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Synthetic Biochemistry: The Bio-inspired Cell-Free Approach to Commodity Chemical Production

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Abstract

Metabolic engineering efforts that harness living organisms to produce natural products and other useful chemicals face inherent difficulties because the maintenance of life processes often runs counter to our desire to maximize important production metrics. These challenges are particularly problematic for commodity chemical manufacturing where cost is critical. A cell-free approach, where biochemical pathways are built by mixing desired enzyme activities outside of cells, can obviate problems associated with cell-based methods. Yet supplanting cell-based methods of chemical production will require the creation of self-sustaining, continuously operating systems where input biomass is converted into desired products at high yields, productivities, and titters. We call the field of designing and implementing reliable and efficient enzyme systems that replace cellular metabolism, synthetic biochemistry.

Keywords: Metabolic Engineering, Enzyme Cascade, cascade, biocatalysis, multienzyme systems Biofuel, Bio manufacturing, green chemistry natural products, commodity chemical.

Introduction

Considerable efforts have been devoted to engineering living organisms to produce useful chemicals ranging from high-value natural products like cannabinoids to low-value products such as, fuels, plastics, and building block Microbial generation of natural products would free us from the problems with extraction from native sources, such as agricultural boom and bust cycles, as well as resource-intensive and expensive purification requirements. Moreover, microbial production can be more environmentally benign than chemical syntheses. Perhaps the greatest environmental impact of metabolically engineered microbes will be in replacing high-volume petroleum products like fuels and commodity chemicals There are numerous potential environmental benefits: the biomass starting materials are renewable; replacing petroleum-based starting materials can lower the release of global warming gases; and the products are generally biodegradable. However, replacing high-volume, low-value products is also the most economically challenging because cost is critical. To make bio derived products economically competitive will require high-efficiency conversion of the input biomass into useful compounds [1].

However, efficient conversion of biomass is difficult to achieve in biological organisms the complexity of cellular physiology makes it difficult to optimize volumetric productivity because many regulatory mechanisms exist in the cell to control pathway flow rates. Life processes distribute resources away from the desired products, lowering yield. Moreover, product or intermediate toxicities can limit product titter. Although engineering of regulatory mechanisms and competing pathways have proven to be useful, the complexity of the problem is hard to overcome. Even successful efforts to engineer industrially scalable strains typically took large teams and hundreds of person years of effort Indeed there may be an effective 'speed limit' for biological production in microbes defined roughly by ethanol production. Ethanol production has had effectively thousands of years of optimization and is only a few enzymes away from the central glycolytic pathway. In contrast, next-generation biofuels and other commodity chemicals generally require more complex pathways. Ethanol can be produced at ~2 g/l/h productivity, ~100 g/l titter and ~90% yield Perhaps the greatest metabolic engineering success stories for commodity chemical production so far are 1,3 propanediol and 1,4-butanediol, both produced in engineered Escherichia coli. The reported production parameters for 1, 3 propanediol are 3.5 g/l/h productivity, a final titter of 135 g/l, but at only 65% of the theoretical yield from glucose (0.51 g 1, 3 propanediol/g glucose) For 1, 4 butanediol the reported production parameters are 2.1 g/L/h, 99 g/L and a yield at 70% of theoretical Moving beyond the range of production parameters approximately defined by ethanol will likely require major technological innovations.

While living organisms can clearly integrate complex inputs and perform remarkably sophisticated tasks, for engineering purposes it can make more sense to take inspiration from biology rather than try to harness life itself For example, we can see that birds fly and learn how they do it, but airplanes do not mimic bird flight mechanisms – and the result is more useful for our needs and more readily subject to engineering. The cell-free, synthetic biochemistry approach similarly seeks to remove the constraints of living organisms to develop simpler, more streamlined versions of biologically inspired systems that can be engineered efficiently for practical uses. Although considerable challenges remain for cell-free approaches, their potential has only begun to be explored. Here, we discuss the potential of cell-free production for bio manufacturing of high-volume chemicals. The possible advantages and challenges of the synthetic biochemistry approach are summarized discussed in detail below. While there is considerable interest in using cell-free methods to prototype new metabolic pathways with the idea of putting the systems back into cells we concentrate on efforts that seek to move chemical manufacturing away from cells. Cell-free approaches for producing high-value chemicals like pharmaceuticals or for making

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biologics like proteins face different opportunities and challenges and are not the focus of this review

Approaches to Setting up Cell-Free Systems

Cell Lysate Method

In the cell lysate method the pathway of interest is expressed in the cell as in any metabolically engineered microbe Rather than using the cells as the bio factory, however, they are lysed, releasing the contents into a bioreactor. The lysate approach has a number of advantages. First, it is simple and potentially inexpensive. Second, the cytoplasmic contents may contain sufficient cofactors so that additional cofactors do not need to be added, further reducing cost A cell lysate approach is particularly effective for complex systems where it may be advantageous to use endogenous systems to make the desired product. Indeed the lysate approach is the one favoured for commercial cell-free protein production [2].

Purified Activity

In this approach, the enzyme activities are more extensively purified This does not necessarily mean, however, that highly purified enzymes must be used. For example, with stable enzymes, activity purification could be as simple as just heating extracts thereby enriching the desired enzyme activity and eliminating other problematic activities. The level of purification required depends on the system. Moreover, each enzyme does not necessarily need to be purified separately. Enzymes could be expressed together and purified together or expressed separately, then mixed and purified together. The advantages of the purified activity approach are a higher level of system control, the elimination of unwanted pathways, and the ability to express proteins in different organisms which affords considerable flexibility. To some extent the purified activity approach will involve additional complexity and preparation costs, however.

Hybrid

Another approach is to use a lysate for most of the enzymes, but then supplement the lysate with other enzymes expressed separately. This approach is of course the most flexible and could be particularly useful when most of the enzymes express in one organism, but others can only be expressed in a different organism.

Possible Advantages of the Cell-Free Approach

Potential for Better Production Parameters

There is potential for cell-free systems to more readily achieve high yields, titters, and productivity – key production parameters that in part define commercial viability. To obtain high yields, all of the input biomass (e.g., sugar) would ideally be converted into the desired product. In the cell-free approach, there is only one pathway in the bioreactor, so it is possible to achieve nearly 100% yields, limited only by enzyme specificity and thermodynamics.In cells, there are myriad biochemical pathways diverting sugar into unwanted side products that are required for maintaining life processes. Indeed, much of metabolic engineering involves deleting these side pathways, which can be a difficult challenge if one wants to maintain cell viability. An alternative strategy, called dynamic metabolic control, seeks to decouple growth and production phases so that unwanted cellular pathways can be shut down during the production In many ways, the cell-free approach is the height of dynamic metabolic control because the growth phase (making enzymes) is completely separated from the production phase (bioreactor). There are indications that it will be possible to obtain higher productivity in cell-free systems. For example, Kay and Jewett reported a remarkable productivity of 11.3 ± 0.1 g/l/h for the cell-free production of 2,3 butanediol The ability to precisely optimize reaction conditions is likely one factor in obtaining high productivity (discussed below). Another factor that could favour cell-free productivity in the future is the ability to concentrate the relevant pathway enzymes to higher levels than would be possible when expressed in a cell culture. Finally, since there is no need to maintain life processes, there are no concerns about intermediates or products that are toxic to cells, a major benefit of cell-free systems. Thus, it may be possible to obtain much higher titters of toxic chemicals than is possible in living cells [3].

Challenges of the Cell-Free Approach

Enzyme Production Costs

Clearly there will be an added cost to preparing a cell-free reactor compared to growing cells in a fermenter. How significant this cost is depends on many factors If a cell lysate approach is used, the added cost should be minimal as it is simply a matter of harvesting cells and lysing them after growth. If purified enzymes are used, the cost depends on expression levels, the host used, whether the enzyme is expressed intracellular or into the medium, and stability.

High enzyme stability is a key factor, operating at many levels to ease the implementation of synthetic biochemistry systems. Purification of highly stable enzymes can be as simple as heating an extract Moreover, the longer the enzymes last, thereby increasing the total turnover number per unit enzyme, the lower the net cost contribution of preparing the enzymes Stability can also make additional process investments such as attaching enzymes to solid supports make sense

For most enzymes, it can be straightforward to obtain stable variants by various means. In particular, genome mining for thermophiles enzymes can be effective. Because thermophiles enzymes are often most active at higher temperatures, they have often been used in cell-free systems at high temperature While high temperature can be useful for suppressing contaminating bacterial growth, it has the disadvantage that all the enzymes must be stable at high temperature and increases the degradation of cofactors (see below). Thus, an alternative is to screen for enzymes from thermophiles and hyper thermophiles that maintain sufficient activity at low temperatures although the specific activities can be reduced, it is possible that the advantages of high stability can outweigh the loss of specific activity. Directed evolution is a particularly effective method for increasing thermo stability; generally only requiring a rapid screen for activity finally, effective design methods have now been developed for enzymes with a known structure or a close homolog of known structure [50].

There are, of course, enzymes that are more difficult to stabilize than others. For example, oxygen-sensitive enzymes that may have sensitive cofactors can be a challenge Redox enzymes with mechanisms involving radical intermediates have a tendency to self-immolate Although, membrane enzymes can be engineered to be highly stable adding purified membrane proteins to a cell-free system is a challenge. Membrane proteins could be introduced in the presence of detergent, but the remaining components of the system would need to be compatible with the detergent. It might be possible to reconstitute membrane proteins into proteoliposomes or Nano discs or to use amphipods but it may be hard to keep the costs low enough for high-volume chemicals. Lysate-based systems provide a way to introduce membrane proteins because they can retain intact cellular membrane vesicles with active membrane proteins [4].

Problem Metabolites

Reactive metabolites in a cell-free system may inactivate enzymes. For example, high concentrations of input-reducing sugars that may glycylate proteins or aldehydes that are generated can react with nucleophiles on proteins. These problems may be mitigated by engineering of enzymes to remove vulnerable sites, or using process or system optimization to keep sugar and aldehyde concentrations low. Cells often compartmentalize toxic intermediates and it is possible that a similar bioinspired strategy could be used **in vitro**, perhaps by channeling toxic substrates to the next enzyme , thereby keeping its concentration low. Reactive oxygen species that are generated must be removed expeditiously; for example, by including catalases to eliminate peroxide. Like cells, high titters of product compounds may destabilize and inactivate enzymes. These problems can often be reduced by improving enzyme stability (see above) or through continuous product removal strategies such as an organic overlay. The advantage of cell-free systems is that problematic enzymes can generally be readily identified and the full panoply of engineering tools can be used to fix them.

Enzymes can make errors and some metabolites are prone to spontaneous chemical alteration. Thus, unwanted, dead-end side products may build up, leading to a decrease in yield. Perhaps more thorny, the side products may inactivate enzymes in the system. In cells, there can be metabolite repair mechanisms to deal with these unwanted metabolites. In a cell-free system it may be possible to adjust conditions to minimize side reactions or it is necessary to introduce repair systems, thereby adding to complexity. For example, Opgenorth and colleagues introduced a two-enzyme metabolite salvage pathway in a cell-free system to deal with the undesired products of a promiscuous enzyme.

Cofactors

Another important issue in cell-free systems is cofactor costs [ATP, NAD(P)H, CoA, etc.]. For low-cost production, it is essential that cofactors are regenerated **in situ** and used many times. A crude back of the envelope calculation illustrates the challenge in the example of converting isoprenol into limonene. The pathway requires 4 ATP per limonene made. At a bulk ATP price of $1000/kg, the ATP cost for producing limonene is then ~$15 000/kg limonene/TTN, where TTN is the total turnover number for ATP. Thus, in this scenario, ATP must be recycled in the reaction at least 1500 times to lower the ATP cost contribution below $10/kg limonene. Lowering cofactor costs as well as effective cofactor utilization is a major challenge for low cost bio manufacturing by synthetic biochemistry [5].

Acknowledgement

None

Conflict of Interest

None

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