



## Complex natural product production methods and options

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### ABSTRACT

Natural products have had a major impact upon quality of life, with antibiotics as a classic example of having a transformative impact upon human health. In this contribution, we will highlight both historic and emerging methods of natural product bio-manufacturing. Traditional methods of natural product production relied upon native cellular host systems. In this context, pragmatic and effective methodologies were established to enable widespread access to natural products. In reviewing such strategies, we will also highlight the development of heterologous natural product biosynthesis, which relies instead on a surrogate host system theoretically capable of advanced production potential. In comparing native and heterologous systems, we will comment on the base organisms used for natural product biosynthesis and how the properties of such cellular hosts dictate scaled engineering practices to facilitate compound distribution. In concluding the article, we will examine novel efforts in production practices that entirely eliminate the constraints of cellular production hosts. That is, cell free production efforts will be introduced and reviewed for the purpose of complex natural product biosynthesis. Included in this final analysis will be research efforts made on our part to test the cell free biosynthesis of the complex polyketide antibiotic natural product erythromycin.

### 1. Introduction

Natural products span a wide range of compounds that demonstrate similarly broad bioactivity [1–5]. Though generally small molecules, these compounds often derive from complex biosynthetic systems that feature fascinating enzymatic pathways [6–8]. It is the genetic and biochemical pathways responsible for natural products that then provide the basis for various production efforts. Resulting natural product classifications include polyketides, nonribosomal peptides, ribosomally synthesized and post-translationally modified peptides (RiPPs), and isoprenoids [9].

As the name implies, natural products derive from environmental cellular hosts that also span a variety of morphological features [10–13]. Microbial sources are common, including bacterial and fungal species, which are traditional hosts for polyketide and peptide natural products [14]. However, plants are also prolific producers of natural products (especially isoprenoids) [15,16]. In conjunction with the underlying natural biosynthetic pathway, the native host system will also greatly influence the directions in scaled production efforts.

Fig. 1 depicts the biosynthetic production sequence for two historically important natural products. The first is penicillin, produced

natively by *Penicillium* fungal species, which ushered in the widespread use and impact of antibiotics and is representative of a nonribosomal peptide natural product [17–19]. The second example provided is erythromycin, natively generated by the soil bacterium *Saccharopolyspora erythraea*, another antibiotic but derived from a more complex modular polyketide biosynthetic pathway [20–24]. Both pathways feature metabolite precursors required to support the enzymatic conversion to the final natural product. The enzymes help to distinguish the classification of the natural product and, as indicated in Fig. 1, highlight the complexity of the biosynthetic process. The penicillin case presents a relatively simple process requiring three enzymes in the construction of a beta-lactam peptide product, with one of these enzymes featured as a nonribosomal peptide synthetase. The erythromycin example highlights a more complex biosynthetic process that can be divided between core polyketide synthase enzymes (here, a multi-domain and high molecular weight complex) and tailoring enzymes responsible for adornment of the polyketide intermediate to functionalize and activate the final antibiotic product. Though only representative examples, the penicillin and erythromycin cases demonstrate key features of natural product biosynthesis, such as the need for key metabolic precursors and the coordinated activity of multiple enzymes either within or separate from

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their native production hosts.

The penicillin and erythromycin examples also serve as key case studies in natural product bio-manufacturing, as they will straddle the approaches and methodologies outlined in the rest of this article. Production efforts are driven by the societally valuable properties of natural products and the desire for more ready access to such products. Scaled production attempts also parallel the timeline of advances in molecular biology. For example, key advances in the discovery and development of penicillin occurred prior to the completely elucidated structure of DNA, with such circumstances prompting production efforts focused on the native system. Alternatively, efforts in the heterologous production of erythromycin depended on advances in molecular biology to enable reconstitution in a surrogate host system capable of providing advantages relative to the native host [25–27]. More recently, elements of both molecular biology and microbiology are considered in efforts to establish biosynthesis in a cell-free format, completely unconstrained from traditional cellular confines. Fig. 2 provides a schematic representation of the native, heterologous, and cell free production systems to be covered, with accompanying commentary connecting production efforts to evolving technical advances. Finally, the chapter also offers a tribute to the central figure of this particular special issue, Dr. Arnold Demain, who made invaluable contributions to established and ongoing efforts in natural product bio-production [28–33].

## 2. Natural product production from native hosts

As introduced, natural products derive from environmental locations [13,34]. Generally, soil and aquatic environs have been fruitful sources of natural products [10,12,35–40]. Within these environs, commonly identified production hosts include plants and microorganisms with natural products possessing biological functions spanning chemical communication to a weaponized form of competition for resources [41–43]. In the latter functionality, compounds like anti-fungals or anti-bacterials would be produced as a form of competitive advantage in warding off microbial rivals for environmental nutrients. Regardless, the bioactivity of natural products served as a key motivator in subsequent efforts to scale production and distribution.

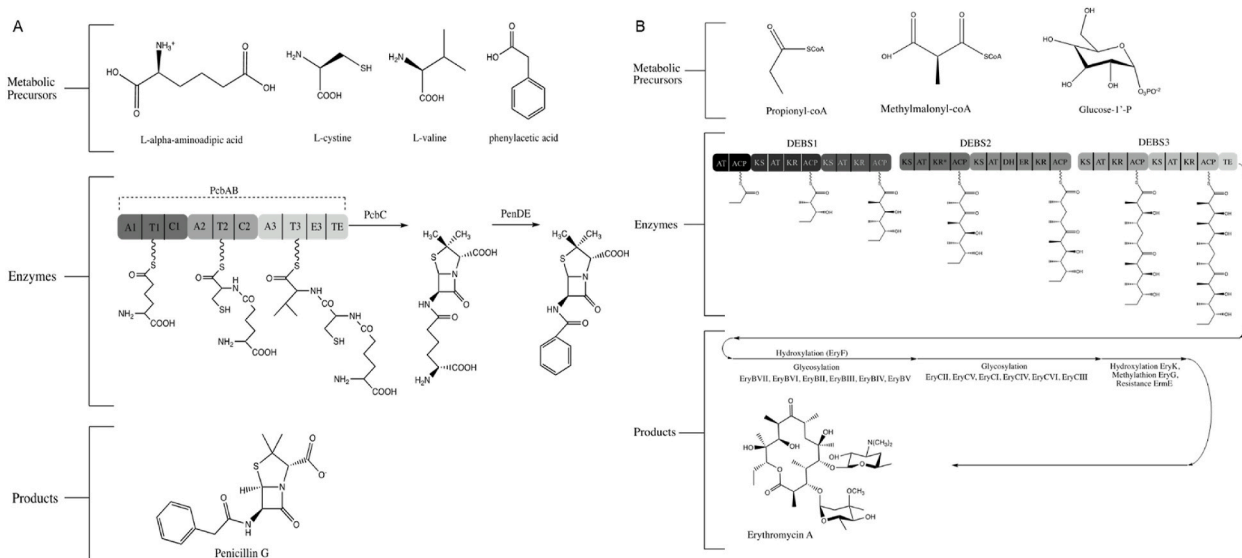
Once a bioactive natural product was identified from an environmental cellular host, a key challenge was to then sufficiently access the active compound [31,32,44]. One option was to generate the same

compound using a chemical synthetic approach [45]. The primary obstacle to this approach was the chemical complexity of natural products (featuring numerous functionalities and chiral centers). Thus, though impressive synthetic efforts resulted in natural product generation, doing so in an economic and scalable fashion remained challenging.

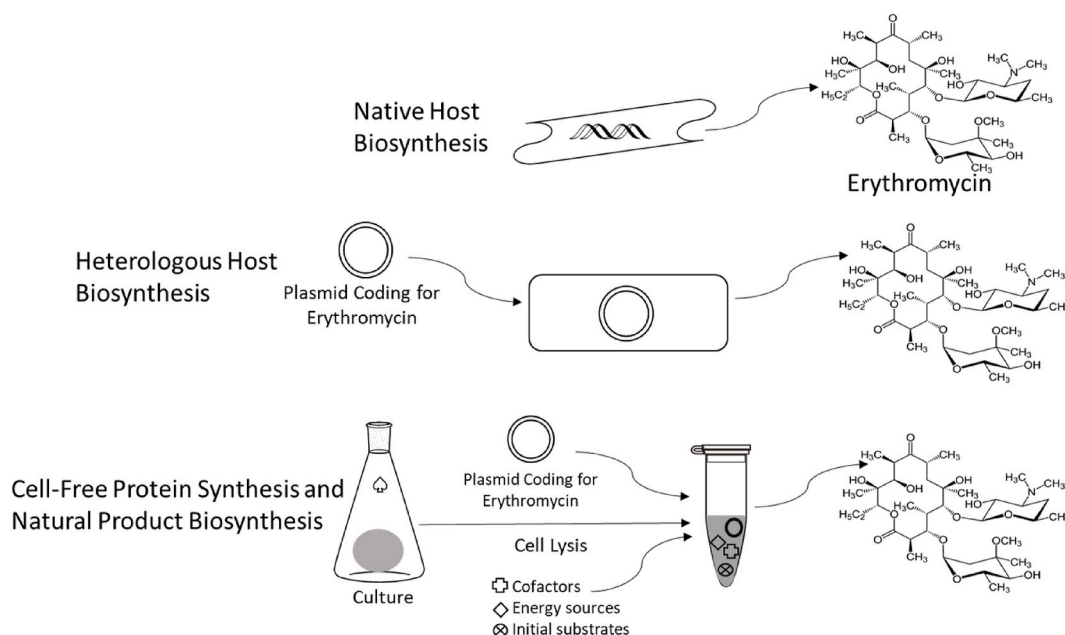
As a result, scaled production options then turned to the native hosts responsible for the bioactive natural product [46–48]. Conceptually, this approach resembled the long history of Eastern herbal therapies reliant on nature. The key difference in the cases highlighted in the middle portion of the 20th century (with penicillin as the prime example) was the focus on industrial scale [18,19,49]. From this standpoint, the influence of the Industrial Revolution was available to aid production efforts. Also coinciding with such efforts were the World Wars that painfully emphasized the damage infectious disease inflicted under such circumstances, thus, propelling efforts to overproduce penicillin for battlefield hospitals.

Native sources, again highlighted via herbal medicines, include many plant species [50–52]. However, these particular hosts are less amenable to scaled production relative to single-cell microorganisms (as often observed for fungi and bacteria), and discovery efforts underway were successfully isolating the microbial species (largely from soil samples) demonstrating bioactive natural product formation [35,53]. Thus, bio-production or bio-manufacturing evolved as a combination of the industrialization of microbial growth and natural product production [54].

Such efforts are best illustrated in the case of penicillin, which has been covered extensively in previous reviews [18,19,48]. Importantly, the template for scaled penicillin production was generally adopted in efforts to isolate, identify, and produce subsequent bioactive natural products in their so-called Golden Age (ca. 1940–70) [47,55–57]. During this period, it is important to emphasize the nascent development of the field that would be called molecular biology. Again citing the penicillin example, a fully scaled bio-manufacturing process was established prior to the complete structural elucidation of DNA and, thus, prior to the molecular biology tools available to current engineering efforts [58]. Table 1 outlines efforts to bio-manufacture several historically-relevant (based upon health and economic impact) natural products from native production hosts. In several cases, key production milestones (processes and production levels) were reached prior to the



**Fig. 1.** Biosynthetic pathways for the natural product antibiotics penicillin (a) and erythromycin (b). Highlighted are the multi-domain biosynthetic enzymes for nonribosomal peptide (penicillin) and polyketide (erythromycin) biosynthesis. A: adenylation; T: thiolation; C: condensation; E: epimerization; TE: thioesterase; DEBS: Deoxyerythronolide B Synthase; AT: acyl transferase; ACP: acyl carrier protein; KS:  $\beta$ -keto-acyl synthase; KR:  $\beta$ -keto reductase; KR\*: non-functional KR; DH: dehydratase; ER: enoyl reductase.



**Fig. 2.** Natural product production options. Featured is the natural product polyketide antibiotic erythromycin, produced natively from *Saccharopolyspora erythraea*. Heterologous production of erythromycin has been completed using *Streptomyces* spp. and *Escherichia coli* as surrogate hosts; whereas, cell-free biosynthesis removes the need for a dedicated host (except for systems that rely on expression machinery provided via cellular lysis, as depicted).

establishment of molecular biology in the 1970s. Industrial microbiology is a field that best describes efforts in this time period. Early in the process, there was a need to identify environmental bioactivity, the responsible microbial source, and microbiological details about the source. In tandem, efforts commenced to both isolate and culture the identified microbe for advanced study, including the need to confirm natural product production and to elucidate the chemical structure of the natural product. A key scientific element missing from such efforts was the genetic and biochemical analysis of the biosynthetic process, mainly due to emerging understandings of such intracellular workings. As such, efforts to scale production of confirmed bioactive natural products from isolated microbes relied upon both physical and biological techniques available at the time.

From a physical perspective, optimization of culture parameters (medium components, process controls [temperature, aeration, pH, etc.]) offered a readily available means of improving production titers. Likewise, the refinement of culture conditions that embraced process control and unit operations (in particular, the bioreactor and its formats across batch, fed-batch, and continuous operations [86–88]) further contributed to improved and scalable production. Biological approaches leveraged current and emerging data about cellular biology. In particular, the concept of mutation and screening allowed improvements in titer by effectively treating the cellular system as a black box in which a perturbation was applied (in the way of a mutational event) followed by laborious screening of single cell mutants demonstrating enhanced production. Though less directed than genetic engineering alternatives that would emerge over the last 50 years, such approaches proved ultimately effective in establishing both scalable natural product production and an industry built around this approach.

### 3. Natural product production from heterologous hosts

Though native host production proved a viable method for access to a series of impactful natural products, the approach also had limitations. First, the process could only begin upon the successful isolation of a native natural host. The natural environments hold an immeasurable amount of biological content [42,89], and only a small fraction of natural product producing microbes have been isolated through

conventional culturing methods [43], which was required for eventual scaled native host production. Because the same conventional methods of source organism culture were used repeatedly for natural product discovery, the issue of replication emerged, where researchers would continually isolate the same production hosts producing the same compounds. Once genotyping technology emerged [90–92], it became obvious that there was a massive gap between those microbes that exist in nature and those that could be successfully isolated and cultured using traditional microbiology [42,89]. Thus, access to a potentially massive repository of novel natural products was being neglected.

In addition, those organisms that were successfully isolated and culturable often possessed less than ideal innate biological properties, such as growth rate, cellular morphology, and the potential for precise genetic manipulation. From a practical standpoint, such innate properties complicated production efforts due to the challenges in culturing the native hosts, long time scales, and the potential for contamination. Moreover, even as genomic analysis emerged and a better understand of the genetic and biochemical workings of these microbes became clear, the lack of molecular engineering tools limited any directed efforts to improve cellular level production metrics.

These issues associated with native production hosts were in sharp contrast to workhorse microbes gaining traction in recombinant DNA practices that emerged in the 1970s. In contrast, these organisms had rapid growth rates (compared to most native natural product producers), single cell morphologies that supported scaled culture, and rapidly emerging genetic manipulation protocols. Example microbes included *Escherichia coli* and *Saccharomyces cerevisiae*. In addition, those working more closely within the natural products community were developing similar tools in hosts such as *Streptomyces* spp., noting that this bacterial group possessed native metabolism able to support complex natural product biosynthesis [53,93–96]. Doing so enabled the first efforts at heterologous natural product biosynthesis, in which the genetic content responsible for a natural product was transferred from the original host to a host like *Streptomyces coelicolor* [97,98]. In so doing, the heterologous host enables the application of molecular biology manipulation of both the heterologous host and the implanted natural product pathway [99–101].

Successful heterologous natural product biosynthesis in *Streptomyces*

**Table 1**  
Natural product production from native microbial sources.

Natural Product (Activity)	Native Host	Enhanced Production Methods	Production Metrics	References
Penicillin (antibiotic)	<i>Penicillium notatum</i>	<ul style="list-style-type: none"> <li>• Semi-solid medium using wheat bran</li> <li>• Addition of cellophane to the medium and pH adjustment</li> <li>• Use of corn steep liquor as a constant carbon and nitrogen source</li> <li>• Studied 20 parameters of batch system for industrial model</li> </ul>	Titer: 5.5 g/L Productivity: 34 mg/L/h (160 h)	[59–61]
Vancomycin (antibiotic)	<i>Streptomyces orientalis</i>	<ul style="list-style-type: none"> <li>• Tested different media to yield the best titers</li> <li>• Addition of inorganic phosphate</li> <li>• Phosphate limitation in a batch process</li> </ul>	Titer: 337 mg/L Productivity: 0.37 mg/gDCW/h	[62–64]
Erythromycin (antibiotic)	<i>Saccharopolyspora erythraea</i>	<ul style="list-style-type: none"> <li>• Addition of sodium propionate with strain NRRL2338</li> <li>• Manipulating fermentation conditions (pH and T)</li> <li>• Genetic engineering by expressing a hemoglobin gene</li> </ul>	Titer: 7.5 g/L Productivity: 10 mg/gDCW/h	[65–67]
Lovastatin (cholesterol lowering)	<i>Aspergillus terreus</i>	<ul style="list-style-type: none"> <li>• Applied glucose/glycerol to the fermentation process</li> <li>• Maintaining pH value above 4.5 during fermentation</li> <li>• Repeated fed-batch process</li> <li>• Use of lactose and potassium nitrate as carbon and nitrogen sources</li> </ul>	Titer: 1.3 g/L Productivity: 9.6 mg/L/h	[68–70]
Cyclosporin A (immunosuppressive)	<i>Tolypocladium inflatum</i>	<ul style="list-style-type: none"> <li>• High yielding strain variants by random mutagenesis</li> <li>• Different nutrient media especially those containing magnesium, iron and zinc ions</li> </ul>	Titer: 3.2 g/L Productivity: 9.4 mg/L/h	[71]
Rapamycin (immunosuppressive)	<i>Streptomyces hygroscopicus</i>	<ul style="list-style-type: none"> <li>• Carbon utilization agar used to standardize carbohydrate utilization</li> <li>• L-lysine improved production by 150%</li> </ul>	Titer: 147 mg/L Productivity: 0.62 mg/gDCW/h Yield: 27.8 mg/g ammonium sulfate	[72,73]
Actinomycin D (anticancer)	<i>Streptomyces antibioticus</i>	<ul style="list-style-type: none"> <li>• L-glutamic acid and L-valine increased production</li> <li>• Sarcosine deprivation improved production</li> </ul>	Titer: 93 mg/L Productivity: 0.5 mg/gDCW/h	[74,75]
Doxorubicin (anticancer)	<i>Streptomyces peucetius</i>	<ul style="list-style-type: none"> <li>• Mutagenic treatment to create variant of <i>S. peucetius</i></li> </ul>	Titer: 12 mg/L Productivity: 7.4 mg/gDCW/h	[76]
Artemisinin (antimalarial)	<i>Artemisia annua</i>	<ul style="list-style-type: none"> <li>• Introduction of <i>ipt</i> gene with leaf explants infection and increase of precursor feed</li> <li>• Addition of selected secondary precursors with elicitor during fermentation process</li> </ul>	Titer: 110.2 mg/L Productivity: 0.15 mg/gDCW/h	[77,78]
Tetracycline (antibiotic)	<i>Streptomyces aureofaciens</i>	<ul style="list-style-type: none"> <li>• Decrease in chloride content and application of a new isolation method</li> <li>• Use of sweet potato residue for solid-state fermentation</li> </ul>	Titer: 12.1 mg/L Productivity: 0.08 mg/gDCW/h	[79,80]
Tobramycin (antibiotic)	<i>Streptomyces tenebrarius</i>	<ul style="list-style-type: none"> <li>• Bioreactor parameter adjustment (pH, T)</li> <li>• Genetic mutation by deleting <i>aprHM</i> gene that is related to apramycin production</li> </ul>	Titer: 480 mg/L Productivity: 6.6 mg/L/h	[81,82]
Daptomycin (antibiotic)	<i>Streptomyces roseosporus</i>	<ul style="list-style-type: none"> <li>• Daptomycin production by <i>S. roseosporus</i> and <i>Actinoplanes</i> co-culture</li> <li>• Genetic mutation using a helium-neon laser</li> <li>• Exogenous addition of precursor (sodium decanoate)</li> </ul>	Titer: 812 mg/L Productivity: 2.8 mg/L/h	[83–85]

spp. helped to validate the approach and led to a wave of impactful studies that deftly combined the new recombinant tools for these hosts with the unique biosynthetic pathways of complex natural products. A primary result was the directed production of unique compounds [102, 103]. This outcome spurred an ongoing movement towards the application and refinement of heterologous natural product biosynthesis.

However, there was a key remaining drawback to the *Streptomyces* spp. used for heterologous natural product biosynthesis. Namely, they still retained nonideal growth properties relative to an alternative bacterial option such as *E. coli*. Unlike *Streptomyces* hosts, however, *E. coli* did not possess the native metabolism capable of supporting complex natural product biosynthesis. Hence, effort was required to both prep the cellular background in anticipation of complex natural product biosynthesis and modify established molecular biology tools, such as expression plasmids, to account for the size and number of heterologous genes needed to be transferred [104,105]. After overcoming a series of such technical challenges, *E. coli* was demonstrated capable of supporting complex natural product biosynthesis, with the erythromycin compound successfully produced from this host [106]. Table 2 highlights other successful examples of heterologous biosynthesis applied in the context of natural product production. Of note, the options of hosts have expanded across the bacterial and fungal species with a general heuristic being that the heterologous host match the type of native host (e.g., transfer from a eukaryotic native host to a eukaryotic heterologous host).

Table 2 also highlights the evolution of the research fields of metabolic engineering and synthetic biology as they relate to heterologous natural product biosynthesis [118–120]. Technically, both fields exists separate from heterologous biosynthesis, but this particular approach offers an excellent means of implementing each of these fields. Before being formally conceptualized, the approaches used to design and transfer natural product pathways from native to heterologous hosts employed now common tenants of synthetic biology. All of the detail that must be considered in gene and pathway expression design were features of the heterologous biosynthetic process to later be merged with the explosion in tools and concepts associated with synthetic biology (itself closely tied to breakthroughs in gene sequencing and synthesis). Similarly, metabolic engineering emerged as a field dedicated to understanding and directing intracellular metabolism for applied purposes. This approach directly resonates with the cellular level optimization available to natural product heterologous biosynthetic efforts. Both of these fields require host systems with amenable genetic manipulation tools. Hence, the progression of heterologous biosynthesis through genetically-amenable systems like bacteria and fungi served to tighten the relationships to metabolic engineering and synthetic biology.

#### 4. Cell free complex natural product biosynthesis

With the proficiency of sequencing technology developing over the last 20 years, there has been a concomitant emergence of natural

**Table 2**  
Natural product production from heterologous microbial sources.

Natural Product Class	Representative Examples (Activity)	Native Host	Heterologous Host	Enhanced Production Methods	Production Metrics	References
Polyketide Type I	Salinomycin (anticancer)	<i>Streptomyces albus</i>	<i>Streptomyces coelicolor</i>	<ul style="list-style-type: none"> <li>Biosynthetic gene screening on PAC library of <i>E. coli</i></li> <li>Cloning of artificial chromosome into <i>S. coelicolor</i></li> </ul>	Titer: 2.4 g/L Productivity: 14.3 mg/L/h Yield: 80 mg/g glucose	• [107]
	Lovastatin (cholesterol lowering)	<i>Aspergillus terreus</i>	<i>Pichia pastoris</i>	<ul style="list-style-type: none"> <li>Monacalin J biosynthetic genes amplified from <i>A. terreus</i> and transformed into <i>P. pastoris</i> to make first strain</li> <li>An additional plasmid allowing conversion of monacalin J to lovastatin to produce second <i>P. pastoris</i> strain</li> <li>Both strains were co-cultured</li> </ul>	Titer: 250.8 mg/L Productivity: 2.6 mg/L/h Yield: 125.4 g/g inositol (malonate pathway)	• [108]
Type II	Chlorotetracycline (antibiotic)	<i>Streptomyces aureofaciens</i>	<i>Streptomyces rimosus</i>	<ul style="list-style-type: none"> <li>Oxytetracycline gene cluster deleted in <i>S. rimosus</i> using Dre/rox system</li> <li>Chlorotetracycline gene cluster from <i>S. aureofaciens</i> integrated into plasmids and transformed into <i>S. rimosus</i> through conjugation via <i>E. coli</i></li> </ul>	Titer: 3.8 g/L Productivity: 32.8 mg/gDCW/h Yield: 1.5 g/g glucose (malonate pathway)	• [109]
	Mithramycin A (anticancer)	<i>Streptomyces argillaceus</i>	<i>Streptomyces lividans</i>	<ul style="list-style-type: none"> <li>Mithramycin (MTM) gene cluster amplified using PCR from <i>S. argillaceus</i> and cloned into a single plasmid</li> <li>Transformation assisted recombination (TAR) utilized <i>S. cerevisiae</i> to generate enough DNA to transform into <i>E. coli</i></li> <li>Double crossover based selection was utilized to delete secondary metabolite gene clusters from <i>S. lividans</i></li> <li><i>E. coli</i> with the MTM gene cluster was used as an intergeneric conjugation host for <i>S. lividans</i></li> </ul>	Titer: 3 g/L Productivity: 17.9 mg/L/h Yield: 1.2 g/g glucose (acetyl CoA pathway)	• [110]
Nonribosomal peptide	Epothilone (anticancer)	<i>Sorangium cellulosum</i>	<i>Schlegelella brevitalea</i>	<ul style="list-style-type: none"> <li>Epothilone gene sequences were used to PCR target two known plasmids to obtain a single plasmid with all biosynthetic genes transformed into <i>E. coli</i>.</li> <li>Two genes, <i>epoA</i> and <i>epoK</i> were ligated using In-Fusion cloning and the entire plasmid transformed into <i>S. brevitalea</i>.</li> <li>Yield improved through malonyl-CoA flux by integrating genes from <i>S. coelicolor</i></li> </ul>	Titer: 82 mg/L Productivity: 0.44 mg/gDCW/h Yield: 32.8 g/g cysteine	• [111]
	Daptomycin (antibiotic)	<i>Streptomyces roseosporus</i>	<i>Streptomyces coelicolor</i>	<ul style="list-style-type: none"> <li>Daptomycin gene cluster amplified by PCR from <i>S. roseosporus</i> and ligated into cloning vector.</li> <li>This vector was cloned into pSA, isolated from pSBAC with a gene, <i>attP-int</i>, that stabilizes heterologous expression of daptomycin</li> <li>Transformed into <i>E. coli</i> S17-1 and conjugated directly with <i>S. coelicolor</i></li> </ul>	Titer: 28.9 mg/L Productivity: 0.24 mg/L/h Yield: 2.89 g/g glucose	• [112]
Isoprenoid	Dihydroxy artemisinic acid (precursor to artemisinin-antimalarial)	<i>Artemisia annua</i>	<i>Saccharomyces cerevisiae</i>	<ul style="list-style-type: none"> <li>HMGS gene from <i>S. cerevisiae</i> replaced by <i>mvaA</i> (HMGR) gene from <i>S. aureus</i> for improved precursor flux</li> </ul>	Titer: 1.7 g/L Productivity: 14.2 mg/L/h Yield: 42.5 mg/g glucose	• [113]
	Naringenin (anti-inflammatory)	<i>Arabidopsis thaliana</i> / <i>Rhodotorula glutinis</i>	<i>Yarrowia lipolytica</i>	<ul style="list-style-type: none"> <li>Genes from <i>R. glutinis</i> and <i>A. thaliana</i> were PCR amplified and integrated into plasmids for expression in <i>Y. lipolytica</i></li> <li>Genomic integration of plasmids using CRISPR-Cas9 further improved yield</li> <li>Xylose utilization was induced by expressing <i>E. coli</i> gene through plasmid in <i>Y. lipolytica</i> followed by genomic integration</li> <li>Xylose concentration and timing of exogenous feeding further optimized product titer</li> </ul>	Titer: 715.3 mg/L Productivity: 5.0 mg/L/h Yield: 11.9 mg/g xylose	• [114]
	Curcumin (anti-allergy and anticancer)	<i>Arabidopsis thaliana</i>	<i>Escherichia coli</i>	<ul style="list-style-type: none"> <li>Cloning four biosynthetic genes from <i>A. thaliana</i> and other plasmids into first strain of <i>E. coli</i> BL21 (DE3) to make ferulic acid</li> <li><i>lacZ</i> gene was disrupted in another <i>E. coli</i> BL21 (DE3) strain using scarless Cas9 assisted recombination for blue-white screen of two co-culture strains</li> </ul>	Titer: 563.4 mg/L Productivity: 8.9 mg/L/h Yield: 14.1 mg/g glucose (chorismate pathway)	[115]

(continued on next page)

Table 2 (continued)

Natural Product Class	Representative Examples (Activity)	Native Host	Heterologous Host	Enhanced Production Methods	Production Metrics	References
	Guaia-6,10 (14)-diene (Precursor to Englerin A-anticancer)	<i>Phyllanthus engleri</i>	<i>Saccharomyces cerevisiae</i>	<ul style="list-style-type: none"> <li>Biosynthetic genes for conversion of ferulic acid to curcumin are transformed into <math>\Delta lacZ</math> strain</li> <li>Co-culturing of two strains</li> <li>CRISPR/Cas9 used to engineer MVA pathway genes (including <i>ERG10</i> and <i>tHMG1</i>)</li> <li>Increase in copy number of <i>tHMG1</i> gene, rate-limiting step in the MVA pathway</li> <li>Galactose requirement eliminated by <i>GAL80</i></li> <li>Cloning of gene cluster from BAC library of <i>S. annulatus</i></li> <li>Desired gene fragments cloned into a vector introduced to <i>E. coli</i> and desired clone selected from colony PCR.</li> <li>Promoters introduced for necessary gene expression within <i>S. avermitilis</i></li> <li>Transformation of expression plasmids into <i>S. avermitilis</i></li> </ul>	Titer: 0.8 g/L Productivity: 0.04 mg/gDCW/h Yield: 0.04 g/g glucose	[116]
RiPP	Telomestatin (anticancer)	<i>Streptomyces annulatus</i>	<i>Streptomyces avermitilis</i>		Titer: 5 mg/L Yield: 2 mg/g glucose	[117]

product pathways (either confirmed or putative) [121–125]. This information has greatly impacted prospects for heterologous biosynthesis as key blueprint is now available to seed reconstitution efforts. Furthermore, with the continuing emergence of DNA synthesis technology, natural product genetic pathways designed for optimal expression within a target heterologous host is becoming a realistic option [25, 126].

While the above technical developments support continued efforts at heterologous biosynthesis, there are other emerging paradigms for the rapid reconstitution of target natural product pathways. One such concept is cell free biosynthesis. In this approach, which stems from cell free protein synthesis [127], one would completely eliminate the cellular framework.

Instead, a more controlled, fabricated reaction would be assembled from the key components of the biosynthetic process. Genetic code would be input to such a system, together with the transcription and translation machinery required for protein formation. In this case, however, the resulting proteins would be expected to convert additional metabolic building blocks into a final natural product.

While heterologous biosynthesis leverages the positive traits of a host system, there is still extensive background metabolism that at best is divorced from the biosynthetic process. However, other times, there is unwanted cross-talk between native and heterologous metabolism that detracts from final heterologous biosynthetic success. There are also post-translational steps, including protein folding [106,128], that will be strongly influenced by the native cellular environment. Whereas, in a cell free setting, the environment could be more finely controlled towards such ends as improved protein solubility and activity. Finally, the heterologous process is also constrained by associated host properties such as growth rate and mutational potential, issues that can be eliminated in a cell free format.

A cell free system, however, would require a degree of complexity to mimic the gene expression process that happens intracellularly. Many ongoing commercial and academic approaches utilize cellular components or extracts from popular heterologous host options, most notably, *E. coli*. This of course dictates genetic pathway designs that are congruent with the transcription and translation mechanics associated with the host from which they have been isolated.

Table 3 highlights ongoing efforts to generate complex natural products using cell free platforms. Further, Fig. 3 highlights our group's efforts to generate the erythromycin product using a cell free approach. These examples and initial data show promise for the approach. Future considerations must include the ultimate end goal of cell free biosynthesis. Challenges will be encountered in efforts to scale cell free production efforts (just as they exist for production from native and

Table 3

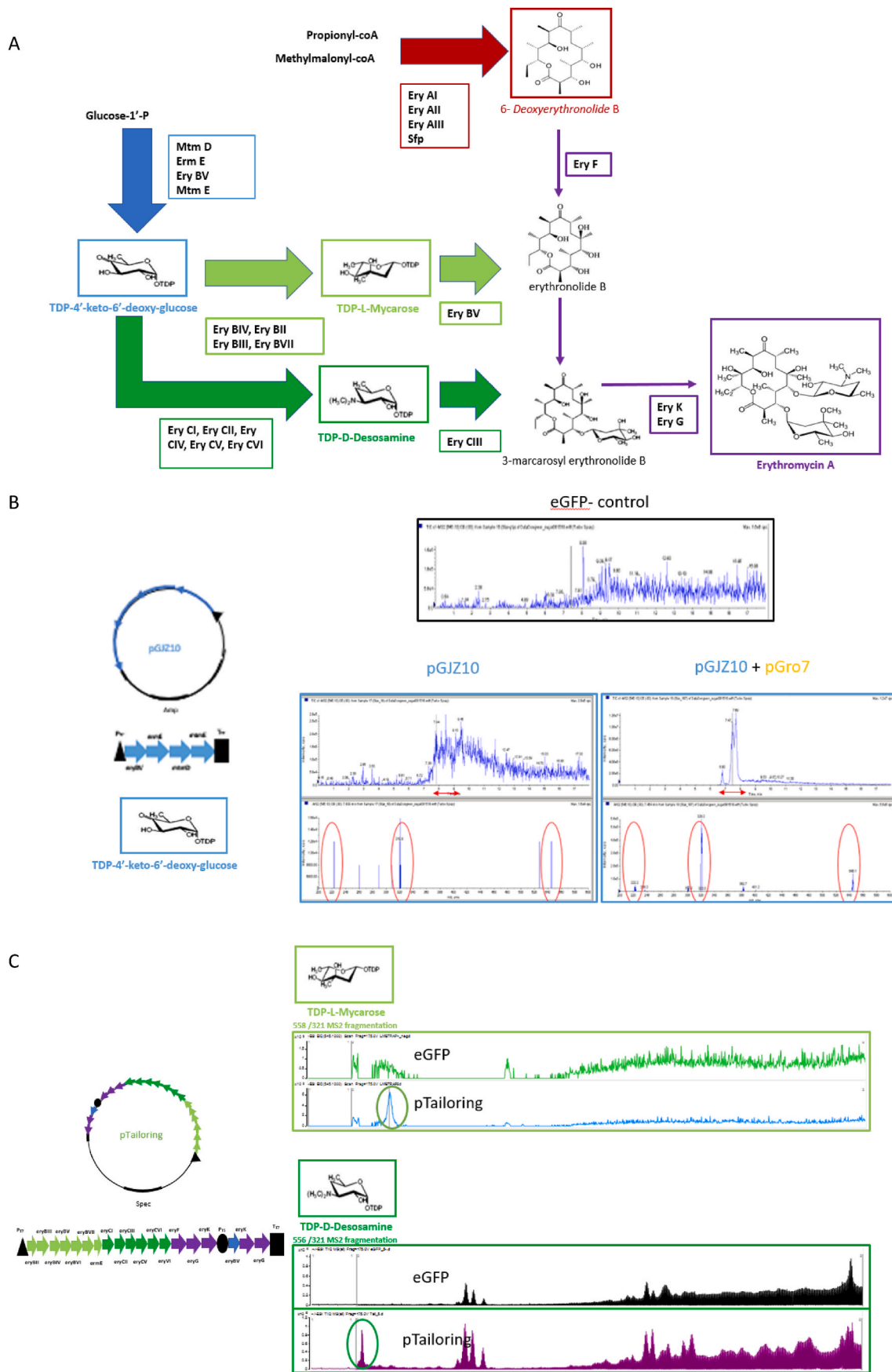
Natural product production using cell free biosynthesis.

Natural Product	Natural Product Class	Production Methods	Production Metrics	References
Diketopiperazine	NRPS	<i>E. coli</i> BL21 Star (DE3) extract	Titer: 12 mg/L Productivity: 0.6 mg/L/h (20 h) Yield: 50 mg/g phenylalanine	[129]
Z-5-Bromo-3 (2-isocyanovinyl)-1H-indole	RiPP	New England Biolabs PURExpress system	Titer 6.6 g/L Productivity: 330 mg/L/h (20 h)	[130]
Limonene	Terpene	<i>E. coli</i> BL21 Star (DE3) extract	Titer: 610 mg/L Productivity: 25.4 mg/L/h (24 h) Yield: 17 mg/g glucose	[131]
Lanthipeptide (NisinZ)	RiPP	Multiple <i>E. coli</i> strains	Titer: 4–5 mg/L Productivity: 0.83 mg/L/h (6 h s)	[132]

heterologous hosts). However, the platform may better serve to support screening efforts of natural product discovery from newly identified sequence information as a way to confirm biosynthesis of a new compound.

## 5. Conclusions

The production methods for complex natural products outlined in this article span native production hosts to cell free platforms, each possessing various advantages and drawbacks, as summarized in Table 4. Over time, the transition from native natural product production hosts to heterologous host options to cell free systems has been spurred by technical advancements in parallel with ongoing engineering efforts to address production challenges. These same technical advances have produced stand-alone academic fields of metabolic engineering and synthetic biology, both widely utilized across the natural product production platforms outlined herein. Moving forward, technical advances will continue to influence the choice of natural product biosynthetic system, with researchers having the option of native host, heterologous host, or cell free systems. The ultimate choice will be



**Fig. 3.** Cell free biosynthesis for erythromycin A. The erythromycin A biosynthetic pathway (a) color-coded for steps leading to complete compound formation. Cell free biosynthesis for TDP-4'-keto-6'-deoxy-glucose using plasmids pGJZ10 (indicated [27]) and pGro7 (providing the GroEL/ES chaperonin genes), using a plasmid

expressing eGFP as a negative control and compared to samples with pGJZ10 and pGJZ10+pGro7 as analyzed by LC-MS (b). Cell free production for TDP-L-mycarose and TDP-D-desosamine using pTailoring previously described [25] (c). Cell free biosynthesis of 6-deoxyerythronolide B (6dEB) using plasmids pDEBS [25] assessed by LC-MS (d). Combining the pTailoring plasmid with the 6dEB molecule indicated erythromycin A formation by LC-MS (e). Cell free extract was prepared as follows: 10 mL of overnight-cultured *E. coli* BL21 Star (DE3) was inoculated in 1 L of 2 × YTPG medium (10 g/L yeast extract, 16 g/L tryptone, 3 g/L K<sub>2</sub>HPO<sub>4</sub>, 7 g/L KH<sub>2</sub>PO<sub>4</sub>, 5 g/L NaCl, and 18 g/L glucose) and grown at 37 °C with 250 rpm shaking. The culture was induced with 0.5 mM IPTG at an OD<sub>600nm</sub> of 0.6–0.8, and the cells were grown until OD<sub>600nm</sub> 3.5. The cells were harvested by centrifuge (8000 rpm, 10 min) and washed thrice with S30 Buffer (10 mM Tris-acetate, 14 mM Mg(OAc)<sub>2</sub>, 60 mM KOAc, 5 mM DTT) and stored at –80 °C after flash-freeze using liquid nitrogen. The cells were resuspended in S30 Buffer (1.2 g/mL) and aliquoted to 1.5 mL. Aliquots were sonicated with a FB50 (Thermo Fisher) sonifier with a 3 mm microtip (10 s on/10 s off) 5 times. The extract was prepared by collecting supernatant after 12,000 rpm of centrifugation. Cell free natural product biosynthesis was performed by using eGFP plasmid DNA as a control and a reaction buffer with the following contents: 1.2 mM ATP; 0.85 mM each of GTP, UTP and CTP; 34 µg/mL folinic acid; 171 µg/mL T7 RNA polymerase; 2 mM each of the 20 translatable amino acids, 0.33 mM nicotinamide adenine dinucleotide (NAD), 0.26 mM coenzyme A (CoA), 33 mM PEP, 130 mM potassium glutamate, 10 mM ammonium glutamate, 12 mM magnesium glutamate, 1.5 mM spermidine, 1 mM putrescine, 57 mM HEPES, 4 mM sodium oxalate, and 1 µL of 20 mg/mL Sfp [133]. To the reaction buffer, 0.25 volume of cell extract (described above) and 20 µg/mL plasmid DNA were added to reach a final volume of 25 µL. The reaction was held for 20 h at 30 °C and the required natural product metabolic substrates were added: glucose-1-phosphate (10 mM) and dTTP (10 mM) for tailoring sugar biosynthesis, and propionyl-CoA (1 mM) and methyl malonyl-CoA (6 mM) for 6dEB biosynthesis. After 20 h, the samples were moved to –20 °C prior to SDS-PAGE and LC-MS analysis. Samples were prepared for analysis by diluting three times in LC-MS grade methanol and centrifuging at 13,000 rpm for 15 min with supernatant used for analysis. Erythromycin and 6dEB samples were applied to a Thermo Scientific Orbitrap XL with a C-18 analytical column. TDP-deoxysugar samples (mycarose, desosamine) were measured by Agilent G6545A quadrupole-time-of-flight (Q-TOF) using an XBridge Shield RP18 3.5 µm, 3.0 mm × 150 mm column from Waters. All MS analyses were conducted in positive ion mode. A linear gradient of 80% buffer A (95% water/5% acetonitrile/0.1% formic acid) to 100% buffer B (5% water/95% acetonitrile/0.1% formic acid) was used at a flow rate of 10 µL/min for the LC.

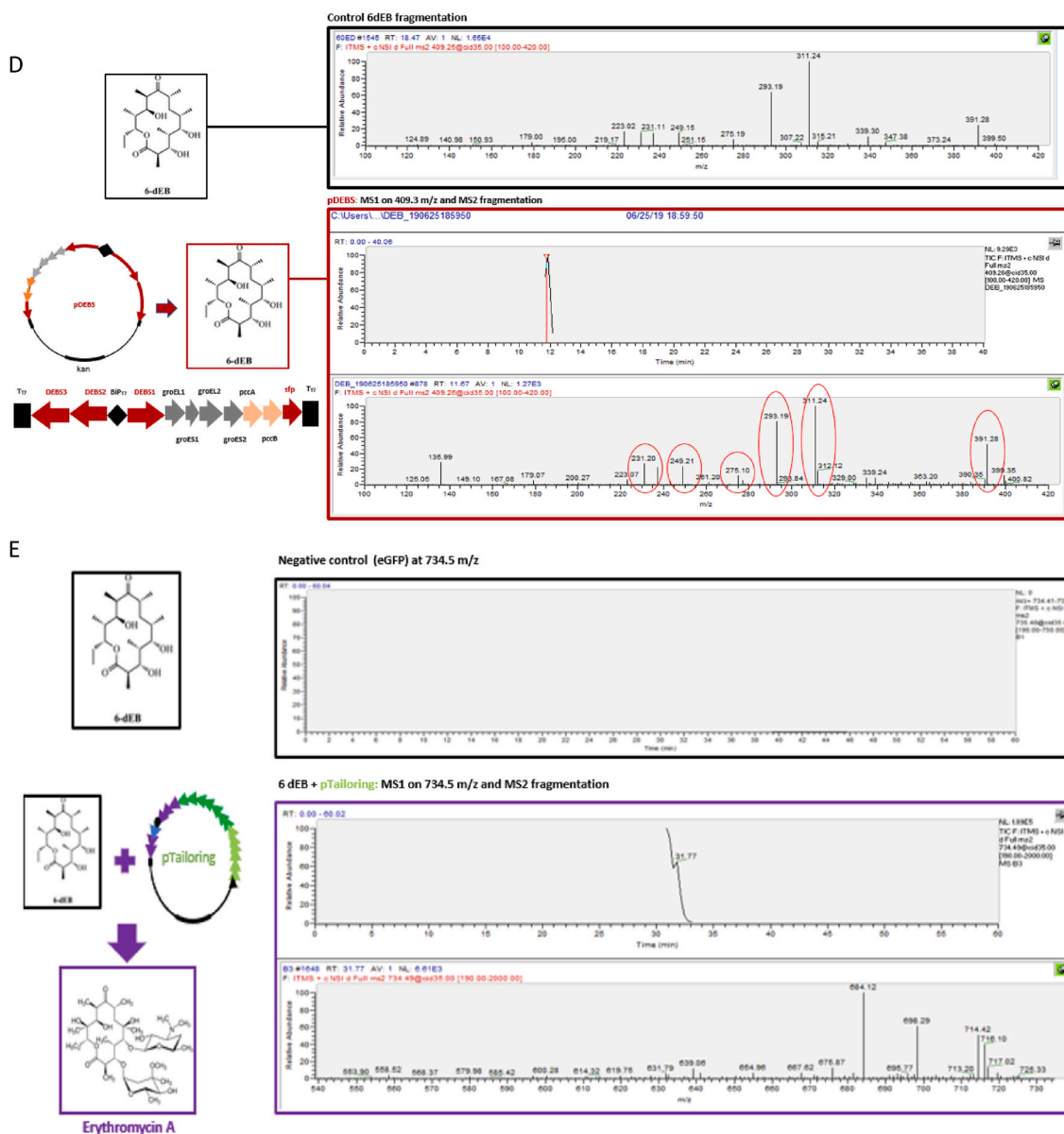


Fig. 3. (continued).



**Table 4**  
Summary and comparison of natural product production options.

	Pros	Cons	
Native Host Biosynthesis	<ul style="list-style-type: none"> <li>• Contains full biosynthetic pathway</li> <li>• Evolutionarily optimized production</li> </ul>	<ul style="list-style-type: none"> <li>• Potential lack of genetic manipulation tools</li> <li>• Potential slow growth rate</li> <li>• Potential “cross-talk” between desired product metabolism and background cellular metabolism</li> </ul>	
Heterologous Host Biosynthesis	Prokaryotic	<ul style="list-style-type: none"> <li>• Simple growth medium requirement</li> <li>• Rapid growth</li> <li>• Amenable genetic manipulation/mutation</li> <li>• Nonpathogenic/safe</li> </ul>	<ul style="list-style-type: none"> <li>• Issues with gene expression</li> <li>• Lack of post-translational modification</li> <li>• Lack of required biosynthetic metabolism support</li> <li>• Prone to phage infection</li> </ul>
	Eukaryotic	<ul style="list-style-type: none"> <li>• Safe</li> <li>• Advanced protein folding/post-translational modification</li> <li>• Resistant to phage infection</li> </ul>	<ul style="list-style-type: none"> <li>• Potentially less advanced genetic manipulation tools and/or range of gene expression elements</li> <li>• Lack of required biosynthetic metabolism support</li> </ul>
Cell-Free Protein Synthesis and Natural Product Biosynthesis	<ul style="list-style-type: none"> <li>• Focused biosynthetic process (no competing cellular metabolism)</li> <li>• Unconstrained by cellular growth rates</li> <li>• Controllable environment (pH, T, reduction)</li> </ul>	<ul style="list-style-type: none"> <li>• Potentially more challenges with large protein production</li> <li>• Challenges with production scale</li> <li>• Challenges with production levels</li> </ul>	

dictated by the economical and sustainable discovery, production, and distribution of a target natural product for societal benefit.

#### Declaration of competing interest

The Authors Declare No Conflicts.

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