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The emerging impact of cell-free chemical biosynthesis

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Biomanufacturing has emerged as a promising alternative to chemocatalysis for green, renewable, complex synthesis of biofuels, medicines, and fine chemicals. Cell-free chemical biosynthesis offers additional advantages over *in vivo* production, enabling plug-and-play assembly of separately produced enzymes into an optimal cascade, versatile reaction conditions, and direct access to the reaction environment. In order for these advantages to be realized on the larger scale of industry, strategies are needed to reduce costs of biocatalyst generation, improve biocatalyst stability, and enable economically sustainable continuous cascade operation. Here we overview the advantages and remaining challenges of applying cell-free chemical biosynthesis for commodity production, and discuss recent advances in cascade engineering, enzyme immobilization, and enzyme encapsulation which constitute important steps towards addressing these challenges.

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Introduction

Biomanufacturing presents a renewable alternative to petroleum-based chemocatalysis for commodity production. In contrast to conventional industrial manufacturing, living cells utilize serially organized enzymatic cascades to drive many vital physiological processes. Such enzyme cascades are a compelling means for biomanufacturing, as they are: (1) highly selective and specific, (2) biodegradable and non-toxic, and (3) optimized to function at similar conditions, minimizing unit ops and the need for intermediate purification. Harnessing these strengths, microbial cells with engineered pathways have been applied towards pharmaceutical and fine chemical manufacturing [1–3]. Despite these successes, it is still challenging to engineer and optimize synthetic pathways in live cells. In particular, mass transfer and pathway optimization are constrained by cell membranes and intracellular processes [4–6]. A promising alternative is 'cell-free' chemical biosynthesis (CFCB) for biomanufacturing, which seeks to exploit the advantages of whole-cells outside of the constraints of live cells.

In CFCB, enzymes and cofactor components are assembled and optimized towards enzymatic pathways with great engineering freedom, thanks to the absence of cell membranes and cellular processes. Such *in vitro* networks have been used to produce a variety of products, such as hydrogen [5,7], electricity [8], medicines [9,10], and fine chemicals [9]. Several excellent reviews have discussed cell-free metabolic engineering and its many applications [6,11–13]. Here we present an overview of CFCB as a platform for industrial chemical biosynthesis, discussing its advantages, challenges and current applications.

Advantages of CFCB

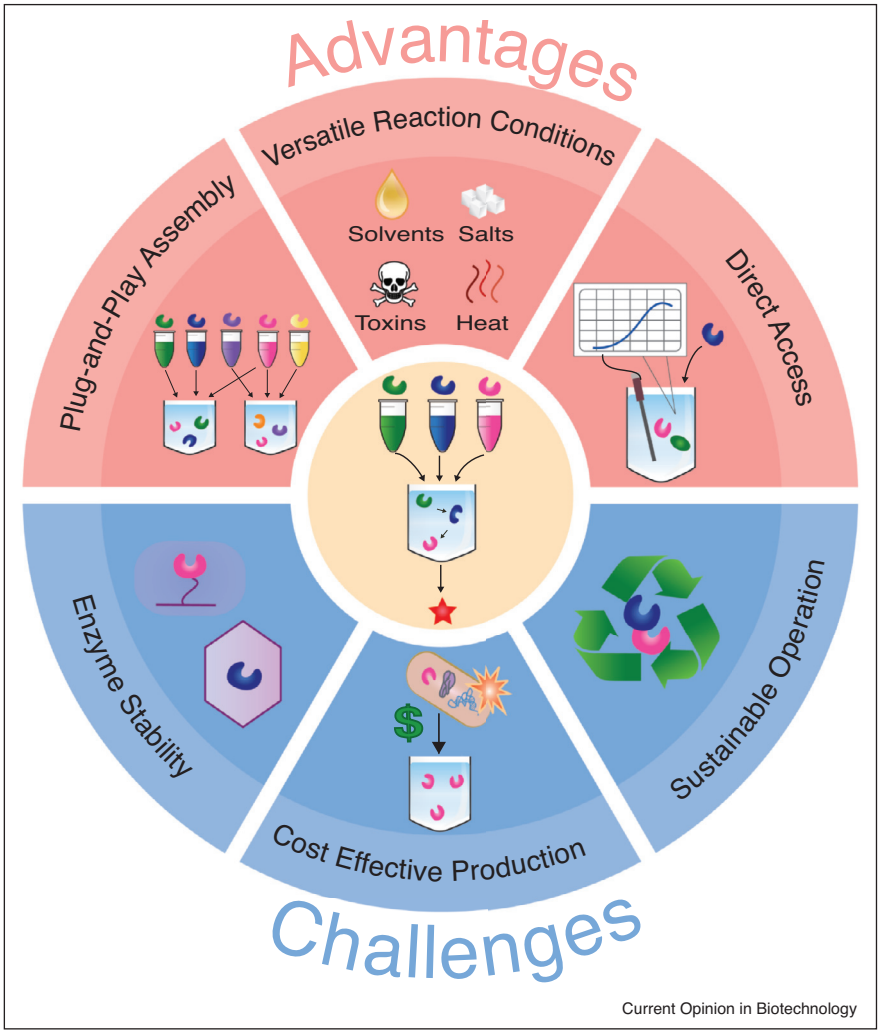
As a chemical production platform, CFCB provides several key advantages over *in vivo* synthesis, including (1) facile plug-and-play cascade assembly, (2) versatile reaction conditions, and (3) direct access to the reaction environment for monitoring, manipulation, and maintenance, as depicted in Figure 1.

Plug-and-play assembly

The CFCB system simplifies cascade production because each enzyme can be produced separately using various hosts and growth conditions, simplifying the optimization of overall biocatalyst production efficiency [14]. Thereafter, enzyme concentrations and combinations can be screened in a modular and high throughput manner [15,16], improving yield and overall titers [16].

These optimized designs can then be scaled up to larger production volumes with minimal re-optimization, as demonstrated recently [17]. Individual enzyme building blocks can also be grouped into modules with known functions, which can be mixed and matched to produce a variety of products [8,18]. Recently, crude lysates have been demonstrated as a useful tool in cascade design [11,19••], enabling rapid iteration through design-build-test cycles by eliminating enzyme purification and allowing biocatalyst synthesis from linear PCR templates [19••,20,21]. Finally, the simpler, more linear setup of CFCB promises simpler computational modeling,

Figure 1



Advantages and challenges of cell-free chemical biosynthesis. Advantages include (1) plug-and-play cascade assembly for construction and optimization of novel cascades, (2) versatile reaction conditions due to the absence of cell viability constraints, and (3) direct access for monitoring and manipulation of the reaction environment. Challenges include (1) cost-effective biocatalyst production, (2) improving enzyme stability, and (3) enabling economically sustainable cascade operation through cofactor and biocatalyst recycling.

broadening the potential for computational design of cascades [5,7,22,23].

Versatile reaction conditions

A primary challenge of *in vivo* biomanufacturing is the balance between product synthesis and the constraints of cellular viability. These constraints introduce two primary limitations: (1) a portion of the energy supplied must be used in cellular growth and metabolism, reducing system efficiency, and (2) the scope of acceptable reaction conditions is restricted to environments tolerated by the cell, limiting solvent options, ionic strength, titers of toxic products or intermediates, and reaction temperatures. Because CFCB eliminates the constraints of cellular viability, CFCB expands available reaction conditions [24–26], enables high reaction rates [8], and allows higher titers of some products and intermediates [9,16,27••], providing greater opportunity for cascade optimization. For example, a CFCB process enabled saccharification of chitin to pyruvate at a higher optimal temperature of 70 °C, which is difficult to achieve *in vivo* [24]. Similarly, the absence of cellular toxicity and crossover with native metabolism recently allowed CFCB processes to exceed *in vivo* titers of various monoterpenes [27••] and fructose 1,6-diphosphate [9].

Direct access to reaction environment

The absence of cell walls in CFCB also eliminates many transport limitations. This enables greater control over the reaction environment and more affordable product

purification [16], and is especially advantageous for products with low secretion rates [9]. In addition, this facilitates continuous product removal, which improves yields of cascades that cannot accommodate an irreversible last reaction step. The open environment of CFCB also facilitates continuous replenishment of cofactors, for example through the application of continuous exchange cell-free synthesis formats [28,29] or through various cofactor replenishment strategies (Figure 2) [9,10,15,16,24,27••,30]. Longer reactions enabled by these strategies increase yields and with continued optimization could enable continuous reaction operation, which would be economically advantageous for chemical production.

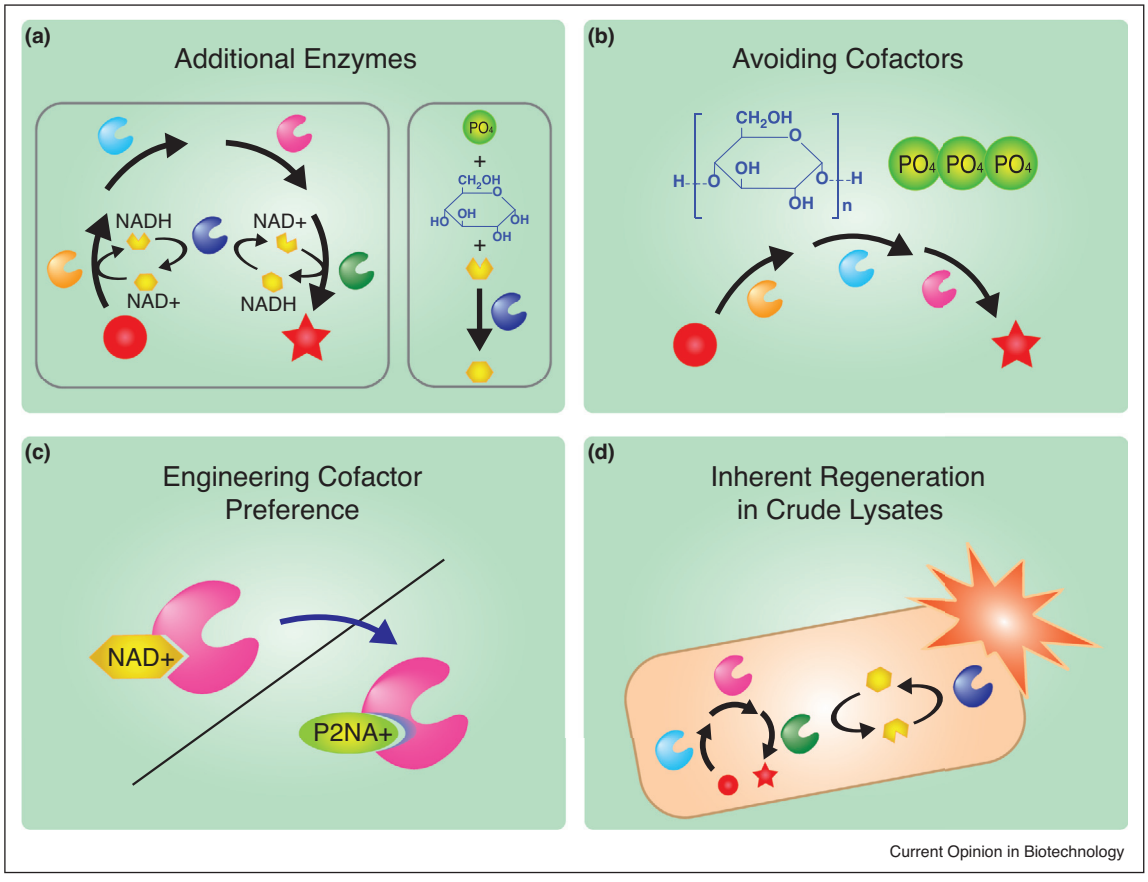
Absence of a cell wall also allows for innovative optimization strategies [19••,31,32••,33]. For example, the kinetics of the reaction can be characterized through manipulation of various inputs and real-time measurement of the outputs [32••]. Cofactor concentrations can also be manipulated towards an optimal concentration [19••]. Direct access to the reaction environment could also improve controllability of the process in industrial applications, as enzymes could be added or removed to eliminate buildup of intermediates or unwanted side products.

Lastly, the open format of CFCB provides opportunities for on-demand synthesis through lyophilization and rehydration of the system on-site [19••,34,35]. Crude lysates systems can also enable on-demand pathway activation through cell-free synthesis of a key cascade enzyme [20].

Challenges and relevant advances in CFCB

Despite the advantages of CFCB for industrial chemical production, its application in industry hinges on solutions to several key challenges: (1) cost-effective generation of biocatalysts, (2) stabilization of enzyme building blocks for long-term use, and (3) economically sustainable cascade operation, as depicted in Figure 1.

Figure 2



Cofactor management methods for enabling continuous cell-free cascade reactions. **(a)** Enzymes can be added into a cascade to recycle cofactors or to regenerate them from low-cost sources such as polyphosphate and glucose. **(b)** Enzymatic cascades can be designed to avoid certain cofactors altogether, for example by deriving energy and phosphate groups directly from low-cost sources such as maltodextrin and polyphosphate. **(c)** Enzyme cofactor preference can be engineered to utilize lower-cost biomimetic cofactors, such as P2NA+/P2NAH, or to change natural cofactor preference in order to stoichiometrically balance a cascade. **(d)** Crude lysates provide inherent machinery for cofactor regeneration, eliminating the need for including regeneration machinery in the enzymatic cascade.

Cost-effective production of biocatalysts

Purified enzymes are often the highest contributor to biomanufacturing cost [13]. This cost has partially been mitigated by efficient purification procedures, such as one-step immobilization and affinity tag purification [16,36,37], made even simpler by using magnetic supports [37].

One approach which is gaining momentum is the use of thermophilic enzymes in CFCB cascades to allow purification of target enzymes by simple heat precipitation. Heat treatment simultaneously lyses the host cells and denatures endogenous enzymes. Using this approach, thermophilic cascades have been designed to produce a variety of important products and intermediates such as pyruvate [24], fructose 1,6-diphosphate [9], glutathione [16], and myo-inositol [17] from low-cost substrates such as chitin [24] and maltodextrin [9,17]. However, this approach is limited by the scope of known thermophilic enzymes.

Another approach involves *in vivo* encapsulation of enzymes into synthetic vesicles such as virus-like particles (VLPs), which can then be purified based on their larger size [38•]. This approach could be used to separately generate multiple purified, encapsulated enzyme 'modules' at low-cost which could be combined in one pot to synthesize different products, provided that the synthetic vesicle can accommodate incoming and outgoing substrates, intermediates, and products.

The simplest approach uses crude lysates for CFCB, eliminating enzyme purification from cascade assembly. In this method, crude lysates overexpressing different enzymes can be produced in parallel and mixed to constitute a new optimized pathway [11,19••,20]. This approach demonstrates that high enzyme purity is not necessary for CFCB, indeed, maintenance of cellular pathways for cofactor regeneration may even be beneficial. However, additional engineering may be required to prevent diversion of energy and intermediates into other enzymatic pathways present in the crude lysate.

Stabilizing enzymes for long-term use

Degradation of enzymes by proteases, heat or harsh solvents causes loss of enzyme activity and necessitates replacement, increasing the cost of CFCB. In addition to promoting long-term enzyme use, enhanced enzyme stability can broaden the scope of CFCB. For example, it can enable high-temperature reactions for breakdown of cellulosic feedstocks [39•], or use of volatile solvents [10] for simpler downstream purifications. Various methods have been employed in order to stabilize enzymes for continuous use and diverse reaction conditions. Conveniently, the same approaches that simplify biocatalyst purification, as described above, are the predominant methods for stabilizing enzymes: (1) application and engineering of thermostable enzymes, (2) immobilization, and (3) encapsulation.

Thermophilic enzymes are often robust against high temperatures, detergents and solvents [30], making them a promising starting point for engineering stable cascades [9,16,17,24,30]. However, there may not be known thermophilic enzymes for a desired cascade, necessitating additional approaches. One strategy is to engineer thermally stable enzymes using computational methods and high-throughput experimental screening using approaches such as directed evolution. Coupling these two approaches recently led to engineering of a thermostable keto acid decarboxylase [39•]. As computational models of protein stability continue to improve, rational engineering of enzymes with improved stability will become increasingly more feasible.

Immobilization is a well-established method for stabilizing proteins and is highly applicable to CFCB. As other reviews have recently discussed major immobilization techniques [40], here we will only briefly discuss advances in immobilization. Immobilization to solid surfaces or nanoparticles [41] or to other enzymes in cross-linked enzyme aggregates (CLEAs) [42] can significantly improve thermal, pH, and storage stability. However, preserving enzyme activity through the immobilization can be challenging; harsh conditions needed for some immobilization reactions can denature enzymes [43], and natural amino acids in enzymes afford insufficient control over conjugation site for optimizing stabilization and accessibility. As a promising alternative, unnatural amino acids have recently been utilized to immobilize enzymes with remarkable control over conjugation site and active-site orientation [44], which can both be rapidly optimized through lysate-based screening systems [45]. This approach could also be applied towards polymer conjugation, which has also been demonstrated to stabilize proteins [46,47].

Another promising method of stabilizing enzymatic cascades is enzyme encapsulation. Enzymes can be encapsulated in a variety of structures, including VLPs [38•], hydrogels [48,49], metal organic frameworks (MOFs) [50•,51•], and DNA cages [52]. Encapsulation in these structures stabilizes the enzymes against proteases [38•,52], heat [38•,48,50•], pH [38•,49], organic solvents, and other denaturants [38•,50•]. Two particularly promising approaches are encapsulation in VLPs and MOFs. Virus capsids self-assemble, simplifying encapsulation, and can protect their enzymatic cargo in a wide variety of environments [38•]. The highly tunable structures of MOFs can be adapted to optimize the structure to the desired enzymes [50•,51•].

Each of the above strategies shows promise, but can also increase the cost of biocatalyst production through

additional materials and preparation steps. Therefore, continued efforts are necessary to allow maximum impact of stabilizing alterations while minimizing production costs and negative effects on specific activity.

Economically sustainable continuous cascade operation

An attractive aspect of CFCB for industrial production is the potential for continuous operation, which allows high yields and minimizes down-time of process equipment. For such a process to be economical, however, pathways must run independent of costly cofactor supplementation, allow for facile retention and reuse of cascade enzymes, and synthesize sufficiently high product yields from low-cost sources.

While continuous exchange is effective at producing high yields of proteins, continuous addition of cofactors is expensive and can lead to changes in the redox potential of the reaction. Recently, efforts to address the challenge of cofactor depletion have centered on cofactor balance and regeneration through thoughtful cascade design. Some of the main approaches are depicted in Figure 2. One approach is to include additional enzymes in the cascade to recycle cofactors [10,24,30] or to regenerate cofactors such as ATP [16,27••], NAD+/NADH [15,27••], NADP+/NADPH [15,24,27••], and CoA [15,27••], using low-cost phosphate sources [16,24,30] and energy sources [15,27••]. Some of these pathways have been engineered to function at high temperatures [9,16,24,30] in order to facilitate optimal CFCB product yield. Another promising future option for cofactor regeneration involves harnessing energy from light [53]. Cascade pathways can also be designed to avoid certain cofactors. For example, pathways have been designed to avoid using ATP by directly utilizing bond energy and phosphate groups from low-cost sources such as maltodextrin [9] and polyphosphates [8,9]. Alternatively, pathways can avoid dependence on natural cofactors by engineering enzymes to use cheaper biomimetic cofactors such as MNAH, BNAH, or P2NAH, which can also be regenerated *via* enzymatic pathways [54]. Another strategy is to engineer some enzymes in a pathway to prefer one cofactor over another in order to balance cofactor turnover [55]. Recent work using crude-lysates for CFCB has also demonstrated that these systems can regenerate cofactors using the host cell's inherent machinery [19••,20]. This approach is significantly less common, but coupled with the advantages for cascade assembly, may prove to be an important asset for CFCB processes in the future.

The challenge of biocatalyst retention and recycling can be addressed through immobilization and encapsulation. While these same methods also conveniently simplify biocatalyst generation and improve biocatalyst stability as described above, biocatalyst recovery and reuse is arguably the most impactful application for these technologies. For example, immobilization to magnetic particles [41,56] or encapsulation in magnetic MOFs [51•] greatly simplifies enzyme recovery. DNA tethers as a means of immobilization [56] also provides a facile method for enzyme replacement *via* strand displacement, enabling reuse of immobilization supports.

Immobilization and encapsulation can also simultaneously improve product yields by improving substrate channeling and increasing enzyme specific activities [41,52]. For example, immobilization to DNA structures affords close control of enzyme proximity [55], cascade geometry [57], and cofactor access [58] in order to optimize flux through the desired pathway. Encapsulation can improve pathway flux *via* compartmentalization [59], which can be further refined using MOFs by optimizing the spatial organization of the various enzymes within the MOF [51•]. Other promising methods to improve product yield and reduce side products include engineering the system around the rate-limiting step [15] and 'metabolic proofreading' to recycle unwanted side products [14,15]. Additionally, cascades have been implemented to use low-cost feedstocks such as glucose [20,60], sucrose [61], chitin [24], xylose [62], and maltodextrin [17] for improved economics.

Current progress in the areas of cofactor regeneration, enzyme recovery, and substrate channeling represent important steps towards implementing continuous CFCB cascades, however additional work is needed. Specifically, additional engineering is necessary for the application of these approaches to longer, more complex cascades, particularly in the areas of enzyme reuse and substrate channeling where work has focused primarily on small cascades of 2 or 3 enzymes.

Conclusions and future directions

Cell-free chemical biosynthesis provides modularity, flexibility and control for cascade design and optimization, towards the manufacturing of commodity chemicals from inexpensive feedstocks. However, to realize such potential, the approach still needs to reduce cost of biocatalyst generation, improve enzyme stability, and enable cost-effective continuous cascade operation. Promising solutions include rational engineering of thermostable enzymes, and precise immobilization and encapsulation strategies. Further progress in the areas of computational modeling may also provide valuable tools for *de novo* design of efficient CFCB pathways and effectively stabilized enzymatic components.

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The authors use spatial co-location in a magnetic MOF to optimize activity of the model system glucose oxidase and horseradish peroxidase, demonstrating that positioning of the second enzyme at the center of the framework with the first enzyme at the outside improves kinetics relative to free enzymes. This study demonstrates the potential of MOFs to both optimize pathway flux and to provide facile separation for catalyst reuse, as high activity was demonstrated after 10 cycles.

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