Review

Cell-free metabolic engineering: Biomanufacturing beyond the cell

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Industrial biotechnology and microbial metabolic engineering are poised to help meet the growing demand for sustainable, low-cost commodity chemicals and natural products, yet the fraction of biochemicals amenable to commercial production remains limited. Common problems afflicting the current state-of-the-art include low volumetric productivities, build-up of toxic intermediates or products, and byproduct losses via competing pathways. To overcome these limitations, cell-free metabolic engineering (CFME) is expanding the scope of the traditional bioengineering model by using in vitro ensembles of catalytic proteins prepared from purified enzymes or crude lysates of cells for the production of target products. In recent years, the unprecedented level of control and freedom of design, relative to in vivo systems, has inspired the development of engineering foundations for cell-free systems. These efforts have led to activation of long enzymatic pathways (>8 enzymes), near theoretical conversion yields, productivities greater than 100 mg L^{-1} h^{-1}, reaction scales of >100 L, and new directions in protein purification, spatial organization, and enzyme stability. In the coming years, CFME will offer exciting opportunities to: (i) debug and optimize biosynthetic pathways; (ii) carry out design-build-test iterations without re-engineering organisms; and (iii) perform molecular transformations when bioconversion yields, productivities, or cellular toxicity limit commercial feasibility.

1 Introduction

Today's chemicals and materials are almost entirely derived from fossil fuels and their products [1]. For example, the petrochemical industry has historically focused on seven low-cost, high-volume commodity chemicals (methanol, ethylene, propene, butadiene, benzene, toluene, xylene), from which nearly all other materials are synthesized. The consequences of the petrochemical/materials paradigm and the resulting world dependence on fossil fuels include: (i) supply limitations due to increasing and fluctuating prices and finite availability [2], (ii) undesirable climate effects [3], and (iii) constraints on materials innovation stemming from a limited set of petrochemical building blocks [4]. While the petrochemical/materials paradigm will not be quickly replaced (if at all), advances in our foundations for engineering biology are poised to open access to novel chemicals and materials based on a potentially richer palette of starting points (i.e. more starting building blocks than the seven typically used today) [4]. This could lead to the production of chemicals and materials with new attractive properties and functions. In turn, novel, sustainable, and cost-effective technologies based on biological synthesis could be realized that help meet the world's increasing need for fuels, pharmaceuticals, and commodity chemicals.

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Abbreviations: CFME, cell-free metabolic engineering; **CFPS**, cell-free protein synthesis; **EMP pathway**, Embden–Meyerhof–Parnas glycolytic pathway; **G6P**, glucose 6-phosphate; **DHAP**, dihydroxyacetone phosphate
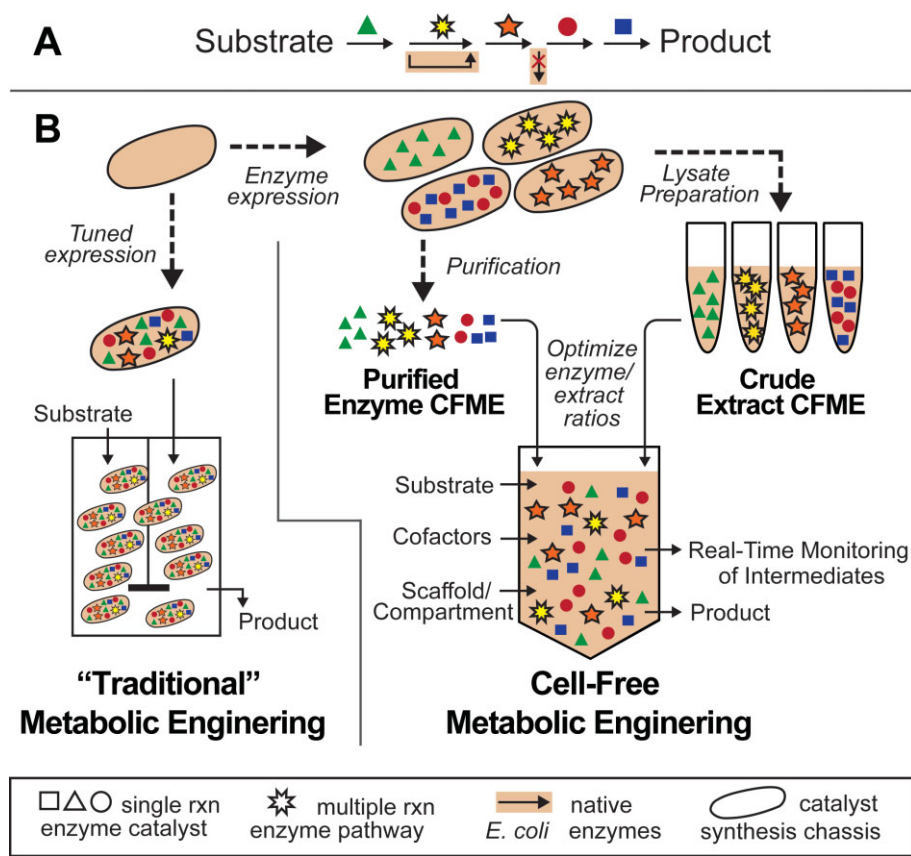
Figure 1. Paradigms for metabolic engineering (**A**) sample pathway (**B**) traditional and cell-free approaches.

In recent decades, the growth of industrial biotechnology and metabolic engineering has shown a tremendous potential to help meet the need for new chemicals and materials [4]. Harnessing enzymes as robust, specific, and efficient catalysts of chemical reactions [5, 6], cellular factories are being constructed to make complex reaction cascades within living organisms to produce a variety of useful small molecules, peptides, and polymers [7–10]. In addition to the achievements of first-generation bioethanol [11], successful commercial bioprocesses have been recently developed for the production of 1,3-propanediol (Dupont Tate & Lyle) [12], polylactic acid (Cargill), and isoprenoids (Amyris) [13], among others. While the number of microbial metabolic engineering success stories is rapidly growing [14], the fraction of biochemicals amenable to economical production is still limited, requiring hundreds of person-years of work to bring a single pathway to market [15–17].

A major challenge to current microbial biomanufacturing practice lies with the inherent limitations imposed by cells. It is virtually impossible to balance intracellular fluxes to optimally satisfy a very active synthetic pathway while maintaining the growth and maintenance needs to the host. This leads to a variety of challenges afflicting the current state-of-the-art. Yields of target molecules are limited to non-toxic levels (e.g. ~2.5% v/v butanol) [18] and

unwanted byproducts are common. Product excretion and/or separation are constrained by intracellular transport barriers (i.e. membranes). Scaling of lab-scale cultures to the industrial level is hampered by heterogeneous fermentation conditions [19]. The inability to control and direct resource allocation (e.g. electron flux or ATP) towards the exclusive synthesis of the target product can result in poor volumetric productivities that limit economic process viability. Additionally, the unwieldy complexity of cells makes rational design unpredictable and difficult to engineer [15, 20]. Put another way, current biomanufacturing efforts are limited by the need to balance the tug-of-war that exists between the cell's physiological and evolutionary objectives and the engineer's process objectives.

Many efforts are underway to address the aforementioned challenges. In one example, the growing field of synthetic biology offers new advanced tools and generalized capabilities to modify living organisms for process engineering objectives. For example, our newfound ability to rapidly read, write, and edit DNA is accelerating the pace of design-build-test loops for product development [21].

Cell-free metabolic engineering (CFME) offers an alternative, yet underutilized, approach. The foundational principle of CFME is that precise complex biomolecular

synthesis can be conducted without using intact cells (Fig. 1) [22, 23]. Instead, purified enzyme systems or crude cell lysates are used, which can be accurately monitored and modeled.

In this review, we focus on the emergence and growth of CFME. Many overlapping terms have been discussed to describe one-pot, cell-free, enzymatic pathways including "synthetic pathway biotransformations (SyPaB) [24]," "cell-free biosystems for biomanufacturing (CFB2) [25]," "multi-enzyme processes [26]," "synthetic metabolic engineering [27]," and "cell-free pathways/minimized reaction cascades [28]." CFME encompasses many of these concepts while emphasizing long pathways (>4 enzymes), economical substrates, potential for industrial-scale production, and rapid pathway prototyping and debugging [23]. Other perspectives on tools for engineering complexity in cell-free systems [29], as well as a broader discussion of applications for cell-free biology [17, 23] are discussed elsewhere. We begin with a brief introduction of the technological capabilities of the field and its potential advantages for pathway debugging and biomanufacturing. In the next section, we discuss state-of-the-art cell-free systems for small molecule metabolite production and pathway optimization. Finally, we examine frontier applications, as well as associated challenges and opportunities.

2 Background

Activation of cell-free enzyme pathways has been possible for over a century. Indeed, seminal biochemical discoveries of glycolysis (by Eduard Buchner) [30] and the genetic code (by Nirenberg and Matthaei) [31] used cell extracts, or lysates, from yeast and *Escherichia coli*, respectively. In other examples, the catalytic proteins of metabolic pathways have been purified from living cells to elucidate and understand their biological activity, as well as to harness them for industrial purposes. To date, commercial usage of enzymes in vitro is primarily for single reactions (or very short pathways). Usage of these enzymes is often in a specialized context where enzymes have distinct advantages over chemical methods, including reduced reaction time, increased product yield, increased product specificity, reduced cost, and reduced environmental impact. Examples include isomerases in starch processing, proteases/lipases in clothing detergents, cellulases/amylases for bioethanol pretreatment, and many others [32].

Only within the last ~2 decades have long enzymatic pathways been activated in vitro with the specific intent to both maximize product yield from low-cost substrates as well as characterize the reaction elements. In this "engineering" context, two approaches to CFME have emerged: purified enzymes and crude extracts.

Purified systems use enzymes that have been overexpressed and purified individually, then recombined to assemble a pathway of interest (Fig. 1). These systems allow exquisite control of reaction conditions and pathway fluxes since the concentration and activity of every component is known. However, cofactor cost and regeneration is a challenge. The majority of CFME research to date has utilized purified systems.

Crude extracts, on the other hand, are prepared by simply lysing the cell, using centrifugation to remove cellular debris, and collecting the supernatant as "lysate" or "extract" (Fig. 1). Lysates are cheap to generate and contain thousands of catalytic proteins naturally present in cell metabolism. The activation of these native pathways in the extract can regenerate cofactors or energy to provide the support system necessary to fuel highly active metabolic conversions. For example, Jewett et al. demonstrated that formation of inverted lipid vesicles during extract preparation facilitates oxidative phosphorylation which converts reducing equivalents from central metabolism into ATP [33]. While this native metabolism can be looked upon as an advantage, this "background" metabolism, if unwanted, can be difficult to characterize, eliminate, or accurately model. The most prominent examples of crude extract systems involve cell-free protein synthesis (CFPS). Yields of lysate-based systems (now >1 g L^{-1} for bacterial systems) [33–38] have exceeded purified ones (e.g. the PURE system) [39] and commercial applications have been established [40, 41]. CFPS will not be discussed further in this paper, but several other outstanding reviews are available [42–44].

In comparing emerging biotechnology platforms to established ones, purified and crude extract-based CFME systems have individual merits, however trade-offs between yield, cost, and other requirements must be carefully considered. That said, CFME systems provide a complement to in vivo technologies; yet offer several distinct advantages for the design, modification, and control of biological systems (Table 1). From a biomanufacturing perspective, cell-free systems separate catalyst synthesis (cell growth) from catalyst utilization (metabolite production). This contrasts the prevailing paradigm of enclosed, cell-based, microbial "reactors." By eliminating the requirement of cell growth and diverting all carbon flux to product, CFME allows for higher theoretical yields and productivities, a wider range of products, and easier manipulation of reaction conditions. For example, cell-free production of 1,3-propanediol from glycerol (0.95 mol/mol) highlights CFME's ability to avoid byproduct losses associated with traditional fermentation (0.6 mol/mol) [45]. Further, cell-free systems avoid many toxicity constraints arising from substrates, pathway intermediates, or products. It has been noted that biological processes (unlike most chemical reactions) experience heterogeneous (and often deleterious) conditions at larger scales [19]; cell-free systems may experience more chemistry-like scale-up. In one example, cell-free protein synthesis has achieved a yield and rate expansion factor

Table 1. Advantages (+) and disadvantages (–) of cell-free systems

Metric	Living cells	Cell-free systems
Pathway engineering	– Engineer's goal (overproduction) is typically opposed to microbe's goal (growth) – Endogenous regulation is difficult to predict and modify + Ability to use directed evolution	+ Easy to use chimeric enzyme pathways sourced from multiple organisms + Allow mixing with chemical catalysts/hybrid solvents that would otherwise be cytotoxic
Yield	– Carbon flux diverted to cell maintenance and byproducts	+ All carbon/energy can be directed to product
Cell wall	– Selective barrier; intercellular characterization, and product excretion can be challenging + Membrane proteins can be used	+ Direct substrate addition and product removal; easy sampling
Effect of yield toxicity	– Viability constraints (e.g. 1–2% v/v isobutanol [124])	+ No viability constraints (e.g. 12% v/v isobutanol for some pathway enzymes [49])
Stability	– Fermentation conditions affect intercellular environment	+ Reaction conditions controlled by engineer
Cost	+ Low; pending high yields, and low product separation cost	– Must incorporate cost of "catalyst" synthesis (e.g. cell growth and/or enzyme purification) – Enzyme and cofactor costs dominate
Scale-up	– Fermentation conditions are heterogeneous at industrial scale – Contamination can be catastrophic	+ Linear scale-up?
Maturity	+ Years of practical experience; well established methods	– Recently established

of 10^6 with nearly identical performance fidelity at 100 L [40]. Finally, CFME systems may enable a new opportunities in logistics and on-demand, point-of-use manufacturing.

From a prototyping perspective, cell-free systems are well suited to rapid design-build-test cycles because they do not require the re-engineering of the entire organism with each design; merely the exogenous addition of the desired protein, cofactor, or metabolite. Consequently, there is a high degree of flexibility to model the kinetics of individual enzymes, measure metabolite fluxes in multi-step pathways, determine stability of catalysts, study the effects of redox potential on pathway performance, and experimentally isolate many other process properties that are confounded in living organisms [17]. One of the largest application of cell-free biology to date has been in the context of pathway debugging [46].

Although cell-free technology offers many exciting advantages, challenges remain that provide opportunity for improvement. For example, most cell-free systems are not yet commercially available as production platforms. In addition, costs of in vitro systems currently exceed those of in vivo approaches, which limit scale-up. Despite these challenges and others (more thoroughly described in Section 4), the benefits of CFME are inspiring new applications. In the next section, we examine achievements in the capacity to synthesize metabolites using CFME.

3 Achievements in cell-free metabolic engineering (CFME)

One of the early examples of purified enzyme pathway reconstruction was by Welch and Scopes in 1985 [47]. Yeast glycolytic enzymes were isolated and ethanol produced at rate of 0.042 mmol L^{-1} h^{-1}; it was also shown that overloading of substrate (glucose) sequestered inorganic phosphate in the early reaction steps preventing subsequent ATP generation and effectively stopping ethanol synthesis. These seminal results led others to extrapolate that cell-free ethanol production is theoretically capable of higher productivities and yields than living cells [48]. While this has yet to be realized industrially, a range of other CFME pathways have since been explored. These efforts have illuminated general rules for achieving high metabolic yields and productivities in vitro. To briefly summarize, requirements for optimal metabolic conversion include: robust and stable enzymes, appropriate enzyme ratios and substrate/cofactor/buffer concentrations, and a means for providing a favorable thermodynamic push towards a product (i.e. cofactor regeneration, product removal, repeated substrate feeding). These requirements are characteristic properties of the in vivo state of a rapidly growing cell. Thus, a guiding principle that has emerged in the development of CFME systems is
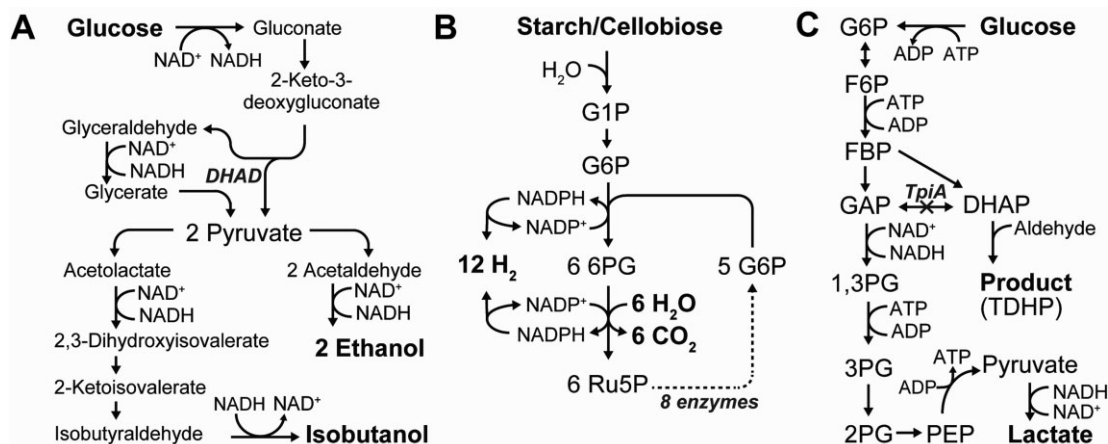
Figure 2. Selected metabolic pathways (**A**) glucose to ethanol/isobutanol [49], (**B**) sugar to hydrogen [61], and (**C**) glucose to DHAP or TDHP [71].

that cytoplasmic mimicry enables highly productive systems [17, 23, 33, 35, 42].

3.1 Purified enzyme systems

3.1.1 Ethanol and isobutanol

A landmark paper for purified CFME describes the conversion of glucose to ethanol (and isobutanol) using 6 (or 9) purified enzymes in a "minimized reaction cascade" [49] (Fig. 2A). Novel use of promiscuous enzymes from hyperthermophilic archaea shortened the ubiquitous 10-enzyme Embden–Meyerhof–Parnas (EMP) pathway to four enzymes using a modified non-phosphorylative Entner–Doudoroff pathway. Glucose was converted to pyruvate while generating two NADH; ATP and additional reducing equivalents generated by the EMP pathway are not needed in this specific pathway. The catalytic activity and tolerance to product concentration of each purified enzyme was assayed, a task not easily accomplished in vivo. The 20 mL complete reaction contained all 6 (or 9) enzymes normalized to an equivalent activity (10 μmol min^{-1}); if all enzymes were active at this rate, the reaction would be theoretically capable of producing ~30 mmol L^{-1} h^{-1} ethanol. The reaction produced an impressive 57% molar yield of ethanol from glucose at 1.51 mmol L^{-1} h^{-1} productivity. This is one of the best-reported examples to date of using CFME as a stand-alone bioproduction strategy. Higher enzyme concentrations and pathways with multiple cofactors (beyond NAD$^+$ alone) are future directions, but this study demonstrates the ease of characterization and the "plug-and-play" nature of CFME optimization.

3.1.2 Pathway prototyping and non-oxidative glycolysis

Despite significant advances in traditional metabolic engineering, most successes to date have been through combinatorial screening as opposed to rational design. Indeed, the semi-synthetic production of the anti-malaria

drug artemisinin has been one of the great success stories of the metabolic engineering and synthetic biology communities, yet the high cost and substantial work (>150 person years) was mostly needed for "pathway balancing" [16]. Cell-free platforms offer a "breadboarding" capability that can be easily monitor and control complex pathways; this can augment the lack of information regarding in vivo steady-state kinetics and metabolic flux imbalances. For instance, mRNA-enzyme fusions bound to DNA were used to explore enzyme ratios in the glucose to trehalose pathway [50]. This concept is extremely valuable for rational metabolic engineering, but is also useful in building engineerable genetic circuits for other synthetic biology applications [46, 51].

Cell-free methods were also recently used in development of a novel non-oxidative glycolysis pathway [52]. The goal of this effort was to develop an alternative to the classic EMP pathway and produce a maximal carbon yield from glucose (i.e. three acetyl-CoA instead of two). This higher carbon efficiency comes at the expense of reducing equivalents (i.e. no NADH is produced) and would be extremely useful in applications that have an external electron source or no need for reducing power. The Liao group was able to achieve ~100% in vitro conversion of glucose to acetyl phosphate at relatively high titer (30 mM) using seven His-tag purified enzymes plus seven purchased enzymes. This result allowed them to validate their proposed pathway and the efficacy a heterologous phosphoketolase enzyme prior to subsequent expression in vivo. The elimination of CO$_2$ as a byproduct of central metabolism is a potentially game-changing result for the metabolic engineering community at large; both inside and outside the cell.

3.1.3 Isoprenoids

In light of intrinsic commercial value and the in vivo efforts regarding artemisinin highlighted above, several groups have reconstituted isoprenoid pathways in vitro to

inform further engineering. Farnesene was produced from acetyl-CoA using nine purified enzymes plus two NADPH reducing equivalents and three ATP [53]. Optimization of in vitro enzyme concentrations informed in vivo addition of a second copy of the *idi* gene that encodes isopentenyl diphosphate isomerase; this resulted in a doubling of in vivo titers. A similar experimental design had been used earlier to improve production of fatty acid-derived compounds [54]. Chen et al. exploited several unique capabilities of CFME to reconstitute a seven-enzyme pathway from mevalonate to amorpha-4,11-diene, an artemisinin precursor [55]. In this work, design of experiments via Taguchi orthogonal arrays were used to quickly explore a broad sample space of relative enzyme concentrations and revealed that the fifth enzyme (IspA, which encodes for farnesyl pyrophosphate synthase) had an inhibitory effect and a lowered concentration improved yields. However, the yield reduction was due to precipitation the IspA product farnesyl pyrophosphate (FPP); subsequent experiments also showed the importance of pH and Mg^{2+} in final titer. These results exemplify the flexibility of CFME to advantageously modify and tune the reaction conditions while also demonstrating CFME's limitations as an in vivo prototyping model since pH and ion concentration are not easily controlled within the cell.

In another example of isoprenoid synthesis, isoprene was made from glycolytic intermediates phosphenolpyruvate (PEP) and pyruvate via a twelve-enzyme pathway at ~100% molar yield [56]. The PEP substrate provided adequate ATP for isoprenoid synthesis; NADPH was recycled from glucose-6-phosphate using glucose-6-phosphate dehydrogenase (G6PDH). A productivity of ~0.2 mmol L^{-1} h^{-1} demonstrates the successful activation of the three-subunit pyruvate dehydrogenase complex and points to the feasibility of a complete pathway from glucose if the glycolytic pathway can be constructed so as to regenerate $NADP^+$ (whereas NAD^+ is the more common glycolytic cofactor).

3.1.4 Specialty chemicals

CFME has biochemically innovative abilities for isotopic and chiral precision. The specificity of enzymatic catalysis has been leveraged to make radiolabeled nucleotides de novo in which specific atoms can be radiolabeled depending on the substrate isotope. In an exemplary *tour de force* example, a 28-enzyme pathway was implemented for the purines GTP and ATP [57], while 18 enzymes are required for the pyrimidines UTP and CTP [58]. Purified enzyme systems have also been used to make chiral molecules difficult to synthesize due a variety of possible enantiomers. Examples include D-fagomine [59] and ethyl (S)-2-ethoxy-3-(p-methoxyphenyl)propanoate (EEHP), a precursor to family of type 2 diabetes drugs, which required discovery/purification of necessary catalytic enzymes resulting in a 100-fold higher productivity compared to whole yeast cells [60].

3.1.5 Hydrogen and fuel cells from sugar

The Zhang group has made significant progress in sugar to hydrogen conversion. H_2 has the potential to be a useful secondary energy carrier in a proposed hydrogen economy; however, it has a relatively low storage density as a gas. An alternative proposal would use the high storage density of sugar to power an efficient, enzymatic sugar-to-hydrogen process with subsequent fuel cell conversion to electricity. The hypothetical energy conversion efficiency of a robust, mobile, enzyme-based sugar-to-hydrogen-to-electricity system would be very high relative to conventional (combustion) and proposed (electric battery or fuel cell) energy systems [24]. Impressive molar conversion of hydrogen has been observed from starch [61], cellobiose [62], xylose [63], sucrose [64], and glucose/G6P [65] (Table 2). Notably, the ~12 mol H_2 mol^{-1} glucose-equivalent yield is triple the in vivo Thauer limit [66] of 4 mol H_2 mol^{-1} glucose-equivalent. The typical continuous flow reaction pioneered by the Zhang group uses thirteen purified enzymes with H_2 and CO_2 monitored in the gas stream (Fig. 2B). Two dehydrogenases are used to reduce $NADP^+$ via two-step oxidation of G6P to Ru5P; the majority of remaining enzymes are used to rearrange the five carbon sugars to six carbons in order to repeat the cycle. Meanwhile, H_2ase generates H_2 from the NADPH. This work demonstrates the utility of a complex pathway, the flexibility to use cheap, five-carbon substrates (i.e. xylose), and the increased efficacy of thermostable enzymes [63, 65]. It should be noted, however, that the xylose system requires phosphorylation of xylulose to xylulose-5-phophate which requires ATP or less costly polyphosphate [63]. In general, comparably high molar productivities point to advantages of a gas-phase target molecule whose separation presumably drives thermodynamic equilibrium towards H_2. Further flexibility was demonstrated when the H_2ase was replaced by xylose reductase to make xylitol from xylose while consuming cellobiose for energy [67]. While achieving (near-) theoretical yields, a challenge for CFME highlighted in these works is the need for costly cofactors (see Section 4.3). For example, to produce ~60 mmol H_2 over 120 h, 2 mmol of $NADP^+$ is needed (a turnover of ~30) [62].

Further work by Zhang's team to utilize recalcitrant feedstocks resulted in a simultaneous enzymatic biotransformation and fermentation system [68]. Here, a short four-enzyme pathway converted cellulose to starch and glucose where the free glucose was metabolized by living yeast to ethanol. This system promises to makes a non-food biomass much more readily available for useful purposes.

In order to further develop the envisioned pathway of sugar to electricity, the pathway in Fig. 2B was repurposed for direct electricity generation using a NADPH-dependent diaphorase enzyme capable of reducing vitamin K3 (menadione) which was immobilized to a fuel-cell anode coated with carbon nanotubes [69]. This allowed

Table 2. Yields and productivities of selected cell-free systems

Target molecule	Substrate	Substrate Conc.	Reaction Time (h)	Product Final titer	Volumetric Productivity	Percent of Theor. yield	Turnover NAD(P)+	Turnover ATP	Ref.
Purified enzyme-based systems									
Ethanol	Glucose	25 mM	19	28.7 mM	1.51 mmol L⁻¹ h⁻¹	57 %	6	–	[49]
Isobutanol	Glucose	19.1 mM	23	10.3 mM	0.45 mmol L⁻¹ h⁻¹	53 %	2	–	[49]
1,3-Propanediol	Glycerol	11.7 mMa)	20	6.3 mMa)	0.73 mmol L⁻¹ h⁻¹	54 %	3	–	[45]
n-Butanol	Glucose	4.2 mMb)	7	3.5 mM	0.49 mmol L⁻¹ h⁻¹	83 %	7	18	[81]
Lactate/lactic acid	Glucose	6 mMb)	10	12.5 mM	1.25 mmol L⁻¹ h⁻¹	~100 %	10	31	[80]
Malate/malic acid	Glucose	2.4 mMb)	4	3 mM	0.75 mmol L⁻¹ h⁻¹	63 %	2	8	[27]
Amorpha-4,11-diene	Mevalonate	10 mM	6	1.7 mM	0.28 mmol L⁻¹ h⁻¹	50 %	–	0.3	[55]
Farnesene	Acetyl-CoA	1 mM	9	~39 µM	0.004 mmol L⁻¹ h⁻¹	35 %	0.07	0.03	[53]
Isoprene	PEP	1.5 mM	~4.3	~0.5 mM	0.13 mmol L⁻¹ h⁻¹	~100 %	0.5	3	[56]
Acetyl phosphate	F6P	10 mM	1.5	~30 mM	20.0 mmol L⁻¹ h⁻¹	~100 %	–	–	[52]
Hydrogen (H₂)	Starch	2 mMc)	~16	–	0.44d) mmol L⁻¹ h⁻¹	43 %	3	–	[61]
Hydrogen (H₂)	Cellobiose	2 mMc)	~10	–	0.48d) mmol L⁻¹ h⁻¹	93 %	10	–	[62]
Hydrogen (H₂)	Cellopentaose	8 mMc)	~6	–	3.92d) mmol L⁻¹ h⁻¹	68 %	18	–	[62]
Hydrogen (H₂)	Xylose	2 mM	~14	–	1.95d) mmol L⁻¹ h⁻¹	95 %	5	0.7e)	[63]
Xylitol (NADPH)	Xylose/cellobiose	100/3 mMc)	48	~18 mM	0.38 mmol L⁻¹ h⁻¹	18 %	9	–	[67]
Hydrogen (H₂)	Sucrose	2 mM	~100	–	2.98d) mmol L⁻¹ h⁻¹	97 %	15	e)	[64]
Hydrogen (H₂)	Glucose/G6P	2/100 mM	~1	–	8/157d) mmol L⁻¹ h⁻¹	–	6/15	e)	[65]
Crude extract-based systems									
DHAP	Glucose	11.1 mM	~5	9.5 mM	1.90 mmol L⁻¹ h⁻¹	83 %	33	7	[71]
TDHP	Glucose	55.5 mM	~1.3	23 mM	17.25 mmol L⁻¹ h⁻¹	41 %	80	16	[71]
Ethanol	Glucose	217 mM	~8	86.8 mM	10.85 mmol L⁻¹ h⁻¹	20 %	1580	22	[73]

F6P, fructose-6-phosphate; DHAP, dihydroxyacetone phosphate (unstable intermediate); TDHP, 5,6,7-trideoxy-D-threo-heptulose-1-phosphate (final product).
a) Values adapted from "fed-batch" to "batch" system for comparison; conc. determined by final substrate/product amount (mmol) divided by total rxn volume (40 mL).
b) Calculated by multiplying feeding rate by time at final titer.
c) mM sugar substrate refers to mM G6P-equivalent.
d) Peak (not average) productivity.
e) ATP and/or polyphosphate is added and consumed as a substrate.

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direct electricity production from maltodextrin in a batch experiment. The energy storage density of this system is an order of magnitude higher than traditional batteries, though at a lower voltage and current density. While a complete review of enzymatic fuel cell literature is available elsewhere [25, 70], this work demonstrates a promising application and direction for future CFME pathways.

In summary, purified systems to date exhibit high percent molar conversion but relatively low molar (mmol L^{-1} h^{-1}) and mass (g L^{-1} h^{-1}) productivities (Table 2). Cofactor turnover could be improved; it currently ranges from ~1 to 30 for both $NAD(P)^+$ and ATP (Table 2). Finally, though cheaper techniques are in development, cost of cofactors and enzymes remain a barrier to commercialization. Crude extract-based CFME has fewer published examples, but offers an alternative approach that could help address some of these issues at the expense of added complexity.

3.2 Crude cell lysate systems

While a majority of CFME approaches use purified enzymes, lysates of cells have been used in other applications (e.g. protein synthesis) to great effect [42]. One seminal paper for crude extract-based CFME involves the synthesis of dihydroxyacetone phosphate (DHAP) from glucose (Fig. 2C) [71]. Here, engineered *E. coli* cells were pelleted and lysed by a high-pressure homogenizer; subsequently, cell debris, along with insoluble components, was removed via centrifugation. The resulting cell lysate was buffered and fed glucose, ATP, and NAD^+ in a 10 mL batch reaction. The deletion of triosephosphate isomerase (*tpiA*) in the source strain caused DHAP to accumulate in the reaction while the absence of AMP-nucleosidase (*amn*) prevented degradation of the catalytic ADP/ATP. Butanal was added to the reaction to generate 5,6,7-trideoxy-D-threo-heptulose-1-phosphate (TDHP) via an endogenous aldolase (FbaA). The subsequent high yield of DHAP and TDHP demonstrates the viability of the crude extract approach and the ability for strain modifications (*tpiA* and *amn* deletion) and fermentation conditions to affect CFME reactions. Additionally, selective addition of NAD^+ (and not $NADP^+$) funneled glycolysis to DHAP as the residual concentration of $NADP^+$ in the extract was apparently too low to allow activation of the competing pentose phosphate pathway. In order to further optimize reaction conditions, Bujara et al. built a continuously stirred reaction where enzymes are sequestered behind a membrane and fifteen pathway intermediates in the effluent are quantified in real time using ESI-MS [72]. Analysis of intermediate flux after combinatorial addition of commercial enzymes informed construction of an operon to express needed enzymes during the fermentation; this improved DHAP yield approximately three-fold. While the pathway to DHAP is inherently limited by loss of metabolite flux to lactate in order to regenerate NAD^+,

the real time analysis and optimization shows the potential for precise control of metabolite flux despite the presence of unused enzymes in the lysate. Further, this shows a powerful approach that can be used to build detailed kinetic models for the metabolic pathways as defined by values of K_M, k_{cat}, and K_i for each enzyme; these models have utility both in vivo and in vitro.

Recently, a yeast cell-free lysate system to produce ethanol from glucose was demonstrated to be highly productive (10 mmol L^{-1} h^{-1}) and required no pathway tuning or strain modification [73]. Yeast cells growing in waste of beer fermentation broth were isolated, grown, lysed via bead beating, buffered, and mixed with glucose to generate 4 g L^{-1} (87 mM) ethanol. Notably, concentrations of ATP (1.8 mM) and NAD^+ (0.11 mM) in the extract were high enough to sustain the reaction without supplementation. In other words, enzyme turnover was >1500 for NAD^+ and >20 for ATP, thus highlighting a potential advantage of crude-extract CFME as compared to purified enzyme systems.

Crude extract pathways are not limited to endogenous target molecules. Keller et al. generated 3-hydroxypropionate from acetyl-CoA and CO_2 using H_2 for reducing power (this is a component of a proposed, 13-enzyme pathway to fix CO_2 to acetyl-CoA via hydrogen reducing equivalents) [74]. Expression of the novel pathway was done in *P. furianosis*, which has optimal activity at 100°C; when the cell-free reaction was run at 70°C, only the recombinantly expressed enzymes were active at the lower temperature. Crude extracts have demonstrated the ability to synthesize high-value biologics and therapeutics; examples include single-step conversion of diketides to chiral triketide lactones (polyketide building blocks) [75], lysine to ε-poly-L-lysine (an antimicrobial) [76], and 7α-demethoxycefminox to cefminox (an antibiotic) [77]. In another study, ^{14}C-labeled substrates were used to elucidate possible biosynthetic pathways for manufacture of the antitumor agent Azinomycin B [78]. These high-value products are promising directions for economically feasible CFME; particularly since crude extracts do not require costly purification steps.

In summary, extract-based cell-free systems have so far seen relatively few examples for biomanufacturing and pathway prototyping. Thus, it is still early to comment on their utility in this arena, as compared to purified systems. However, the underlying metabolism is able to support high-level cofactor and energy regeneration. Moreover, the approach can enable rapid combinatorial iterations of enzymes during optimization of biosynthetic pathways. Thus, we expect to see substantial growth in the upcoming years.

4 Challenges and opportunities in CFME

CFME is already showing tremendous value for pathway construction and prototyping. While many proof-of-

concept pathways have been developed at the lab scale, it remains unclear to what extent CFME systems might also serve as commercially relevant production factories. Many barriers remain to commercializing cell-free synthetic systems. These include high costs of protein purification, stability and longevity of reactions, cofactor regeneration, limited efforts in modeling, and ability to scale. Following is discussion of current efforts to address these challenges.

4.1 Purification

Large-scale purified enzyme systems are limited by several major obstacles including enzyme cost and stability. Strategies to increase total turnover of enzymes would alleviate some of these issues [24, 67]; in addition, cost-effective strategies to purify multiple enzymes are desirable. Heat purification and immobilization, as well as the use of cheap scaffolds, are some of the most active areas of research being employed to address this challenge.

The Ohtake lab has done significant work in the CFME space by expressing thermophilic enzymes in *E. coli*, lysing the cell, and heating the extract to inactivate all proteins except those needed for the pathway of interest [79]. This effectively "purifies" the relevant enzymes in a single step. This method was used to build a 10-enzyme "chimeric glycolysis" pathway to make lactate [80]. The GAPDH/PGK enzymes of traditional EMP glycolysis are replaced by GAPN; thus, no net ATP is generated by the pathway. In a fed-batch system with a slow feed rate, lactate was produced from glucose at a rate of ~1.25 mmol L^{-1} h^{-1} and nearly ~100% molar conversion. In a similar fashion, a sixteen-enzyme pathway to produce *n*-butanol from glucose was constructed [81]; this specific project used a pyruvate decarboxylase and CoA-acylating dehydrogenase to bypass the large and difficult-to-express pyruvate dehydrogenase complex [82]. Directed enzyme evolution of malic enzymes was needed to optimize a glucose to malate system [27, 83]. Stability of cofactors is a tradeoff when using thermophilic enzymes in CFME; the reaction temperature of 50°C is a compromise between the high temperature needed for maximum enzyme activity and lower temperature needed for NAD^+ and ATP stability.

"Heat" purification was also utilized during efforts to engineer a thermotolerant aldehyde dehydrogenase for glucose-to-ethanol CFME [49]. Here, reaction velocity and NAD^+ affinity were improved via protein refolding [84] and evolution by random mutagenesis [85]. Shorter pathways have also leveraged "heat" purification in conjunction with ammonium sulfate precipitation, a technique where the salt concentration is increased until proteins begin sequentially precipitating [86].

In an effort to improve stability of the thermophilic enzymes themselves, glutaraldehyde was used to cross-link proteins so that the *E. coli* chassis cell remained

intact after heat treatment [87]. The membrane in this state is permeable to small molecules and the "whole cells" could repeatedly convert fumarate to malate. This "permeabilized cell" concept, like crude extracts, can eliminate the need for enzyme purification [88]. A number of permeabilizing agents using a variety of mechanisms are available [88]; for example, Krauser et al. used Triton X-100 in efforts to produce flavolin [89]. However, more research is needed to characterize enzyme stability and kinetics under permeabilized conditions.

In another purification strategy inspired by the cellulosome, dockerin-tagged enzymes are bound to cohesin attachments sites linked together as a scaffoldin subunit. The scaffoldin complex can then be attached to a magnetic nanoparticle [90] or a cellulose-binding domain (which can adsorb to a low-cost regenerated amorphous cellulose) [91]. As an added benefit, the spatial proximity and/or added enzyme stability of these enzymes in the CFME reaction improved activity of a three-enzyme pathway by ~5-fold and ~40-fold, respectively. Thus, the purification technique lowers costs and can lead to higher yields and productivities, particularly if chemical reactions are diffusion limited.

4.2 Spatial organization and enzyme stability

The reaction environment for CFME is inherently different than in vivo. Cells have organelles for spatial organization which can sequester pathways with overlapping intermediates, isolate pathways with toxic intermediates, and accelerate flux through slow or bottleneck enzymes by creating high local concentrations. Additionally, enzyme concentrations in CFME are typically 1–10 mg total protein mL^{-1} in crude extracts [35, 71] and lower still for purified systems, whereas total cytoplasmic protein concentration is ~200 mg mL^{-1}. Enzymes were evolved for maximal activity in the crowded macromolecular environment in vivo. Thus, compartmentalization, scaffolding, and immobilization are but a few of the strategies being used to circumvent these challenges [92, 93].

For compartmentalization, CFME could utilize liposomes (lipids), polymersomes (amphiphilic block copolymers), layer-by-layer capsules (charged polyions), microfluidic encapsulation, and other approaches [94]. For example, polymersome nanoreactors can be engineered for selective porosity (dependent on pH and sugar concentration) as well as positional control of a three-enzyme cascade [94]. Also, in vivo scaffolding strategies [95] using proteins [96] or DNA [97] could be easily adapted to cell-free applications. The success of the scaffoldin-dockerin work by Zhang described in Sections 3.1.5 and 4.1 [90, 91] indicates the reaction is diffusion-limited or that scaffolding improves enzyme stability and activity.

Enzyme stability and longevity are challenges for CFME. With continuous removal of inhibitory byproducts and feeding of substrates, crude extract cell-free protein

synthesis has been shown to last up to ~100 h [98], yet most cell-free batch systems show decreased productivity over time (Table 2), possibly due to enzyme inactivation. Enzyme immobilization is a common industrial practice to increase the longevity of enzymes, often the expense of activity, yet many immobilization strategies for CFME have been proposed including particle entrapment, crosslinking to microchannel surfaces, and immobilization to membranes or nanoparticles [26]. Recent work to improve activity of alcohol dehydrogenase on a variety of solid supports points to a hypothetical reaction scheme in which all pathway enzymes are immobilized [99]. For small quantities of high-value products, chip-based immobilization strategies (such as fusion pro-tags) could be useful [100]. In general, the increased local concentration of substrates and catalysts via scaffolding/immobilization has the potential to increase both the longevity and productivity of cell-free systems.

4.3 Cofactor engineering

While crude extract-based CFME efforts have shown the potential for activating and controlling long-lived, cost-effective cofactor regeneration in lysate systems [33, 73] (Table 2), cofactor costs and instability remains a significant challenge for CFME and presently limits its commercial viability for low-value commodity chemicals [24]. Cofactor engineering could catalyze more cost-effective CFME efforts, especially for purified systems that must supplement the reaction with the necessary cofactor(s). The nicotinamide nucleotides NAD^+ and $NADP^+$ are ubiquitous electron carriers that are utilized by most redox enzymes. These molecules differ only in an additional phosphate group esterified at the 2′-hydroxyl group of NAD^+. To activate de novo or existing enzyme pathways, cofactor balance can be crucial, especially without the regulation of a living cell. A number of protein engineering studies have successfully changed cofactor specificity from $NADP^+$ to NAD^+ [101], NAD^+ to $NADP^+$ [102] or made the enzyme active with either cofactor [103]. A prominent CFME example leverages the cofactor specificity of an engineered pyruvate dehydrogenase complex and oxidase to create a dynamic molecular purge valve that oxidizes excess NADH (but not NADPH) to balance cofactor levels in polyhydroxybutyrate (PHB) and isoprene pathways [104]. As mentioned earlier, $NAD^+/NADP^+$ are unstable in vitro and expensive; several groups have been examining the feasibility of biomimetic cofactors such as 1-benzyl-3-carbamoyl-pyridinium [105]. Other excellent reviews are available for discussion of cofactor-specificity and enzyme engineering [25, 106].

In specific pathways or applications, the ease of access to the reaction space could inspire integration of electronics with CFME where reducing power could be provided directly to enzymes by an external electric current (i.e. electrosynthesis) [107, 108]. These enzymatic bioelectrochemical systems (e-BES) could be extremely useful in adapting CFME to pathways that are not redox balanced [108].

4.4 Modeling

Prediction of intracellular concentrations and fluxes using computational modeling has greatly improved the cofactor management and product yields of conventional metabolic engineering efforts. In silico analysis of cell-free metabolism could thus shed light on pathway dynamics and inform optimization strategies. Removal of transport barriers and microbial growth objectives allows adaptation of in vivo models to cell-free systems; alternatively, new models can be made from scratch. For example, to analyze the cellobiose-to-hydrogen system described previously [62], Ardao and Zeng used a kinetic model incorporating nineteen mass balance equations to predict that modularizing the one-pot system into two reactors could improve productivity two- to eight-fold [109]. As another illustrative example, a genetic algorithm for one-pot multi-objective optimization of the thirteen enzyme loadings plus initial concentrations of cellobiose, phosphate, and $NADP^+$ showed the importance of hydrogenase loading and predicted a possible productivity up to 355 mmol L^{-1} h^{-1} [109]. This paper also identified which enzymes would benefit most from engineering to improve activity or solubility. Metabolic flux analysis (MFA) and associated tools could also be adapted to CFME efforts by removing transport operations and boundaries and reconceptualizing objective functions [110–112]. For example, a well-studied flux balance analysis tool (COBRA) was modified to analyze crude extract production of DHAP [112]. This model was able to correctly predict a previously identified undesired pathway for DHAP synthesis from adenosine phosphate, as well as identify other potentially interfering pathways that could be feasibly modified to further insulate the desired metabolic pathway.

The development of computational design tools such as BNICE [113, 114], PathPred [115], UM-PPS [116], Retropath [117], Metabolic Tinker [118], and others [111] has led to an explosion of proposed novel metabolic pathways and circuits. CFME will be crucial tool in the experimental validation of these pathways. CFME's ability to adjust cofactor/enzyme concentrations and easily sample reaction intermediates/products are significant advantages when troubleshooting and optimizing these pathways.

4.5 Scalability

It is well known in microbial fermentation processes that reaction scale affects molecular diffusion, gas transfer, hydrostatic pressure, mixing rates, temperature gradients, and other parameters [19]. To date, cell-free systems

have relatively few examples of industrial scale production. However, published results from crude-extract cell-free protein synthesis recently demonstrated linear scalability for protein yields from the microliter to liter scale for aglycosylated antibodies (5 L scale) [41] and cytokines (100 L scale) [40]. Highlighting an expansion factor of 10^6, these findings suggest cell-free engineering considerations are more parallel to chemistry than a living system and give promise to the technical scalability of CFME pending economic viability.

5 Outlook

CFME holds tremendous potential for rapidly developing metabolic systems capable of synthesizing useful small molecules. While the number of successfully demonstrated pathways continues to grow, purification and cofactor costs are challenges that have yet to be resolved. By addressing these challenges, as well as others described in Section 4, we anticipate that cell-free systems will enable new opportunities. One such emerging opportunity is the ability to explore combinatorial assembly of pathways by mixing-and-matching lysates or individual enzymes (Fig. 3, shown for lysates). For example, when optimizing a selected pathway, multiple cell extracts could be prepared, each with a different enzyme(s) overexpressed. The extracts provide supporting metabolic enzymes for highly active energy production and co-factor regeneration (e.g. to convert glucose to acetyl-CoA). Subsequently, extracts selectively enriched with these different enzyme(s) could be mixed in different combinations/ratios to build partial or full-length pathways that provide initial estimates of beneficial enzyme ratios, as well as indications of enzyme promiscuity to different starting substrates and the presence of unwanted side reactions. Notably, such an approach does not require the focus on flux balancing and delicate promoter tuning to maintain viability as for in vivo systems [119–121]. Moreover, strain modification techniques, such as MAGE [122], could be leveraged to add protease cleavage sites [23] or affinity tags [123] to essential enzymes; this would allow selective degradation or physical removal of unwanted pathways critical for growth. An additional possibility is mixing lysates from multiple organisms, i.e. organismal amalgams, to construct novel natural product pathways that would be impossible to express in one organism in vivo (Fig. 3).

Looking forward, we expect that CFME will enable new frontiers for rapidly generating and evaluating new enzymes and metabolic pathways. It is unclear to what extent CFME will be used for prototyping systems for in vivo application (e.g. pathway balance or enzyme promiscuity) or as a large-scale biomanufacturing system in its own right. Small-scale, high-value synthesis in chip-like applications may also prove to be useful. Obviously, these
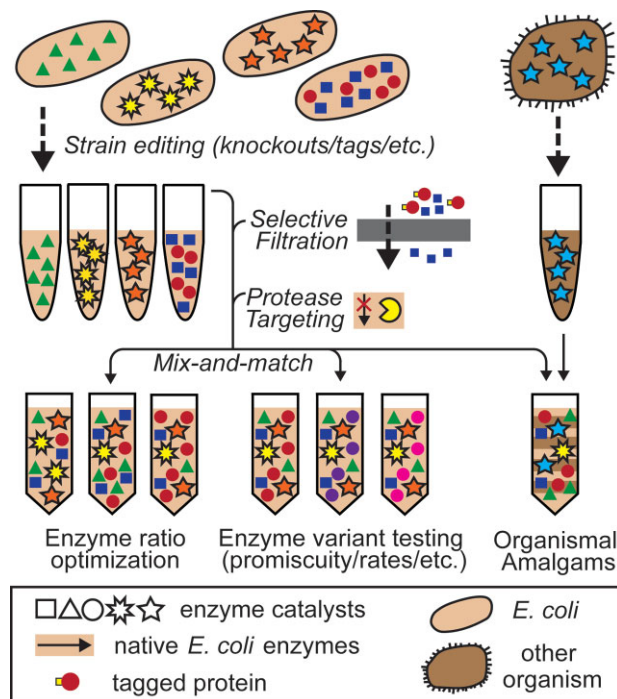
Figure 3. New opportunities to use crude cell lysates for pathway optimization and debugging.

questions will only be answered as viable commercial ventures emerge. That said, by avoiding toxicity constraints, CFME will offer novel ways to create hybrid molecule products composed of elements derived from both chemical and biological synthesis strategies. In sum, CFME holds the potential to expand the definition of biomanufacturing, allowing cell-free biosynthesis to penetrate into new industrial applications and provide immediate and significant solutions to global challenges in materials, medicine, and sustainability.

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