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Current Opinion in Biotechnology

Toward sustainable, cell-free biomanufacturing

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Industrial biotechnology is an attractive approach to address the need for low-cost fuels and products from sustainable resources. Unfortunately, cells impose inherent limitations on the effective synthesis and release of target products. One key constraint is that cellular survival objectives often work against the production objectives of biochemical engineers. Additionally, industrial strains release CO_2 and struggle to utilize sustainable, potentially profitable feedstocks. Cell-free biotechnology, which uses biological machinery harvested from cells, can address these challenges with advantages including: (i) shorter development times, (ii) higher volumetric production rates, and (iii) tolerance to otherwise toxic molecules. In this review, we highlight recent advances in cell-free technologies toward the production of non-protein products beyond lab-scale demonstrations and describe guiding principles for designing cell-free systems. Specifically, we discuss carbon and energy sources, reaction homeostasis, and scale-up. Expanding the scope of cell-free biomanufacturing practice could enable innovative approaches for the industrial production of green chemicals.

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Current Opinion in Biotechnology 2021, **69**:136–144

This review comes from a themed issue on **Chemical biotechnology**

Edited by **Ajikumar Parayil** and **Christine Santos**

For a complete overview see the Issue and the Editorial

Available online 13th January 2021

https://doi.org/10.1016/j.copbio.2020.12.012

Introduction

For decades, microbial hosts have been engineered as an economical and environmentally friendly approach to chemical production. Unfortunately, cellular platforms impose inherent limitations on the effective biosynthesis and release of chemicals [1]. For example, cellular survival reduces the carbon available for desired chemical products and limits the maximum titer to nontoxic concentrations. To avoid these constraints, synthetic biologists have developed cell-free systems for a wide variety of applications, including small molecule biosynthesis [2•]. The cell-free metabolic engineering (CFME) approach can be used to build metabolic pathways *in vitro* using purified enzymes and/or crude cell extracts, which enables fine tuning of reaction conditions and enzyme concentrations [3•]. Extract-based approaches employ the biological machinery harvested from cells through lysis to disrupt the membrane and centrifugation to remove cell wall fragments, genomic DNA, and insoluble components. The resulting cell extract contains ribosomes and cellular proteins that enable *in vitro* transcription and translation, as well as endogenous metabolism, to produce protein products or metabolic enzymes. Similarly, engineered cells expressing metabolic enzymes can produce enzyme-enriched extracts without the need for *in vitro* protein synthesis [4]. In addition, CFME platforms provide open reaction environments with flexibility for purification and varied reaction modes in the absence of cellular barriers. This presents unique opportunities (e.g. direct addition of substrates and cofactors, reaction monitoring, and simplified product purification) as well as disadvantages (e.g. loss of cofactor regulation) [5] (Table 1). Although many strategies for cell-free protein synthesis have been explored in extract-based systems, production of non-protein products has not been pursued extensively beyond laboratory-scale demonstrations [2•].

In this review, we highlight select advances in cell-free approaches for biomanufacturing and outline promising paths toward sustainable, cell-free chemical biomanufacturing (Figure 1). We first set the stage by describing seminal work using crude extract-based systems to prototype biochemical production pathways. Then, we discuss four core advances necessary to realize the potential of cell-free biomanufacturing platforms: addressing sustainable feedstocks, utilizing alternative energy sources, maintaining homeostasis *in vitro*, and increasing reaction scales for industrial biochemical production.

Table 1

Advantages and limitations of cell-free biosynthesis for small molecules

Advantages	Limitations
• Lack of biomass production enables increased carbon flux toward product	• Currently optimized for small-scale prototyping reactions
• Lack of cell viability constraints permits greater product titers and molecule classes	• Cost of cell processing and reagents (e.g. cofactors) render low-value molecules economically unviable at present
• Presence of endogenous metabolism for cofactor recycling and energy regeneration	• Endogenous metabolism may compete with desired pathway
• Absence of cellular barriers enables direct manipulation of reaction conditions, including the addition or removal of essential enzymes and the addition of non-natural substrates	• Catalyst stability may limit reaction longevity
• Pathways can be built for high theoretical carbon efficiency	• Limited range of cofactor-based enzyme classes available for cell-free transformations
• Metabolic proofreading can increase the longevity of cell-free reactions	• Not all dead-end metabolites and cofactors can be easily converted
	• Enzyme inhibition, inactivation, or regulation by reaction intermediates and products

Cell-free metabolic engineering

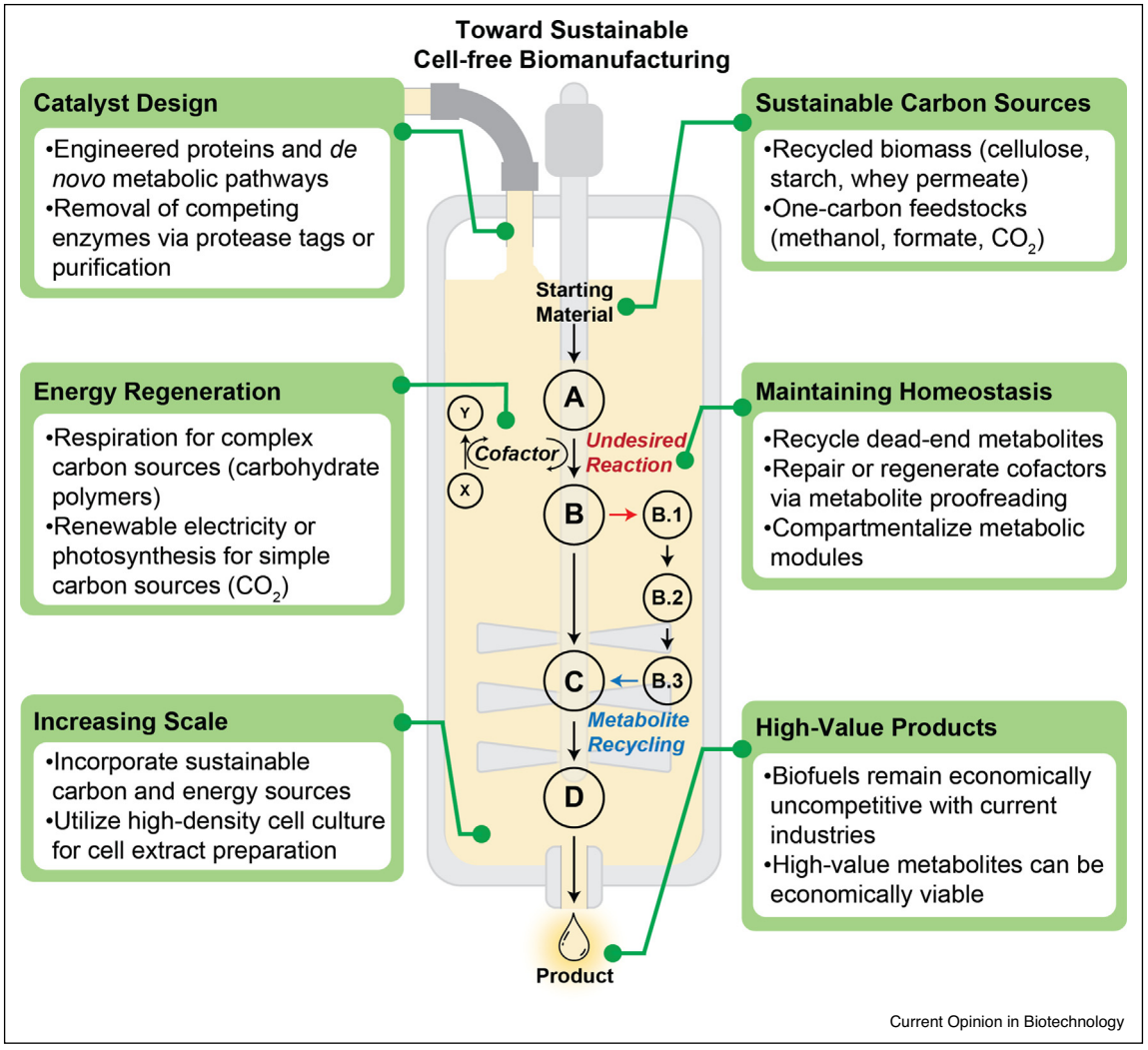
CFME systems provide many advantages for building metabolic pathways, such as enhanced control over the chemical environment and rapid design-build-test cycles [2•,4,6]. Many groups have used kinetic characteristics from purified enzymes studies to better select enzymes for metabolic pathways of interest [7,8]. Compared to these purified *in vitro* systems, crude cell extracts contain native metabolic elements that can provide the added benefit of prototyping enzymes within the context of competing biochemical reactions [9–12]. Complete enzymatic pathways can be constructed *in vitro* by either combining cell extracts containing enzymes produced in host cells before lysis or *in vitro* through cell-free gene expression (CFE) (Figure 1) [2•]. For instance, pathways toward the production of dihydroxyacetone phosphate [11], 2,3-butanediol [13,14], mevalonate [10], *n*-butanol [9,15], limonene [16,17], polyhydroxyalkanoates [12], and styrene [18••] have been constructed successfully in crude cell extracts (Figure 2). From the biochemist's perspective, the cell-free approach offers a high degree of flexibility to model the kinetics of individual enzymes. For the metabolic engineer, the scheme could accelerate the design of new biosynthetic pathways by optimizing pathway parameters (including enzyme variants and relative concentrations of individual enzymes) before they are expressed in cells in commercial fermenters [19•]. While recent work has successfully optimized pathways close to central carbon metabolism, there are still large areas of more complex metabolism that have yet to be explored by extract-based CFME approaches (Figure 2, gray areas). Bioactive molecules in particular, such as cannabinoids [20•] and indole alkaloids, present appealing opportunities for commercially viable targets for cell-free biomanufacturing. In order to access these more complex products, new strategies must be developed to increase the functional repertoire of cofactor-dependent enzyme families in cell-free systems (e.g. P450s, radical *S*-adenosylmethionine enzymes, ferredoxin-dependent oxidoreductases) and to better control carbon flux. Cell-free systems also present the potential to serve as lone-standing, *in vitro* biomanufacturing platforms that can efficiently carry out chemical transformations that are unattainable in living organisms, including greater volumetric productivities [13] or higher concentrations of cytotoxic molecules [18••,21••].

Sustainable carbon utilization pathways

While many recent efforts have sought to expand the product portfolio of cell-free biomanufacturing platforms, feedstock utilization has received less attention. Most cell-free platforms rely on glycolysis with glucose as the starting substrate. However, there is significant potential for cell-free systems to move toward more efficient biochemical transformations using cheaper, more sustainable carbon sources. Biopolymers such as starch, whey permeate, and cellulose can be used as a feedstock by breaking them down to glucose molecules, as seen for both *in vivo* and *in vitro* approaches [12,14,22–24]. Addition of whey permeate to cell-free extracts has been shown to enhance cell-free protein synthesis based on *Escherichia coli* MG1655 extracts by 50% without the addition of any additional enzymes and has been used to increase production of 3-hydroxybutyrate [12]. Utilization of cellulosic biomass has proven to be challenging and slow for organisms, but chemical pretreatment can be used to make more bioavailable hydrolysates. While the chemical compositions of these hydrolysates are often difficult for cells to metabolize due to toxicity, cell-free systems are less affected by these toxic components and the many solvents typically used in commercial operations for biomass pretreatment [21••]. Thus, cell-free biomanufacturing platforms have the potential to utilize many waste feedstocks, that would require extensive processing after chemical pretreatment for *in vivo* conversion.

Redesigning central carbon metabolism *in vitro* could fulfill the desired transformations more efficiently. In

Figure 1

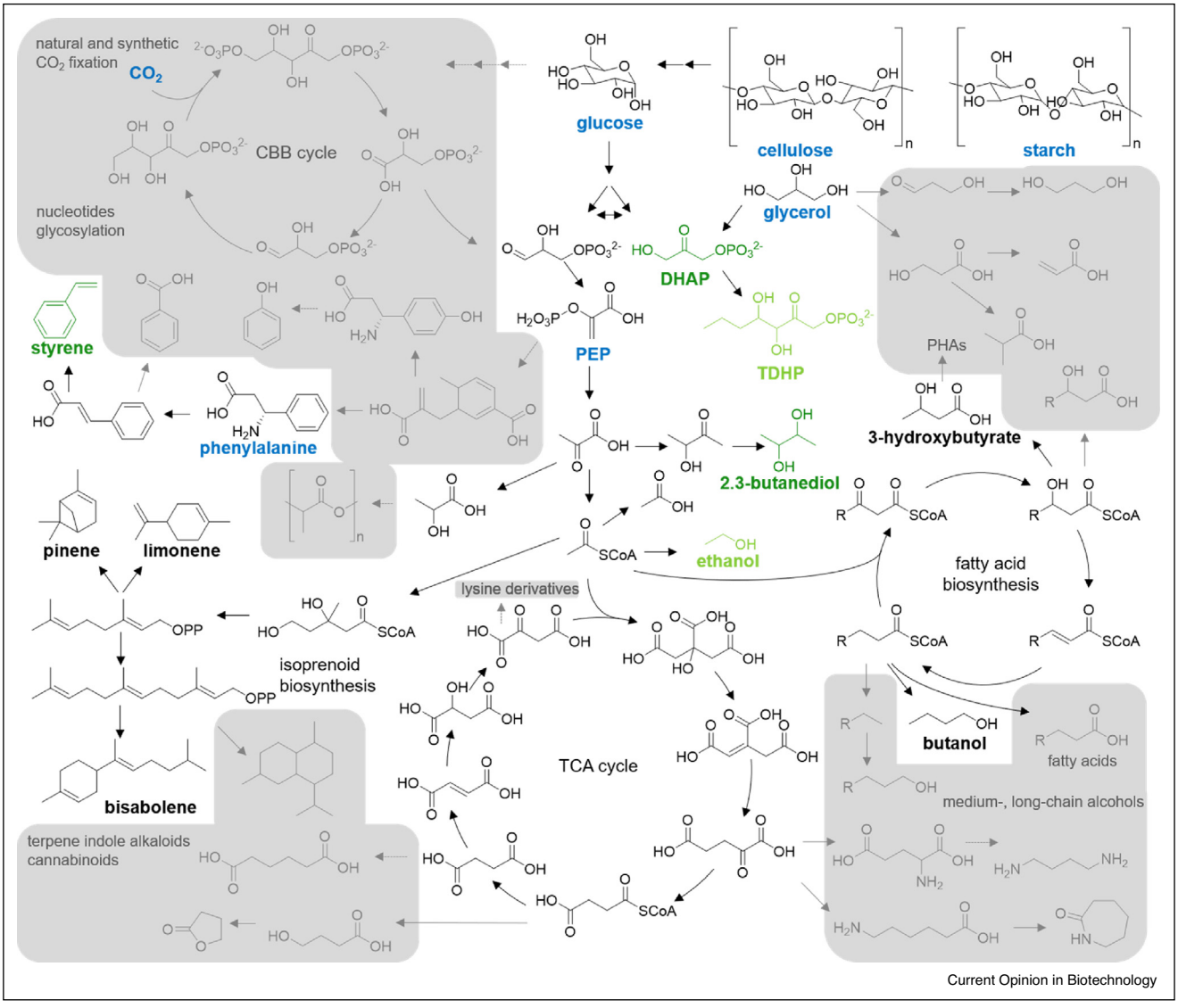


Overview of key considerations for sustainable, cell-free biomanufacturing.
Continued research into these topics will advance cell-free biosynthesis and provide economically viable routes to extract-based biomanufacturing processes in vessels such as the large, idealized reactor shown here.

canonical glycolysis, two molecules of CO_2 are lost in the conversion of one glucose molecule to two acetyl-CoA, limiting the total carbon flux from starting material to product to 66%. The synthetic, non-oxidative glycolysis pathway, on the other hand, manages to retain all the carbon and produce three acetyl-CoA molecules instead [7]. This pathway would not be viable *in vivo* without accessory pathways, but it can be employed *in vitro* using purified enzymes to achieve 100% carbon yield to the desired products. A similar redesign of glycolysis has been successfully implemented in an *in vitro* system using purified enzymes, where the cofactor demand (e.g. ATP, NADH and NADPH) can be adapted to the downstream conversion module and difficult enzyme complexes can be avoided by artificial bypasses [20•,25,26]. Adapting these strategies in extract-based systems will lead to increased carbon conversion and better cofactor homeostasis (see below).

One-carbon feedstocks (e.g. methane, methanol, formate, and CO_2) present another promising substrate class that has not been extensively explored for cell-free biomanufacturing systems [27]. CO_2 fixation modules are particularly interesting, as they carry the potential for the production of sustainable products from an inexpensive and readily available carbon source [28]. Fortunately, cell-free synthetic systems are less constrained on the architecture of these CO_2 fixation modules than microbial biorefineries, as they do not need to function in the complete context of cellular metabolism. This is exemplified by the

Figure 2



Cell-free metabolic map.
This diagram highlights key biochemical pathways that have been established in extract-based cell-free systems from several carbon sources (blue) and the opportunities for further exploration and optimization (gray). End products that have been produced below 10% of maximum theoretical yield are labeled in black, ones between 10 and 40% in lime and ones above 40% in green. We define maximum theoretical yield here as (# of carbons of substrate)/(# of carbons of product) to avoid pathway-specific biases.

recent implementation of the first synthetic CO_2 fixation cycle in an *in vitro* system with purified enzymes, the CETCH cycle [29,30••]. This synthetic pathway is at least as efficient and simple as natural CO_2 fixation pathways but relies on a completely different set of enzymes originating from nine different organisms across all domains of life. Exploring both natural and synthetic one-carbon utilization modules may lower feedstock costs and net carbon emissions for sustainable, extract-based biomanufacturing platforms in the future (Figure 2, gray area).

Efficient energy regeneration modules

Engineering sustainable energy regeneration systems can improve cell-free biomanufacturing, as ATP and NAD(P)H production are essential to power biosynthetic reactions and produce proteins. While most gene expression platforms regenerate ATP with sacrificial substrates (e.g. kinases and polyphosphate systems) to maximize yield, these molecules are expensive and result in inhibitory phosphate accumulation [2•,31]. In contrast, CFME platforms typically generate ATP from natural catabolic pathways (e.g. glycolysis, oxidative phosphorylation) [3•,31,32]. Respiration rates can even

be increased to provide more ATP for biosynthesis by the addition of purified *E. coli* membrane vesicles or artificial vesicles containing cytochromes and ATP synthase that are active *in vitro* [33–35]. However, employing simpler carbon sources could limit the extent of ATP regeneration by oxidative phosphorylation. Therefore, carbon and energy sources for CFME must be considered and tuned in tandem to accommodate the cofactor requirements of the catabolic and anabolic components.

With simple carbon sources, widely available electrical energy could power cell-free metabolism after conversion to chemical energy. For example, CFME systems could utilize external electrical energy by incorporating components from electrically active microbes (e.g. *Shewanella oneidensis* and *Geobacter sulfurreducens*) for direct transfer of energy to biological electron carriers, which has been demonstrated *in vivo* with native [36,37] and heterologous expression [38•,39] of the electrobiological machinery. Electrically powered CFME reactions could consist of heterologously expressed machinery or hybrid reactions containing a defined proportion of cell extract from *Shewanella*, which relies on electroactive membrane proteins, or *Geobacter*, which synthesizes conductive pili [38•,39]. Alternatively, solar radiation is even more abundant than electricity, and biological systems possess well-defined mechanisms to convert sunlight into chemical energy. Solar energy has been harnessed for *in vitro* biosynthesis using thylakoids derived from plants [30••] as well as synthetic vesicles containing bacteriorhodopsin and ATP synthase [40,41••]. These natural and artificial vesicles could be added to CFME reactions to generate ATP from light in a sustainable manner. Implementing such sustainable energy regeneration mechanisms in extract-based platforms could increase the efficiency and economic viability of cell-free biomanufacturing.

Maintaining homeostasis *in vitro*

Cells go to great lengths to maintain homeostasis and enable productive metabolism. To do this, cells degrade nonproductive enzymes, compartmentalize toxic intermediates, repair damaged cofactors, and recycle dead-end metabolites back into metabolism or export them out of the cell [42,43]. Integrating these components into the design of cell-free metabolisms and maintaining their proper equilibria may help to achieve more cell-like longevity for reactions in cell-free systems. In particular, metabolite proof-reading is a critical and historically overlooked aspect in metabolic pathway design. This is the principle by which unwanted side reactions, such as the production of metabolically incompatible cofactor forms or undesired intermediate isomers, are recycled with auxiliary enzymes to maximize production of the desired product. Although metabolite proofreading has recently seen some much-deserved attention [42,44,45•], translating these complex design principles toward a sustainable cell-free biomanufacturing platform remains a grand challenge.

The benefit of including auxiliary enzymes to correct metabolic mistakes has been demonstrated in cell-free systems using purified enzymes. For example, early prototypes of the CETCH cycle suffered from the accumulation of malyl-CoA. Addition of a malyl-CoA thioesterase successfully cleaved the nonproductive metabolite into two intermediates that could be reintroduced into the cycle [29]. Similarly, in a pathway for the production of polyhydroxybutyrate (PHB) from glucose, a key phosphoketolase showed reactivity with multiple substrates and led to the unwanted byproduct erythrose-4-phosphate (E4P). Installation of a 'salvage pathway' consisting of three enzymes reintroduced E4P into the cycle and dramatically enhanced production of PHB (nearly 10-fold) [46]. This illustrates the complementary utility of metabolic proofreading and *de novo* pathway design in the current landscape of cell-free biosynthesis. Continued effort mapping common side reactions and enzyme promiscuity is crucial to identifying additional leaks in metabolism.

Similar strategies have been applied to repair, regenerate, and otherwise maintain proper concentrations of expensive redox cofactors $NAD(P)^+$/NAD(P)H [47]. Several dehydrogenase-based systems have been described that use simple alcohols, glucose, or formate (to name a few) as sacrificial substrates to regenerate NAD(P)H [46,48,49,50••]. In addition, multiple dehydrogenases can be used to construct redox purge valves that are effective in maintaining a proper $NAD(P)^+$/NAD(P)H balance [26,46]. Other innovative photochemical and electrochemical approaches to regenerate NAD(P)H have been described and shed the need for chemical energy [51•,52,53]. Each of these examples demonstrate that homeostatic mechanisms significantly improve the fidelity and efficiency of cell-free metabolic pathways. Employing these principles in extract-based cell-free systems should produce similar improvements, bringing the vision of cell-free biomanufacturing with cell extracts ever closer.

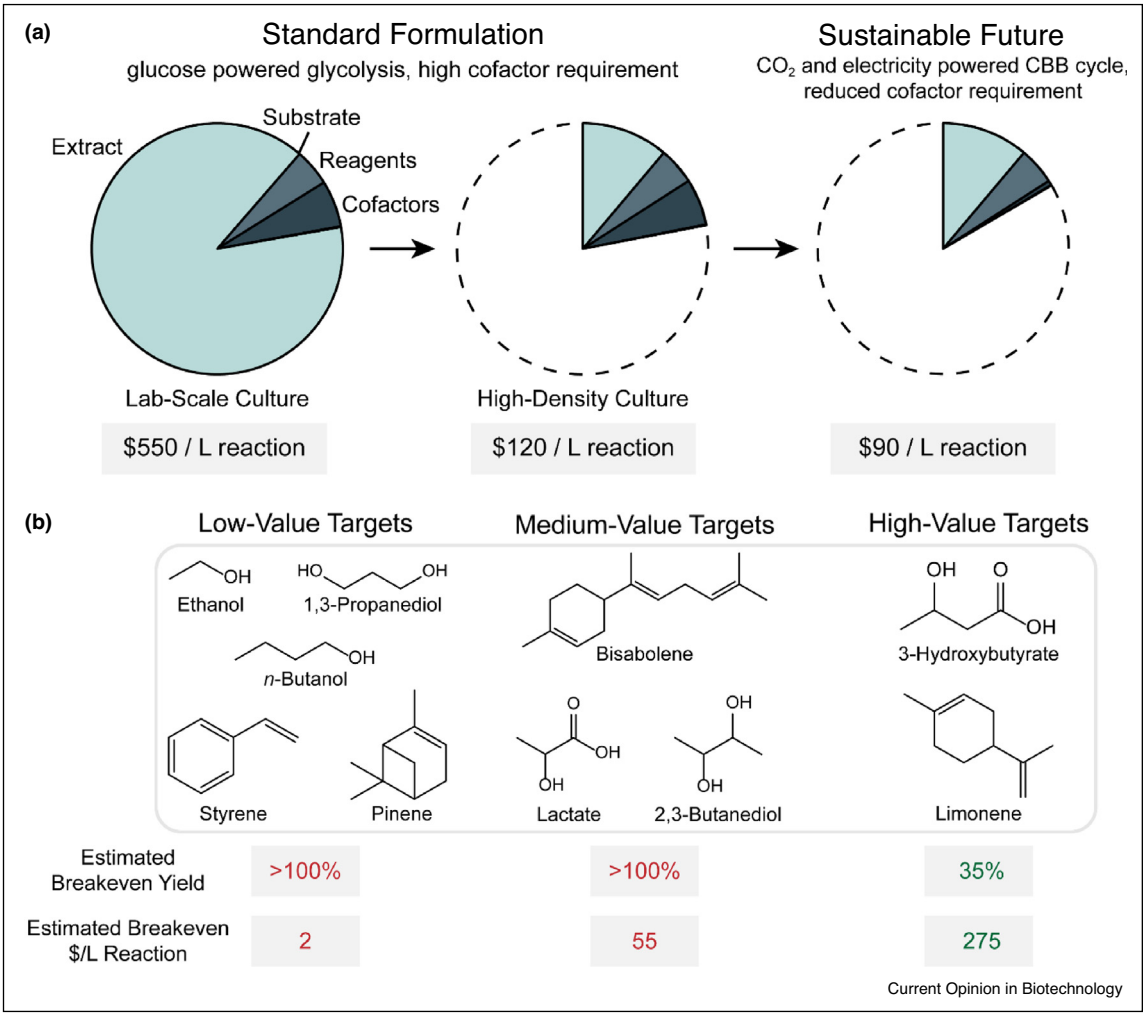
Scaling up cell-free reactions

Despite major advantages of cell-free systems, the transformation of these systems from benchtop to industrial-scale biomanufacturing remains limited by the expenses associated with biocatalyst generation [54]. Common strategies found acceptable for building and prototyping metabolic pathways, such as affinity tag purification, can become cost-prohibitive at large scales. One strategy to overcome this involves building metabolic pathways with enzymes from thermophiles and heat-treating crude extracts to precipitate undesirable endogenous enzymes. This approach has produced a wide variety of chemicals including glutathione [55], fructose 1,6-diphosphate [56], glucaric acid [57], and myo-inositol [58], and it has proven

relevant at industrial scales with successful operation in a 20 000 L reactor [22]. These feats indicate the utility of heat-purification for facile biocatalyst purification with the additional advantage of minimizing side reactions catalyzed by endogenous enzymes. However, this approach is limited by the scope of known thermophilic enzymes and by heat-sensitive cofactor constraints requiring additional pathways to salvage damaged cofactors [59•], batch addition of expensive cofactors [58], or circumventing pathways that require cofactors altogether [56].

Crude extracts represent a simpler approach to industrial scale biomanufacturing as they eliminate enzyme purification from the workflow. Engineered strains overexpressing enzymes can be lysed and mixed to reconstitute a functional pathway [4], providing the opportunity for integrating sustainable carbon and energy platforms described above or simply utilizing the host native metabolism for cofactor regeneration and metabolite production. Regardless of the substrate and energy modules employed, dedicated strain engineering may be required to consolidate the flux of substrates and metabolites through the desired pathway. A scalable solution could be 'enzyme silencing' by inserting genomic tags for affinity purification or protease degradation to remove unwanted enzymes before harvesting cells for extract preparation. In principle, this strategy enables essential proteins to remain in cells during growth but be removed

Figure 3



Cost comparison for cell-free, crude-extract based biomanufacturing of diverse chemical products.
(a) Cost analysis of cell-free reactions. Standard culture methods with rich media are cost-prohibitive and should be replaced with high-density cultures (~20 g/L dry cell weight). Neglecting cofactors (e.g. coenzyme A), labor, and capital costs, the cost of a standard formulation cell-free reaction based on raw materials from traditional chemical manufacturers is approximately US$120 per liter reaction. Replacing glucose as a substrate and decreasing cofactor demands decreases costs to approximately US$90 per liter reaction. **(b)** Approximate minimum possible yields and reaction costs of a sustainable future cell-free reaction that would make cell-free biosynthesis lucrative, neglecting labor and capital costs while considering 1X separation costs. Estimated breakeven yield is defined as percent of maximum theoretical yield or yield under complete carbon conservation (based on a 200 mM glucose input). Estimated breakeven cost considers a 100% maximum theoretical yield scenario.

from cell extract after lysis, thereby achieving the benefits of a gene knockout by reducing carbon flux to side reactions without sacrificing healthy cell growth. Successfully implemented examples of this approach include the addition of an OmpT protease cleavage site into a target interfering enzyme, which would subsequently be cleaved upon cell lysis when the native OmpT outer membrane-bound protein is freed [60], and the insertion of a tobacco etch virus protease cleavage site (TEV-tag) into an enzyme target [61]. While the addition of TEV is required for proteolytic cleavage, processing could be minimized via coculture with a strain producing TEV to conditionally target enzymes after cell lysis. Enzyme silencing strategies allow for the elimination of essential enzymes that would not be removable *in vivo* and can therefore lead to the construction of synthetic metabolic networks that would be untenable in organisms. Equipped with the above strategies, CFME has potential to be an industrial solution for value-added chemical production.

To demonstrate the economic feasibility of these advancements, we consider the large-scale cell-free synthesis of value-added products already established in extract-based systems (Figure 2). Neglecting labor and capital costs, we conservatively estimate that a standard cell-free reaction formulation costs around $550/L, with lab-scale extract preparation contributing the greatest expense (Figure 3). High-density cultures can significantly alleviate these costs to achieve approximately $120/L, with extract preparation and reagent consumption contributing equally to overall reaction costs [62]. However, implementing the strategies presented here could decrease reagent costs by approximately 50% to reduce overall reaction costs to $90/L. Such a reaction could be fueled by CO_2 as a carbon source and electricity as an energy source with cofactors being repaired by auxiliary enzymes. We predict that implementation of these sustainable carbon and energy sources will be feasible without substantial cost consideration for the technology in a cell-free biomanufacturing platform due to successful utilization of gaseous substrates [28] and electrical currents [63] within cellular bioreactors. Our analysis (Supplemental Note 1) suggests that under these conditions the viability of the cell-free production of high-value targets, like limonene and 3-hydroxybutyrate, is encouraging. Currently, lower market value chemicals like biofuels, are not cost-effective targets for cell-free approaches without further innovations.

Outlook

Extract-based cell-free systems present appealing advantages for industrial biomanufacturing, including the presence of native-like metabolism and the ability to encode additional enzymes for catabolism, anabolism, and metabolic proofreading (Table 1). The biosynthetic potential of cell-free systems could be enhanced by replacing contemporary substrates with inexpensive carbon sources (e.g. cellulosic biomass, methanol or CO_2) and ubiquitous energy sources (e.g. electricity or sunlight) to reduce reagent costs and consume waste materials from other industries. Additionally, expressing heterologous enzymes to maintain cofactor pools and reduce byproduct accumulation could increase the yield and longevity of reactions while reducing cofactor costs. While much work remains to realize sustainable, cell-free biomanufacturing of diverse chemical products, innovative methods for producing cell extracts and utilizing renewable carbon and energy sources will overcome current limitations of cell-free biosynthesis to enable rapid advances. These advances will expand the definition of biomanufacturing, thereby allowing cell-free biosynthesis to penetrate into new industrial applications and become a major driver of global innovation and sustainable economic growth.

Author contributions

The authors contributed to all aspects of the article.

Conflict of interest statement

M.C.J. has a financial interest in SwiftScale Biologics and Design Pharmaceuticals Inc. M.C.J.'s interests are reviewed and managed by Northwestern University in accordance with their conflict-of-interest policies. All other authors declare no conflicts of interest.

Acknowledgements

M.C.J. acknowledges support from the Department of Energy Grant DE-SC0018249, the DOE Joint Genome Institute ETOP program, the Office of Energy Efficiency and Renewable Energy Grant DE-EE0008343, the David and Lucile Packard Foundation, and the Camille Dreyfus Teacher-Scholar Program. The work conducted by the U.S. Department of Energy Joint Genome Institute, a DOE Office of Science User Facility, is supported by the Office of Science of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231. B.J.R. was supported by an NDSEG Fellowship (Award ND-CEN-017-095). B.V. was supported by a SNSF Early Postdoc Mobility fellowship (P2SKP3_184036). G.M.L. was supported by an NSF Graduate Research Fellowship (DGE-1842165). The U.S. Government is authorized to reproduce and distribute reprints for Governmental purposes notwithstanding any copyright notation thereon. The views and conclusions contained herein are those of the authors and should not be interpreted as necessarily representing the official policies or endorsements, either expressed or implied, of the U.S. Government.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.copbio.2020.12.012.

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- •• of outstanding interest

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