescence-activated cell sorting) technologies promise even greater capability.

More recently, Lui et al. (2011) reported the discovery of the first improved epidermal growth factor (EGF) mutant despite several previous attempts. The key to success was the use of CFPS to produce bioactive EGF mutants. Radioactive labeling enabled a high-throughput assay of product concentration so that the same dose of each mutated protein could be applied to the cell-based assay. In addition, the high activity of the product allowed approximately 10^6-fold dilutions of the CFPS product mixtures so that the unpurified sample could be added directly to the assay cell culture. Finally, the cell-free format also facilitated an increase in the translational initiation factor concentrations so that all of the candidate library could be expressed and evaluated.

Cell-free metabolic engineering (CFME)

As previously explained, some of the first CFME with crude cell extracts was conducted to improve protein synthesis. For example, multiple chromsosomal deletions in the source cells avoided "degradation" of the amino acid substrates (Calhoun and Swartz, 2006) during CFPS, and modification of the chemical environment activated oxidative phosphorylation (Jewett et al., 2008). More recently, Sven Panke's group has developed more quantitative approaches toward reengineering cell extracts to overproduce a desired metabolite. They have used the accessibility of the CFME reaction chamber for real-time analysis of the concentrations of metabolic intermediates (Bujara et al., 2011). This information enabled optimization of enzyme levels to obtain the predicted 2.5-fold improvement in dihydroxyacetone phosphate (DHAP) production.

A new company, GreenLight Biosciences, has also been developing novel approaches for CFME. Their overall technology concept is illustrated in Figure 6. The underlying hypothesis is that more productive cell extracts can be produced if the cells are not stressed before harvesting and cell lysis, and additionally, if targeted enzymatic modifications are effected only after cell lysis. This involves using the periplasm of *E.coli* as a second compartment for sequestering enzymes that are needed at elevated levels in the cell extract. However, only the enzymes that would threaten cell health need be placed there. For example, if the first enzyme in a dedicated metabolic pathway is placed in the periplasm, the other pathway enzymes can probably be accumulated in the cytoplasm without ill effects since these enzymes will not be supplied with their substrates. Additional advantages that could be gained from further compartmentalization are also illustrated in Figure 6.

More complete reviews of cell-free biology have recently been offered by Hodgman and Jewett (2011) (cell-free

Figure 7. A hypothetical synthetic pathway that combines a metabolic intermediate (MI) with a chemically synthesized precursor (CSP) to stereo-specifically assemble a new molecule which could not have been produced either enzymatically or by chemical synthesis.

synthetic biology), and Carlson et al. (2011) (cell-free protein synthesis).

Examples of Possible Future Applications

Hybrid small molecule synthesis

As mentioned earlier, a very intriguing future application is the multistep enzymatic assembly of complex biomolecules composed of elements derived both from known metabolic pathways and from chemical synthesis. This is illustrated in Figure 7. Chemical synthesis can employ toxic solvents and conditions that would otherwise denature enzymes to produce chemical compounds not practical for enzymatic synthesis. If such a building block is, however, somewhat similar to naturally occurring biomolecules, it is likely that an enzyme could recognize or be evolved to recognize this compound as a substrate for stereospecific combination with other metabolites. This is particularly relevant for metabolic pathways that produce secondary metabolites such as those that are as important as human medicines. In this way, libraries of novel and complex biomolecules could be synthesized for evaluation as therapeutics for a broad variety of diseases.

Artificial "cells"; e.g., a circulating artificial pancreas

Taken full circle, cell-free biology could also be used to produce entities that possess some, but not all of the functions of a living cell. For example, small cell-free protein synthesis capsules could be produced that are programmed only to make and to secrete proinsulin. They would not have the ability to reproduce, or, in fact, to make any other protein. These could be bundled into a larger capsule (for example, about the size of a red blood cell) containing a tethered protease to mature the proinsulin thereby releasing insulin and the C-peptide to diffuse out of the large capsule into the blood stream. If the CFPS capsules are programmed only to import glucose when its concentration is above the desired level, protein synthesis would only have an energy source to make insulin when it was needed. While there are many other design criteria that would need to be satisfied, progress in cell-free biology coupled with other advances in synthetic biology suggest that such approaches might now be feasible.

Flexible cell-free manufacturing formats

The diagram shown in Figure 1 suggests great flexibility in the design of cell-free processes. By its nature, cell-free biology resembles traditional homogeneous catalysis. However, for CFME, the large molecular weight of the catalysts (enzymes) relative to the metabolites indicates that heterogenous catalysis approaches can be used. For example, ultrafiltration could retain the enzymes while continuously removing the product. Energy and reducing equivalent carriers would also be removed, but these could be returned after online extraction of the product. Pathway precursor and intermediate concentrations could be managed to keep the product concentration much larger than those of the other metabolites. Online monitoring of process properties such as the concentrations of precursors, product, reducing equivalent carriers, and energy carriers could also be continuously monitored as a basis for online process control. As needed, enzymes could be evolved for greater stability and troublesome proteases could be avoided by genetic deletions or by post-lysis inactivation to prolong the useful life of the catalytic pathways. Because organisms such as *E.coli* can be grown on simple media, such measures would allow the inexpensive high-volume production of commodity biochemicals.

It is also interesting to reflect on new developments such as the use of immobilized enzymes and synthetic protein scaffolds. Inexpensive methods for incorporating non-natural amino acids would, for example, enable rapid and inexpensive *in situ* immobilization of key enzymes on larger particles, mostly likely without the need for enzyme purification. This could further stabilize the proteins and possibly facilitate enzyme retention for continuous product harvesting. Both protein (Dueber et al., 2009) and RNA (Delebecque et al., 2011) based scaffolds have also been developed to assemble multiple enzymes into an integrated complex. These potentially serve to stabilize the catalysts, help control relative enzyme concentrations, and possibly also facilitate substrate transfer through the pathway. This latter factor might be particularly important, for example, for pathways that transfer reducing equivalents (i.e., electrons) or for pathways with toxic intermediates.

Summary

All of aforementioned examples underscore the incredible versatility of biological systems. Biology really is amazing and, even given our current knowledge, provides an astounding panoply of opportunities to serve society. Now, using cell-free biology, we can access and manipulate biology at the most intimate level, and with this new and direct relationship comes an exciting potential to direct complex biological processes as never before. While these tools and insights must be used responsibly, the intrinsic departure from the use of a living organism provides built-in safeguards. Nonetheless, these approaches will stimulate unprecedented biodesign creativity, and care must still be taken.

Acknowledgment and Disclaimer

The author wishes to thank Michael Jewett for helpful comments and also would like to inform the reader of a financial interest in Sutro Biopharma and GreenLight Biosciences.

Literature Cited

Bancroft, F. C., G.-J. Wu, and G. Zubay, "Cell-Free Synthesis of Rat Growth Hormone," *Proc. Nat. Acad. Sci.*, **70**, 3646–3649 (1973).

Boyer, M. E., J. A. Stapleton, J. M. Kuchenreuther, C.-W. Wang, and J. R. Swartz, "Cell-Free Synthesis and Maturation of [FeFe] Hydrogenases," *Biotech. Bioeng.*, **99**, 59–67 (2008).

Borsook, H., "Protein Turnover and Incorporation of Labeled Amino Acids into Tissue Proteins *In Vivo* and *In Vitro*," *Physiol. Rev.*, **30**, 206–219 (1950).

Bujara, M., M. Schumperli, R. Pellaux, M. Heinemann, and S. Panke, "Optimization of a Blueprint for *In Vitro* Glycolysis by Metabolic Real-Time Analysis," *Nature Chem. Biol.*, **7**, 271–277 (2011).

Bundy, B. C., M. J. Franciszkowicz, and J. R. Swartz, "*Escherichia Coli*-Based Cell-Free Synthesis of Virus-Like Particles," *Biotechnol. Bioeng.*, **99**, 351–367 (2008).
Calhoun, K. A., and J. R. Swartz, "An Economical Method for Cell-Free Protein Synthesis Using Glucose and Nucleoside Monophosphates," *Biotechnol. Progr.*, **21**, 1146–1153 (2005).
Calhoun, K. A., and J. R. Swartz, "Total Amino Acid Stabilization During Cell-Free Protein Synthesis Reactions," *J. Biotechnol.*, **123**, 193–203 (2006).
Carlson, E. D., R. Gan, E. Hodgman, and M. C. Jewett "Cell-Free Protein Synthesis: Applications Come of Age," *Biotechnol Adv.*, in press (2011).
Delebecque, C. J., A. B. Lindner, P. A. Silver, and F. A. Aldaye, "Organization of Intracellular Reactions with Rationally Designed RNA Assemblies," *Science*, **333**, 470–474 (2011).
Dueber, J. E., G. C. Wu, G. R. Malmirchegini, T. S. Moon, C. J. Petzold, A. V. Ullal, K. L. Prather, and J. D. Keasling, "Synthetic Protein Scaffolds Provide Modular Control Over Metabolic Flux," *Nat Biotech.*, **27**, 753–759 (2009).
Findeis, M. A., and G. M. Whitesides, "Protein Synthesis In Cell-Free Reticulocyte Lysates on Multi-Hour Incubation," *Appl. Biochem. Biotech.*, **15**, 169–189 (1987).
Goerke, A. R., A. M. Loening, S. S. Gambhir, and J. R. Swartz, "Cell-Free Metabolic Engineering Promotes High-Level Production of Bioactive Gaussia Princeps Luciferase," *Metabolic Eng.*, **10**, 187–200 (2008).
Goerke, A. R., and J. R. Swartz, "High-Level Cell-Free Synthesis of Proteins Containing Site-Specific Non-Natural Amino Acids," *Biotechnol. Bioeng.*, **102**, 400–416 (2009).
Goshima, N., Y. Kawamura, A. Fukumoto, A. Miura, R. Honma, R. Satoh, A. Wakamatsu, J.-I. Yamamoto, K. Kimura, T. Nishikawa, T. Andoh, Y. Iida, K. Ishikawa, E. Ito, N. Kagawa, et al. "Human protein factory for converting the transcriptome into an *in vitro*-expressed proteome," *Nature Methods*, **5**, 1011–1017 (2008).
Griffiths, A. D., and D. S. Tawfik, "Directed Evolution of an Extremely Fast Phosphotriesterase by *In Vitro* Compartmentalization," *EMBO J.*, **22**, 24–35 (2003).
Hodgman, E. C., and M. C. Jewett, "Cell-Free Synthetic Biology: Thinking Outside the Cell," *Metabolic Engineering*, doi: 10.1016/j.ymben.2011.09.002 (2011).
Jewett, M. C., K. A. Calhoun, A. Voloshin, J. J. Wuu, and J. R. Swartz "An Integrated Cell-Free Metabolic Platform for Protein Production and Synthetic Biology," *Molecular Syst. Biol.*, 57 (2008).
Kim, D.-M., and J. R. Swartz, "Regeneration of Adenosine Triphosphate from Glycolytic Intermediates for Cell-Free Protein Synthesis," *Biotech. Bioeng.*, **74**, 309–316 (2001).
Kim, D.-M., and J. R. Swartz "Efficient production of a bioactive, multiple disulfide-bonded protein using modified extracts of *Escherichia coli*," *Biotech. Bioeng.*, **85**, 122–129 (2004).
Koga, Y., K. Kato, H. Nakano, and T. Yamane, "Inverting Enantioselectivity of *Burkholderia cepacia* KWI-56 Lipase by Combinatorial Mutation and High-Throughput Screening Using Single-Molecule PCR and *In Vitro* expression," *J. Mol. Biol.*, **331**, 585–592 (2003).
Lui, B. H., J. R. Cochran, and J. R. Swartz "Discovery of Improved EGF Agonists Using a Novel *In Vitro* Screening Platform," *J. Mol. Biol.*, **413**, 406–415 (2011).
Madin, K., T. Sawasaki, T. Ogasawara, and Y. Endo, "A Highly Efficient and Robust Cell-Free Protein Synthesis System Prepared from Wheat Embryos: Plants Apparently Contain a Suicide System Directed at Ribosomes," *PNAS*, **99**, 14652–14657 (2000).
Nirenberg, M. W., and J. H. Matthaei, "Characterisitics and Stabilization of DNAase-Sensitive Protein Synthesis in *E.coli* Extracts," *PNAS.*, **47**, 1580–1588.
Patel, K. G., and J. R. Swartz, "Surface Functionalization of Virus-Like Particles by Direct Conjugations using Azide-Alkyne Click Chemistry," *Bioconjug. Chem.*, **22**, 376–387 (2011).
Pfeifer, B. A., S. J. Admiraal, H. Gramajo, D. E. Cane, and C. Khosla, "Biosynthesis of Complex Polyketides in a Metabolically Engineered Strain of *E. coli*.," *Science*, **291**, 1790–1792 (2001).
Sawasaki, T., T. Ogasawara, R. Morishita, and Y. Endo, "A cell-free protein synthesis system for high-throughput proteomics," *PNAS.*, **99**, 14652–14657 (2002).
Schultheisz, H. L., B. R. Szymczyna, L. G. Scott, and J. R. Williamson, "Pathway Engineered Enzymatic *de novo* Purine Nucleotide Synthesis," *ACS Chem. Biol.*, **3**, 499–511 (2008).
Schultheisz, H. L., B. R. Szymczyna, L. G. Scott, and J. R. Williamson, "Enzymatic *de novo* Pyrimidine Nucleotide Synthesis," *J. Am. Chem. Soc.*, **133**, 297–304 (2011).
Shimizu, Y., A. Inoue, Y. Tomari, T. Suzuki, T. Yokogawa, K. Nishikawa, and T. Ueda "Cell-Free Translation Reconstituted with Purified Components," *Nature Biotech.*, **19**, 751–755 (2001).
Spirin, A. S., V. I. Baranov, L. A. Ryabova, S. Y. Ovodov, and Y. E. Alakhov, "A Continuous Cell-Free Translation System Capable of Producing Polypeptides in High Yield," *Science*, **242**, 1162–1164 (1988).
Swartz, J., "Developing Cell-Free Biology for Industrial Application," *J. Ind. Microbiol. Biotechnol.*, **33**, 476–485 (2006).
Wang, Y., W. Huang, N. Sathitsuksanoh, Z. Zhu, and Y. H. Zhang, "Biohydrogenation from Biomass Sugar Mediated by *In Vitro* Synthetic Enzymatic Pathways," *Chem. Biol.*, **18**, 372–380 (2011).
Winnick, T., "Incorporation of Labeled Amino Acids into Protein of Embryonic and Tumor Tissue Homogenates," *Fed. Proc.*, **9**, 247 (1950).
Woodward, J., M. Orr, K. Cordray, and E. Greenbaum, "Enzymatic Production of Biohydrogen," *Nature*, **405**, 1014–1015 (2000).
Zawada, J. F., G. Yin, A. R. Steiner, J. Yang, A. Naresh, S. M. Roy, D. S. Gold, H. G. Heinsohn, and C. J. Murray, "Microscale to Manufacturing Scale-Up of Cell-Free Cytokine Production - A New Approach for Shortening Protein Production Development Timelines," *Biotech. Bioeng.*, **108**, 1570–1578 (2011).
Zhang, Y.-H. P., B. R. Evans, J. R. Mielenz, R. C. Hopkins, and M. W. W. Adams, "High-Yield Hydrogen Production from Starch and Water by a Synthetic Enzymatic Pathway," *PlosOne*, **2**(5), e456 (2007).
Zhang, Y.-H. P., S. Myung, C. You, Z. Zhu, and J. A. Rollin, "Toward Low-Cost Biomanufacturing through *In Vitro* Synthetic Biology: Bottom-Up Design," *J. Mater. Chem.*, (2011). doi:10.1039/c1jm12078f.