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Review Article

Prototyping of microbial chassis for the biomanufacturing of high-value chemical targets

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Metabolic engineering technologies have been employed with increasing success over the last three decades for the engineering and optimization of industrial host strains to competitively produce high-value chemical targets. To this end, continued reductions in the time taken from concept, to development, to scale-up are essential. Design–Build–Test–Learn pipelines that are able to rapidly deliver diverse chemical targets through iterative optimization of microbial production strains have been established. Biofoundries are employing *in silico* tools for the design of genetic parts, alongside combinatorial design of experiments approaches to optimize selection from within the potential design space of biological circuits based on multi-criteria objectives. These genetic constructs can then be built and tested through automated laboratory workflows, with performance data analysed in the learn phase to inform further design. Successful examples of rapid prototyping processes for microbially produced compounds reveal the potential role of biofoundries in leading the sustainable production of next-generation bio-based chemicals.

Introduction

In recent years, there has been renewed interest in transitioning metabolic engineering into a viable manufacturing technology, to meet the growing demand for bio-based built-to-order products by providing speciality chemicals and molecular building blocks. Industrial biomanufacturing is thus required to compete efficiently with established technologies by reducing time-to-market and cost, while assuring the quality of the final product. Therefore, many standardized protocols and metrics have started to appear in order to benchmark the efficiency of the biomanufacturing process, to assess sustainability and to conduct techno-economic analyses. Those challenges have already been addressed in conventional manufacturing technologies, where rapid prototyping (RP) has demonstrated its potential to reduce the time and cost of product development. RP is a process that fabricates physical parts layer-by-layer under computer control directly from three-dimensional (3D) models developed using computer-aided design (CAD). A key benefit of 3D printing technologies is that they allow users to build and test customized parts in a very short time, to meet predefined design criteria in manufacturing technologies. The ability to print electronics from functional materials offers similar advantages. Parallels are now emerging in biofoundries, where the ability to rapidly assemble and test genetic circuits using standard characterized parts is allowing RP of microbial production chassis for industrial biomanufacturing.

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Recent descriptions of standardized Design–Build–Test–Learn (DBTL) pipelines, with multi-level prototyping capabilities, that are applicable to any chemical target and microbial strain, demonstrate that RP is now a technology that can be successfully applied to biomanufacturing. Nevertheless, biofoundries must continue to innovate, and draw inspiration from other manufacturing technologies if they aspire to compete with the conventional industrial production of high-value bio-based chemicals.

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Main body

Rapid prototyping applied to biomanufacturing

Additive manufacturing (AM) is a wide category of manufacturing technologies that fabricate functional objects from 3D models, layer-by-layer. RP can be encompassed inside AM when the process tries to produce prototypes in a short timescale [1]. Originally developed by the traditional manufacturing industry in the late 1980s [2], this approach is quickly being implemented in other research areas. The principles of RP, much like those of the Agile methodology in software development or project management, argue that creating and testing prototypes — functional early versions towards the final goal — and learning from each iteration is more efficient than the traditional alternative (i.e. spending long periods of time designing and building pre-conceptions of the perfect solution).

The AM process requires design methodologies during both the creation and evaluation of the parts produced, especially in the early stages of development, to minimize the extra costs that a late change in the design would require [2,3]. Therefore, it is crucial to know the manufacturing restrictions for a particular project as early as possible. This can be facilitated by RP.

Biomanufacturing uses biological factories (microorganisms, tissues, cell extracts, enzymes) to produce high-value chemical targets, and has several well-publicised early examples of success [4]. The latest advancements made in biotechnology indicate that the biomanufacturing industry may soon become a viable alternative to traditional chemical synthesis [5]. Furthermore, the growing need for green technologies that solve society's problems and reduce the environmental footprint of our activities, whilst driving down currently high development costs, provide a strong argument for innovation in this new industry. For a nice comparison between bio-based and chemical production see [6].

As early as in 2002, the limitations of traditional biomanufacturing were already described. The scale-up problem of current biomanufacturing needs different solutions away from the traditional ones. Building more bioreactors increases the costs of the product, whereas increasing the size of the production vessels is limited by nutrient and heat diffusion constraints [7]. Apart from poor scalability, another issue that needs to be tackled is the long timescale required from design to production [5]. Moreover, the current costs of research and production may appear, at first, overwhelming for new players (academic groups and startups) in the biomanufacturing game.

It is clear that different approaches, away from the traditional bioproduction techniques used in the early stages of this industry are needed. To close the gap between biomanufacturing and chemical synthesis, more research and disruptive developments are required. It is predicted that the discovery and development of new technologies will become a competitive advantage for small starting enterprises that are willing to try riskier, but the higher reward, methods [8] such as the integration of advanced manufacturing platforms with machine learning methods [9,10]. Biofoundries (see below) may act as a sort of intermediary for starting enterprises, applying their expertise in RP to provide these new groups with quick fundamental knowledge, helping them take well-informed decisions.

Biomanufacturing sits at the intersection between biology and engineering. For this reason, biomanufacturing has the potential for the development of truly breakthrough technologies, through the implementation of engineering concepts and methodologies in biotechnology, and is expected to lead into collaborations between currently distant research areas, acting as a platform for new converging innovations [4,11]. Engineers have played, and continue to play, an important role in the development of synthetic biology and metabolic engineering, driving this new discipline to become systematic and goal-oriented, to focus on decreasing costs and to manufacture new products [4].

However, synthetic biology methodologies are no panacea. They open up a wide range of possibilities but can also appear to offer fewer manufacturing advantages compared with traditional chemical synthesis or already established cell factories [4]. For this reason, it is imperative to keep researching new ways to improve the discipline. RP is a prominent example and a candidate for a disruptive technology that may very well change the biomanufacturing industry. However, to achieve this potential the development of new software and biological tools is paramount [5]. These tools can be created to fit any of the four stages of what has become the cornerstone of RP applied to biomanufacturing: DBTL [12]. The DBTL pipeline tries to accomplish a biomanufacturing goal, through the means of a pre-established metric (e.g. yield), iterating as many times as needed through the process. Although the DBTL paradigm aims to accelerate the development of biomanufacturing processes, many bottlenecks remain, including gene synthesis costs and timescales, sequence verification

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of genetic construct libraries and laborious genome editing of host strains. Other shortcomings that need to be addressed include typically low titers (<1 g/l), insufficient information of candidate enzymes and *de novo* pathways, as well as issues related to enzyme expression (protein solubility and toxicity, low levels of metabolite precursors and enzyme cofactors, and a lack of suitable regulatory elements in the native host).

RP in biomanufacturing is achieved by applying prototyping strategies to the different construction layers — or levels — of the engineered microbial strains in each of the DBTL steps (Table 1).

Biofoundries

Biofoundries are specialized laboratories that combine software-based design and automated or semi-automated pipelines to build metabolic pathway constructs and genetically modified strains and then test them for production using microplate readers, HPLC methods and mass spectrometry (MS) techniques. This allows the generation of constructs in a high-throughput manner, together with the gathering and analysis of large amounts of information that can later be used during the learn phase of each DBTL iteration (see Figure 1).

A global alliance of biofoundries (GBA) was launched in 2019 to co-ordinate the activities of non-commercial biofoundries around the world [13]. In the webpage of the Alliance [14], we can find a current list of 23 members and a description of their goals and approaches to tackle the problems of the bioeconomy. We shall describe below some GBA members working in, or close to, the biomanufacturing area that we consider relevant to this review.

The Agile BioFoundry is a consortium of seven U.S. laboratories that works in a distributed manner. It was founded in 2016 by the U.S. Department of Energy's Bioenergy Technologies Office with the aim of accelerating biomanufacturing to boost the bioeconomy. Motivated by the reduction of fossil usage, their efforts focus towards the biomanufacture of bio-based fuels, materials and chemicals. Their expertize covers the whole DBTL pipeline, although their work is particularly robust in building (DNA synthesis, cloning, sequencing and transformation) and testing (strain characterization, microfluidics, proteomics and metabolomics). They also provide special scale-up services, host selection services, and techno-economic and life cycle analyses. For public and private laboratories to utilize their capabilities they provide their own funding opportunities.

During the time this facility has been running, they have developed, among others, an integrated suite of tools for metabolic engineering [15], several tools that (through ML models) facilitate genotype-to-phenotype predictions [16], or recommend strains to be built together with predicted levels of target production [17], and tools such as DNA Scanner to compare DNA-synthesis services in terms of price, time of production and complexity constraints [18]. Furthermore, they have also automated and streamlined processes, such as the extraction of proteins for high-throughput proteomic applications via an automated workflow that processes 96 samples in 2 h and can process 384 samples in parallel [19].

Members of the Agile biofoundry ran two DBTL cycles to improve the dodecanol production in *Escherichia coli*. (Figure 1; [20]). The first cycle was used to gather information to train an ML algorithm capable of suggesting modifications to the initial approach in the second cycle, which managed to increase dodecanol production by 21%. In another engineering campaign [16], collaborators from Agile and The Novo Nordisk

Table 1. Prototyping strategies for biomanufacturing at different levels

Level	Prototyping strategy
Gene	Soluble expression (SDS–PAGE gels) and *in vitro* activity (lysate biotransformation assays)
Pathway module	Pathways split into tractable modules (1–4 genes) with consideration of the stability of pathway intermediates in the selected microbial host and the ease with which they can be quantified (mass spectrometry (MS) standards or suitable assays)
Genetic construct architecture	Combinatorial assembly of parts using design of experiments (DoE)-based plasmid library design
Full pathway	Testing modules together in the microbial host and consolidation into a single construct
Chassis	Relative performance of gene knockout and overexpression strains, either through quantification of metabolite substrates for heterologous biosynthetic pathways or direct assessment of final target production in these strains
Process optimization and scale-up	DoE-based optimization of culture conditions and fermentation process, plasmid stabilization and chromosome integration, downstream processing development

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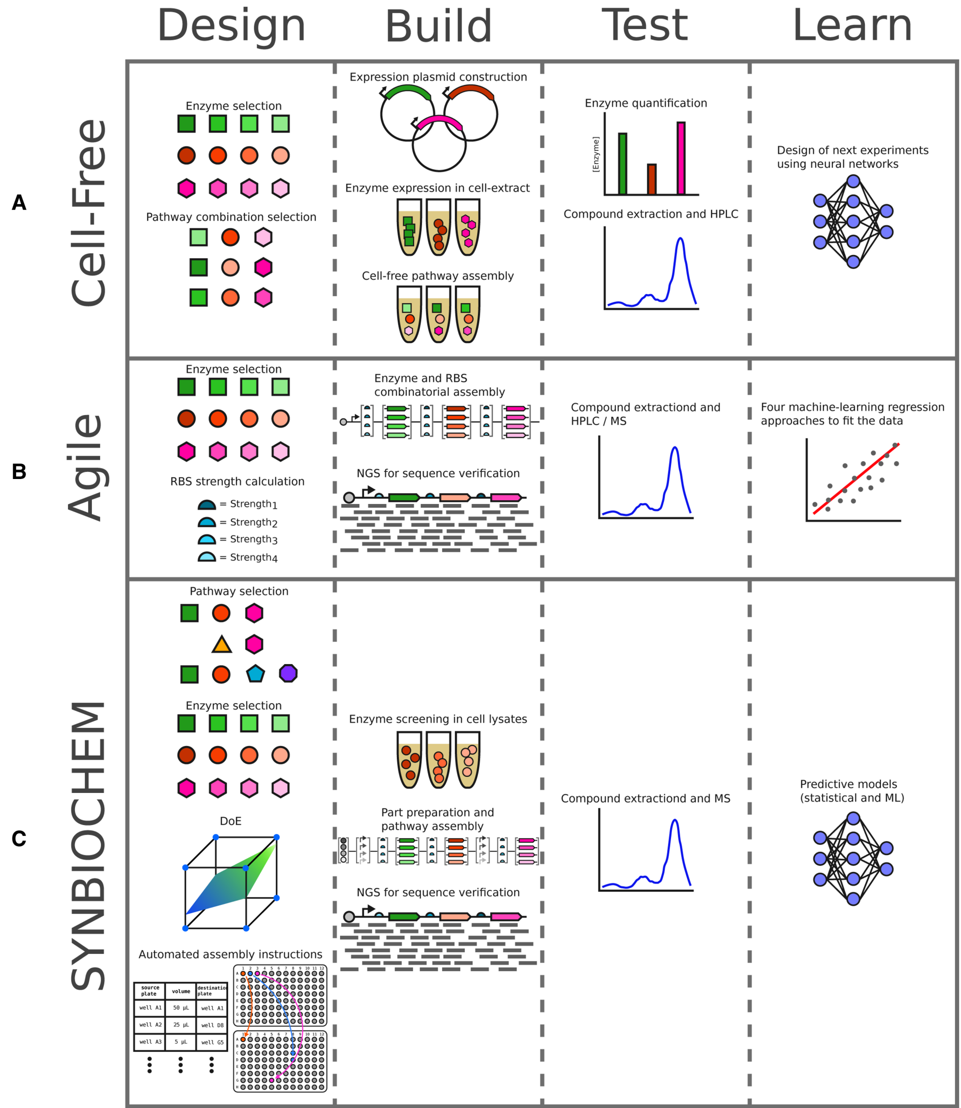

Figure 1. Comparison of three rapid prototyping approaches employed by different biofoundries.
(**a**) Cell-free prototyping of genetic part and metabolic pathway designs in biofoundries such as Northwestern University [51] and SynBiCite [29]. (**b**) Research into dodecanol production in *Escherichia coli* at the Agile BioFoundry [20]. (**c**) Rapid prototyping of microbial strains for the production of materials monomers by SYNBIOCHEM's biofoundry [34].

Foundation Center for Biosustainability (DTU Biosustain) biofoundries combined mechanistic and ML models to predict genetic designs for the production of tryptophan in *Saccharomyces cerevisiae*. The trained models were able to identify genetic circuits that showed up to 74% higher tryptophan yield.

The DAMP (design, automation, manufacturing and prototyping) laboratory is another biofoundry, located in Boston within the biological design center (BDC). It started working at the beginning of 2018 to support synthetic biology research through the development of new biological systems, using a combination of high-throughput liquid handling robots, standardized protocols and computerized software scheduling. They offer 35 molecular biology automated protocols, including DNA assembly, DNA/RNA purification, sequencing, storage, PCR, transformation and several characterization assays. This biofoundry has a special interest in developing microfluidics applications [21], and they have contributed to microfluidics research through the development of a human-friendly microfluidic hardware description language (MHDL) [22]. They also created a DNA assembly metric (Q-metric) — to quantify the advantage of using automation methods over traditional and

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manual approaches — and a software application called Puppeteer to capture those values, provide design assistance and translate the results into human and robot instructions [23].

The SYNBIOCHEM Center biofoundry at the Manchester Institute of Biotechnology focuses on a DBTL pipeline for the RP of microbial factories for the production of fine and speciality chemicals. The facility, launched in 2015, applies a portfolio of design tools such as RetroPath2.0 [24], Selenzyme [25] and PartsGenie [26] for the selection of viable metabolic routes, candidate enzyme screening and DNA part design, respectively. The assembly, NGS-based verification and activity screening of combinatorial libraries of genetic constructs follows a semi-automated pipeline. Directed evolution campaigns and host strain engineering, as well as process optimization and bioreactor scale-up are also performed in-house. The center's DBTL pipeline was showcased through the 500-fold improved microbial production of flavonoids, from early prototype to final production strain [27].

Finally, the London Biofoundry is a core facility of SynbiCITE that launched at the beginning of 2017. Its efforts are focussed towards DNA synthesis and construction, and they provide high-throughput DNA assembly and transformation services, as well as NGS library preparation, screening campaigns, diagnostic assays, CRISPR campaigns and RT-qPCR setups. They also have the facilities for prototyping biomanufacture and have developed an automated workflow able to build and design heterologous metabolic pathways [28], a rapid automated platform for measuring and modeling *in vitro* cell-free reactions to quantify, for example, ribosome binding site variants and new promoters [29], and a workflow to optimize the miniaturized preparation of plasmid DNA libraries for NGS [30].

Other important biofoundries have been established which are not part of the GBA. For example, The MIT-Broad Institute Foundry described in 2018 a timed pressure test administered by the U.S. Defense Advanced Research Projects Agency (DARPA) [31]. The goal was to try to produce 10 different molecules, unknown to them in advance, in 3 months using a range of different microorganisms or cell-free systems. This project demonstrated how the co-ordinated efforts of skilled synthetic biologists can engineer working prototypes of strains to produce new biomolecules in very short periods of time. The researchers managed to, among other feats, increase the production of 1-hexadecanol, pyrrolnitrin and pacidamycin D, find new metabolic routes for the enediyne warhead, and create a cell-free system for the production of monoterpenes.

To remain agile, biofoundries should be capable of tackling any chemical target of interest, even where no prior biosynthetic pathway knowledge exists. Furthermore, in the interests of responsible research and innovation (RRI), the selection of relevant targets should be carefully considered beforehand, as part of prospective analyses which assign priority based on sustainability and techno-economic criteria, in the same manner as the chemical manufacturing industry. Similarly, automated retrosynthetic [24] and enzyme selection tools [25] should be employed to prospectively map viable production pathways for intended targets and perform an appraisal of maximum theoretical yields. Such steps are becoming nowadays more standardized thanks to the use of reaction rules as a representation of the biochemical space, and CAD platforms to map out the design space of parameters. Optimal experimental design [32,33], a technology borrowed from manufacturing, allows quick estimation of the impact that different parameters can have on the performance of the producing strain, without requiring an excessive number of experimental runs.

The SYNBIOCHEM biofoundry approach to rapid prototyping

We have recently benchmarked such an approach in the SYNBIOCHEM center biofoundry, where RP was performed for microbial production of a suite of material monomers within a predefined time limit [34]. Our DBTL pipeline incorporates automation of the DBTL workflows and protocols, for improved performance and reduction of human error, and is designed with the flexibility to handle any feasible chemical target and microbial host [27]. The pipeline provides a reference platform for other laboratories looking to make their project workflows more efficient through automation.

One of the relevant compounds for the biomanufacturing industry, which was prototyped through this approach, was chavicol. Chavicol is a natural product found in betel oil, bay leaves and sweet basil. Chavicol-based benzoxazine monomers can be polymerized into thermoset resins with adjustable thermomechanical properties through controlled cross-linking [35]. An automated pathway and enzyme selection DESIGN workflow [24,25] combined with literature information provided a panel of enzyme candidates with good phylogenetic representation, selecting 6× CCR (coumarate CoA reductase; [36,37]), 5× CAD (cinnamyl alcohol dehydrogenase; [38–40]), 4× CFAT (coniferyl alcohol acyltransferase; [41]) and 4× EGS (eugenol synthase; [42,43]) orthologs [34], see Figure 2. In addition, FjTAL (tyrosine ammonia-lyase from *Flavobacterium*

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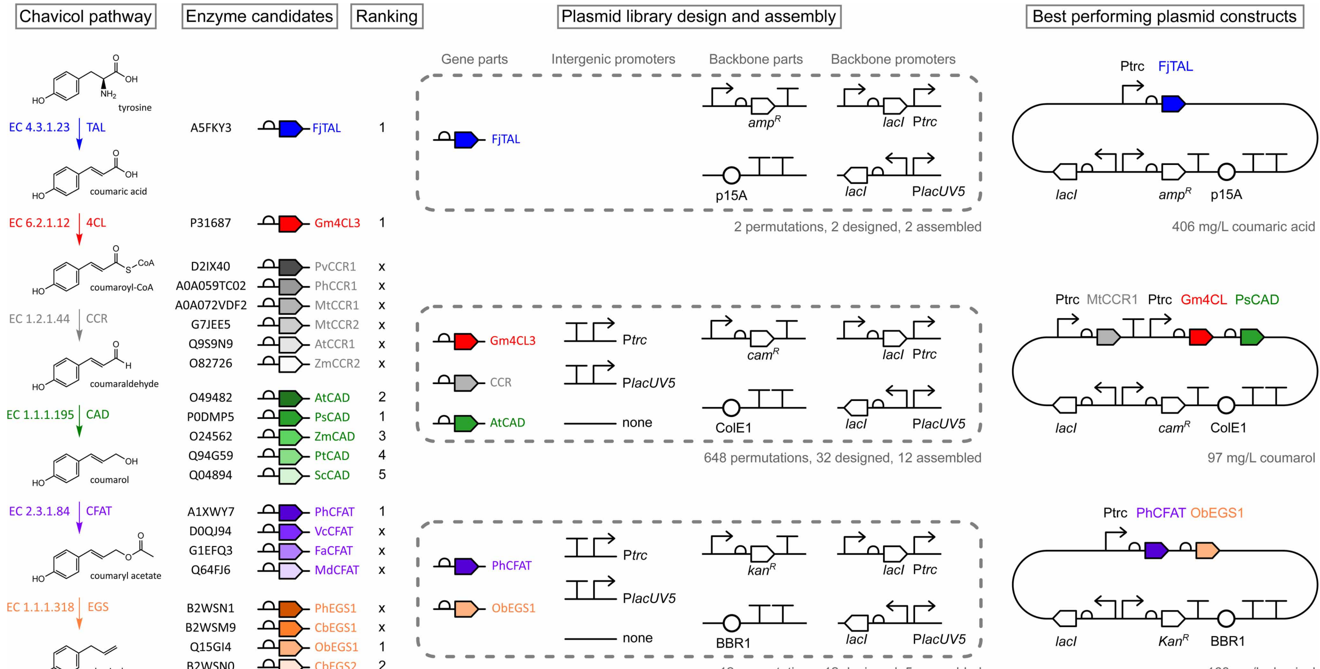

Figure 2. Prototyping a biosynthetic pathway to chavicol in *E. coli*.

RetroPath identified a 6-step pathway from native metabolism to chavicol (Enzyme Commission numbers listed). Enzyme abbreviations: tyrosine ammonia-lyase (TAL), 4-coumarate-CoA ligase (4CL), cinnamoyl-CoA reductase (CCR), cinnamyl alcohol dehydrogenase (CAD), coniferyl alcohol acyltransferase (CFAT), eugenol synthase (EGS). Selenzyme identified enzyme candidates, with 4–6 selected for each step (UniProt accession numbers listed). Efficient TAL and 4CL enzymes were already known from earlier studies in our laboratory [46]. Gene parts were designed with optimized codon usage and RBS sequences. *In vitro* enzyme screening ranked the activity of all candidates except CCR. The 6-step pathway was split into three modules, each producing a quantifiable intermediate. Plasmid libraries were designed using a DoE workflow to intelligently sample the combinatorial space of potential plasmid constructs. Replication origins and selectable markers were chosen to ensure compatibility in the same cell. Plasmids were screened for activity in *E. coli*, feeding respective substrates and monitoring products by MS. The best-performing plasmid constructs are shown, along with their product titers.

johnsoniae; [44]) and Gm4CL (4-coumarate:CoA ligase from *Glycine max*; [45]), were selected on the basis of prior enzyme screening for flavonoid production (3× TAL and 5× 4CL; [46]).

After evaluating the sequences of our chosen enzyme candidates, we proceeded to design our DNA gene parts [26]. Our final gene part designs are typically composed of a codon-optimized CDS and bespoke RBS sequence, flanked by custom-cloning tags that are removed during the assembly of final expression constructs. To assemble gene expression constructs, gene parts are combined with various gene regulatory parts and plasmid backbone parts. The *in silico* design of DNA parts can be completed in a single day by our BUILD workflow, these parts are then synthesized commercially (Twist Bioscience), which takes 3–6 weeks for delivery. Custom-cloning and transformation allows us to prototype our gene parts immediately, using our robotics platforms to inoculate and culture transformants at 1-ml scale in 96-deepwell blocks, and then induce enzyme expression at the target cell density. Enzyme screens are conducted with individual or pooled lysate samples, mixed with appropriate substrates and cofactor samples as required, using automated pipetting worklists. Through this process, our enzyme candidates are ranked for their ability to catalyze the target chemical transformation steps, within 5 days of receiving the gene/transformant samples. Enzyme rankings for the chavicol project are shown in Figure 2.

To prototype DNA construct performance in host strains we have a semi-automated TEST workflow. Once the starter cultures are grown, an automated method is used to normalize the optical density (OD) and then sub-culture into fresh media. Induced cultures are returned to the shaker-incubator and sampled at predefined time points, typically every 24 h. Harvested samples are processed using an automated method to measure final OD, quench growth, lyze cells and dilute clarified lysate in appropriate solvent for analysis by MS. The

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workflow for testing a batch of plasmid constructs is 4 days, from plasmid transformation to charting of analyte titers after 24 h. The LEARN workflow performs a regression analysis on the resulting experimental data to analyze the influence of each factor on relative and absolute titers. The next round of design is then defined based on the predicted titers according to a predictive model.

Our rapid prototyping project towards the production of materials monomers generated a set of functional plasmid constructs, which were *in vivo* validated in *E. coli*. However, these prototype producer strains will still require significant optimization to compete economically with established chemical synthesis routes. The first step should be to investigate further improvements to the plasmid constructs themselves. Several pathways were split into modules (three modules for chavicol, Figure 2) which should later be consolidated into a full pathway on a single plasmid vector, to relieve the burden of maintaining separate plasmids and associated antibiotic selection. Further enhancements in productivity could be achieved by a suitable combination of replication origins, and resistance markers, and by replacing inducible promoters with constitutive equivalents [47]. Similarly, the RBS sequences of genes can be adjusted to fine-tune enzyme levels across the pathway, balancing flux to optimize production using machine learning [48].

A further step towards enhancing target production involves metabolic engineering of the host chassis. There are established strategies for boosting the levels of many central metabolites in *E. coli*, for example, tyrosine [49], which is the substrate for the chavicol pathway. We enhanced tyrosine production 16-fold in *E. coli* DH5α (1.28 g/l of tyrosine, compared with 0.08 g/l for the wildtype strain), by deleting the *tyrR* (tyrosine repressor) and *pheA* (chorismate mutase/prephenate dehydratase) genes, and transforming this strain with a plasmid overexpressing *ppsA* (phosphoenolpyruvate synthase) and feedback-resistant mutants of key genes from the shikimate pathway [34]. We also genome-integrated this plasmid construct at the *lacZ* (β-galactosidase) gene locus of the DH5α double-knockout, although this reduced the tyrosine productivity compared with the plasmid-borne construct.

During our materials monomers project, we also investigated optimization and scale-up of mandelate production, a monomer for degradable thermoplastics with polystyrene-like properties [50]. Prototype production strains were developed at 1-ml scale, that yielded 170 mg/l of (*S*)-mandelate and 251 mg/l of (*R*)-mandelate. Subsequently, through a combination of pathway engineering and bioprocess optimization, we scaled-up to 1-l bioreactor cultures and enhanced mandelic acid titers to 970 mg/l (89% *S*-enantiomer) and 800 g/l (98% *R*-enantiomer) [46].

Perspectives

- Metabolic engineering technologies have been employed with increasing success over the last three decades for the engineering and optimization of industrial host strains to competitively produce high-value chemical targets.

- Continued reductions in the time taken from concept, to development, to scale-up are essential. DBTL pipelines that are able to rapidly deliver diverse chemical targets through iterative optimization of microbial production strains have been established.

- Successful examples of rapid design and prototyping processes for microbially produced compounds reveal the potential role of biofoundries in leading the sustainable production of next-generation bio-based chemicals.

Competing Interests
The authors declare that there are no competing interests associated with the manuscript.

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Author Contributions

C.J.R., J.T.L. and P.C. drafted the initial version of the work. All authors made substantial contributions to the conception of the work, or to the acquisition, analysis and interpretation of data for the work. All authors approved the final version.

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Abbreviations

AM, additive manufacturing; CAD, computer-aided design; DAMP, design, automation, manufacturing and prototyping; DBTL, Design–Build–Test–Learn; GBA, global alliance of biofoundries; MS, mass spectrometry; OD, optical density; RP, rapid prototyping.

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