Engineering biology for sustainable 1,4-butanediol synthesis

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Spotlight

Engineering biology for sustainable 1,4-butanediol synthesis

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Over the past decade, numerous approaches have been taken to replace unsustainable chemical syntheses with green biosynthetic alternatives. In a landmark paper, Yim *et al.* utilised an *in silico*-to-*in vivo* workflow to enable the high-level bioproduction of the unnatural small molecule 1,4-butanediol in the bacterium *Escherichia coli*.

Biocatalysis is a cornerstone of green chemistry positioned to help tackle the environmental impact of the modern chemical industry. Compared with traditional chemical synthesis, biocatalysis bypasses energy intensive reaction conditions, toxic solvents, and downstream processing requirements to facilitate greener synthetic processes [1]. Furthermore, when genetically encoded and interfaced with primary metabolic reactions within a microbial host, multistep chemical syntheses can be performed in one-pot via the fermentation of sustainable bio-based feedstocks, rather than from nonrenewable fossil fuels. Combined with the microbial recycling of 'waste' feedstock such as lignin and polyethylene terephthalate (PET) plastic into primary metabolic substrates, engineering biology for sustainable synthesis is kickstarting a Golden Age in industrial chemistry.

Previous work in the field targeted platform/bulk and fine chemicals that are known metabolites in nature by reconstituting and enhancing their native biosynthetic pathways in laboratory microbes. However, this approach is limited to a fraction of small molecules used by the chemical industry today and, therefore, engineering biological systems to produce valuable chemicals of non-natural origin is a critical challenge for the field moving forward.

Emerging strategies in this area include the use of refactored biosynthetic pathways, the evolution of enzymes to perform non-native chemistry, the computational design of new-to-nature enzymes, and the repurposing of non-enzymatic catalysts to operate in concert with engineered metabolic pathways (termed 'biocompatible chemistry'). A seminal example by Yim *et al.* stands as one of the earliest successes in this field, combining *in silico* pathway design with metabolic engineering to achieve high-level bioproduction of the unnatural chemical 1,4-butanediol (BDO) from glucose [2].

BDO is a prevalent petrochemical used to synthesise important materials (e.g., spandex) and industrial products (e.g., tetrahydrofuran) [3]. Yet traditional manufacturing routes rely on unsustainable fossil resources (acetylene or *n*-butane) and energy-intensive chemical processes. Engineering biology to enable BDO production from sustainable feedstocks by fermentation is therefore an important challenge.

The study began by identifying suitable 'reaction operators' using in-house SimPheny Biopathway Predictor software, resulting in ca. 10 000 feasible routes to BDO via known metabolic chemistry (not necessarily catalysed by known enzymes) from central metabolic intermediates. Iterative ranking based on attributes, including pathway length, number of non-native steps, unfavourable thermodynamics, and maximum theoretical yield, consolidated the available pathway designs to around 10% of the initial hits. Manual curation based on known enzymatic chemistry targeting the shortest number of steps from central metabolism led the authors to two pathway designs pivoting around the intermediate 4-hydroxybutyrate (4-HB), which could be subsequently reduced to BDO by two successive NAD(P)H dependent enzymatic reductions through 4-hydroxybutyryl-CoA. Two routes to 4-HB via succinyl semialdehyde were proposed: from succinate, via succinyl-CoA catalysed by a succinyl-CoA synthase and CoA-dependent dehydrogenase; and from α-ketoglutarate, catalysed by a α-ketoacid decarboxylase (Figure 1). The 4-HB biosynthesis and subsequent reduction pathways were constructed and validated as two separate 'modules' in different *Escherichia coli* MG1655 *lacI*$^{Q+}$ strains before being combined into a single strain. The result was 1.3 mM BDO after 40 h incubation, which, whilst low, marked the first instance of BDO biosynthesis entirely from central metabolites in a microorganism.

Competing pathways for carbon flux were eliminated using the OptKnock algorithm by identifying multiple knockouts (Δ*adhE*, Δ*pfl*, Δ*ldh*, and Δ*mdh*) that coupled maximum BDO production to cell growth, predicting 0.37 g/g yield under anaerobic conditions by blocking native fermentation pathways to ethanol, formate, lactate, and succinate and driving BDO production to balance cellular redox. In practice, the knockouts severely reduced cell fitness, with the Δ*adhE*Δ*pfl*Δ*ldh* intermediate strain unable to grow under anaerobic conditions; likely due to rediverted carbon flux through the native pyruvate dehydrogenase, which displayed reduced activity under the experimental conditions. Although overcome by replacing the limiting subunit (*lpdA*) with a functional homolog from *Klebsiella pneumoniae*, this barrier highlights a general limitation of *in silico* prediction models to account for off-pathway effects in complex metabolic systems yet how these can be mitigated via rational pathway engineering. The recovered strain displayed improved BDO production but only grew under microaerobic

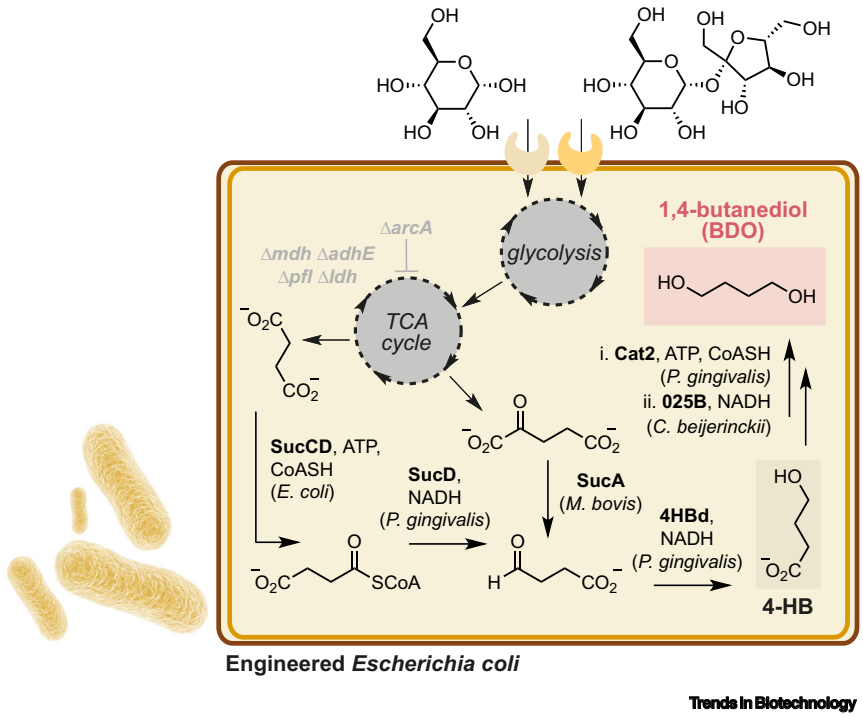

Figure 1. Metabolic engineering of *Escherichia coli* for sustainable 1,4-butanediol synthesis from carbohydrate feedstocks. Abbreviations: *C. beijerinckii, Clostridium beijerinckii; 4-HB, 4-hydroxybutyrate; M. bovis, Mycobacterium bovis; P. gingivalis, Porphyromonas gingivalis.*

conditions and produced significant quantities of unwanted side-products such as acetate, pyruvate, ethanol, stalled pathway intermediates, and γ-butyrolactone (GBL) formed by spontaneous cyclisation of 4-hydroxybutyryl-CoA. To address this, the authors prioritised flux through BDO synthesis by knocking out *arcA* and *mdh* to alleviate transcriptional repression of several aerobically expressed oxidative TCA cycle genes and block entry into the reductive TCA cycle, respectively. Aided by expression of an NADH-insensitive citrate synthase mutant GltA(R163L) to increase TCA cycle intermediates, the resulting strain achieved 95% carbon flux to BDO and a titre of ca. 13 mM after 40 h. A final evaluation of the engineered strain by pulse-labelling with ^{13}C-glucose indicated a late-stage 4-HB bottleneck resulting from inefficient alcohol and aldehyde dehydrogenase activity in the downstream pathway. A codon-optimised aldehyde dehydrogenase from *Clostridium beijerinckii* was implemented that displayed improved reduction of 4-HB-CoA to BDO in concert with endogenous *E. coli* reductase enzymes, with minimal competing background reduction of acetaldehyde to ethanol.

Finally, the authors expanded the pathway to encompass alternative carbohydrate feedstocks. By introducing a sucrose-utilisation operon into the *E. coli* genome, comparable BDO titres were achieved from sucrose, xylose, and impure mixed biomass hydrolysate, the latter providing attractive flexibility from a bioprocess perspective when considering the relatively high cost and environmental impact of using glucose as a feedstock at scale [4].

The final engineered strain produced 18 g/l BDO from 20 g/l glucose over a 5-day fermentation. This achievement at such an early stage in the development of metabolic engineering, synthetic biology, and industrial biotechnology for sustainable chemical synthesis is remarkable and sets a benchmark for what can be achieved through predictive *in silico* metabolic modelling, rational metabolic engineering, and *de novo* biochemical pathway design. Titres achieved here also eclipsed those reported for the overproduction of known metabolites in *E. coli*. For example, isoprene production was achieved in 2011 by fermentation of glucose at only 314 mg/l scale [5].

Continued efforts have built upon this seminal work to design and commercialise an *E. coli* strain capable of producing 125 g/l BDO from renewable feedstocks [6]. Industrial use required extensive host strain and pathway engineering (>50 further genetic edits), in addition to genetic reconfigurations essential for scale-up. Consistent with the original report, new modifications were introduced by rational design aided by ^{13}C flux and transcriptomic analyses. Knockouts Δ*sad*Δ*gabD* prevented flux moving back into the TCA cycle by limiting succinate semialdehyde conversion to succinate at high 4-HB concentrations, increasing BDO titres from 18 g/l to 29 g/l [7]. Further strain improvements focussed on resolving competing side reactions and sources of both rate-limitation and by-product formation. Flux analysis pinpointed a rate-limiting bottleneck in the pathway during the final reduction steps from 4-HB to BDO once optimisations reached an 80–90 g/l scale. As such, Ald and Cat2 enzymes were engineered via directed evolution to suppress competing Ac-CoA reduction pathways and overcome product inhibition at high BDO titres, respectively. Once implemented, the evolved enzymes reduced 4-HB titres by 75% whilst increasing BDO titres by 20% to 110 g/l [6]. To address by-product formation, *ppc* expression was increased to improve overall TCA cycle flux from pyruvate. Additionally, deleting ATP inefficient electron transport chain components

(Δ*ndh*Δ*appBC*Δ*cydAB*), thioesterases (Δ*ybgC*Δ*tesB*), and acetate kinase/phosphotransacetylase (Δ*ackA-pta*) genes decreased CO_2, GBL, and acetate formation, respectively. Such modifications were responsible for reaching a final BDO titre of 125 g/l.

To improve industrial uptake, a final reconfiguration of genetic parts was required to improve genetic stability and reduce processing costs. This was achieved by chromosomal integration of the plasmid-encoded pathway genes under constitutive promoters and the removal of unstable cryptic prophage. Further genetic stability was conferred by mutating phage-associated receptor genes (*tonA*/*lamb*) and removing duplicated genomic regions. This stabilised strain was then subjected to final growth media and bioprocess optimisations to minimise downstream processing steps, affording the final BDO production strain for commercialisation.

The success of this work serves as a milestone in the field of microbial biocatalysis and demonstrates the feasibility of engineered *de novo* biosynthetic pathways at scale. Together with continued enzyme discovery, increasingly rapid enzyme engineering, and synthetic biology methods, there is undoubtedly a bright future ahead for this field as the need and demand for more sustainable chemical manufacturing technologies becomes a reality.

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Declaration of interests

No interests are declared.

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