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# The bright frontiers of microbial metabolic optogenetics

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**Abstract**

In recent years, light-responsive systems from the field of optogenetics have been applied to several areas of metabolic engineering with remarkable success. By taking advantage of light's high tunability, reversibility, and orthogonality to host endogenous processes, optogenetic systems have enabled unprecedented dynamical controls of microbial fermentations for chemical production, metabolic flux analysis, and population compositions in co-cultures. In this article, we share our opinions on the current state of this new field of metabolic optogenetics.We make the case that it will continue to impact metabolic engineering in increasingly new directions, with the potential to challenge existing paradigms for metabolic pathway and strain optimization as well as bioreactor operation.

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Metabolic engineering, Optogenetics, Cybergenetics, Biosensors, Control, Dynamic control of microbial fermentations: Design build test learn cycle, DBTL, Multivariate modular metabolic engineering, MMME.

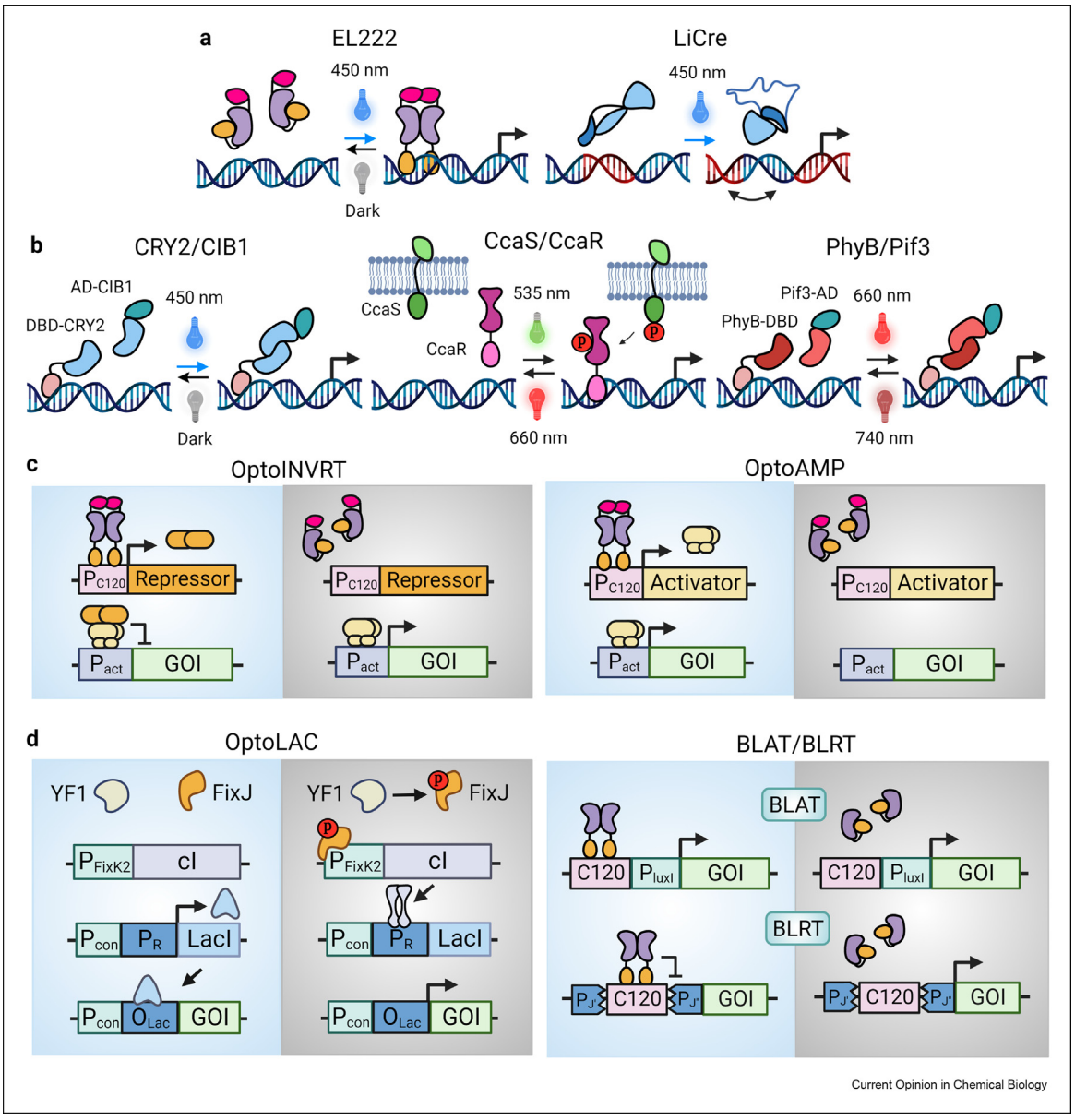
## Introduction

Metabolic engineering aims to improve cellular phenotypes by modifying metabolic pathways, often associated with the overproduction of desired chemicals or consumption of specific substrates. The purpose of metabolic engineering is largely to address challenges in renewable energy and sustainable manufacturing by enabling bioprocesses for the production of fuels, chemicals, and materials that are currently extracted from petroleum, plants, or animals [1]. Moreover, there is great potential to enable the synthesis of products with novel properties, by engineering pathways or enzymes to catalyze new reactions [2,3]. Thus, metabolic engineering enhances our ability to produce value-added products in processes that are compatible with current energy and environmental goals.

In recent years, optogenetics has been applied to metabolic engineering with transformative impact. Optogenetics is a technique that uses light-responsive proteins to control protein and cellular functions. Using light as an inducible agent offers several advantages: light is highly tunable, reversible, and orthogonal to biological processes (it has low toxicity, it cannot be metabolized, and causes little to no side effects in most non-photosynthetic organisms), and it is easy to interface with computers for automated control. These advantages have been demonstrated in the application of various optogenetic systems to control microbial physiology, gene expression, growth, cooperative dynamics, and engineered metabolic pathways [4].

Most applications of optogenetics to metabolic engineering involve light-controlled transcription, which can be classified based on the number of components involved in light reception and signal transduction. A one-component system commonly used in metabolic engineering is the EL222 protein from *Erythrobacter litoralis*, which reversibly dimerizes in blue light (450 nm) causing it to bind to a specific DNA sequence (called C120) [5]. Fusing EL222 to VP16 activation domain allows the induction of gene transcription with light [5,6] (Fig. 1a). EL222 has also been used as a light-dependent transcriptional repressor in bacteria, in which binding to a promoter containing an EL222 binding site prevents RNA polymerase association [7,8]. A popular two-component system used in metabolic engineering is the CcaS/CcaR system from *Synechocystis sp.*, in which the membrane-bound green (535 nm) light-activated and red (670 nm) light-inactivated CcaS histidine kinase regulates the phosphorylation state and thus the activation of the transcription factor CcaR (Fig. 1b), [9].

**Figure 1**




Representative optogenetic systems used in microbial metabolic engineering. **(a)** Examples of single component systems: transcription factor EL222 (purple and orange) fused to VP16 activation domain (pink), used for branched chain alcohol production [21], and LiCre recombinase used for beta-carotene production [10]. Both comprise a light-sensitive LOV domain, which upon exposure to blue light undergoes a conformational change that alters protein activity. **(b)** Examples of two-component systems (CRY2/CIB1, CcaS/CcaR, and PhyB/Pif3) in which pairs of proteins mediate the response to light of different wavelengths, resulting in transcription from a cognate promoter. **(c)** Yeast optogenetic circuits. Inverter circuits (OptoINVRT) use VP16-EL222 to induce in blue light expression of transcriptional repressors of genes of interest (GOIs), which are thus only induced in the dark. Amplifier circuits (OptoAMP) use VP16-EL222 to induce in blue light expression of strong transcriptional activators of GOIs. **(d)** Bacterial optogenetic circuits. In the BLAT/BLRT systems [7], the position of the C120 binding site dictates whether EL222 will behave as a transcriptional activator ($P_{C120}$) or repressor ($P_J$), as EL222 binding within the conserved –35/-10 region prevents RNA polymerase recruitment. In OptoLAC, the reversibly blue-light-regulated YF1 kinase controls the phosphorylation of the FixJ transcription factor, allowing for darkness-induced expression of GOIs. When dephosphorylated (in blue light), FixJ is unable to transcribe cI repressor, which controls the expression of the lacI repressor controlling GOIs, resulting in repression of GOIs. When phosphorylated (in the dark), FixJ activates transcription of cI, which represses lacI expression, thus de-repressing GOIs.

Numerous other optogenetic transcriptional controls exist, which respond to different wavelengths of light through a variety of mechanisms. Examples of such controls include light-induced DNA recombination by the Cre recombinase-AsLOV2 chimera LiCre [10] (Fig. 1a), and light-induced heterodimerization of functional transcription factors, such as Cry2/CIB1 [11] (activated by blue light) or PhyB/Pif3 [12] (activated

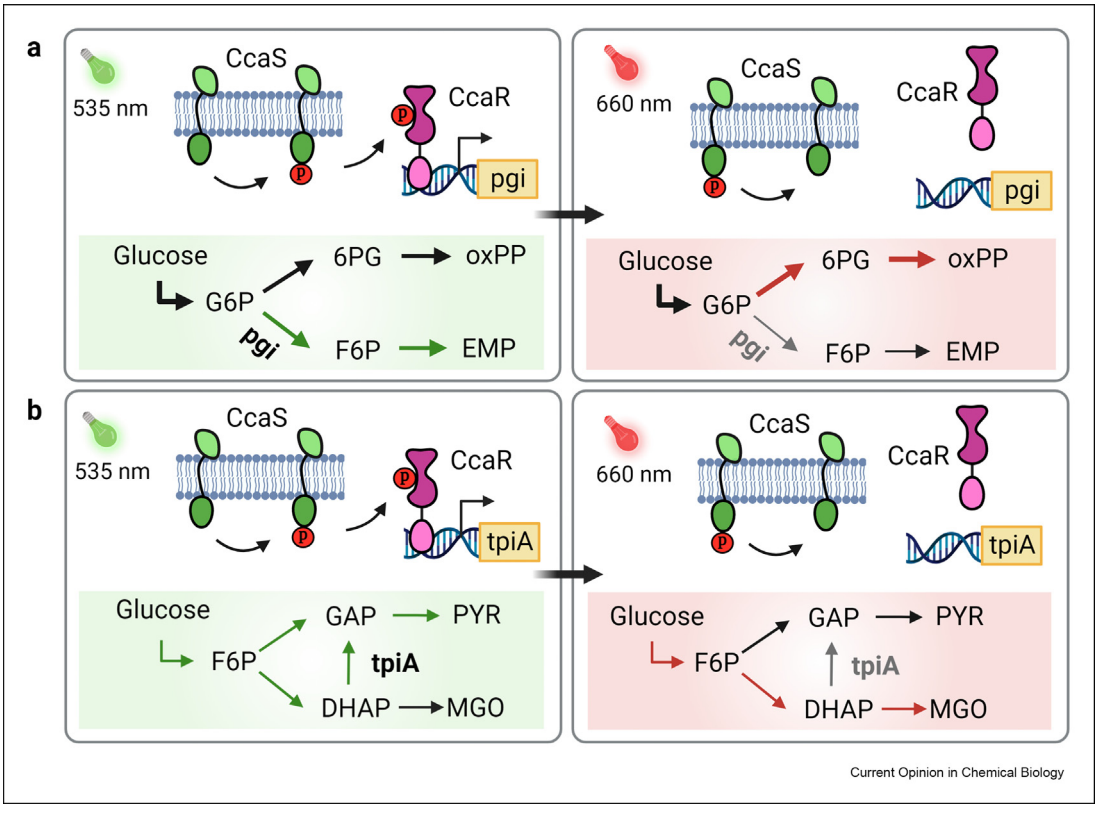
and inactivated by red and near-infrared light, respectively) (Fig. 1b). These optogenetic systems and others that act post-translationally have been thoroughly reviewed elsewhere [4,13,14]. In this opinion article, we discuss the major contributions that optogenetics has thus far made to metabolic engineering, and our vision for how the unique capabilities of light controls could further transform the field.

## Optogenetics for dynamic control of microbial fermentations

When product formation is not associated with cell growth, microbial fermentations are often divided into two temporal phases: one dedicated to biomass accumulation and the other to chemical production. This two-phase fermentation strategy alleviates metabolic competition between cell growth and product synthesis, leading to increased strain stability as well as higher productivities, yields, and titers. The transition from growth to production is traditionally induced with chemical agents and less frequently with changes in nutrients, temperature, or pH [15]. Alternatively, fermentations can be automatically transitioned from growth to production by autoregulatory systems based on mechanisms such as quorum-sensing or metabolite-induced transcription of biosynthetic pathways [16]. While these methods have been successful for many years, they do have some limitations. They can be difficult or impossible to tune or reverse, especially in batch or fed-batch fermentations. Additionally, their interactions with endogenous systems can cause side effects that limit maximal induction. Finally, in autonomous systems, it can be difficult to optimize the timing of induction independently of the strength of induction [17].

Optogenetics overcomes these limitations by enabling new modalities of dynamic control, in which light or darkness act as inducible agents [18]. The first demonstration of optogenetics in metabolic engineering exploited several advantages of light induction, using optogenetic inverter (OptoINVRT) circuits derived from VP16-EL222 (Fig. 1c) to control growth and chemical production in *Saccharomyces cerevisiae* [6]. The

**Figure 2**




Optogenetic control of *E. coli* central carbon metabolism using CcaS/CcaR. **(a)** Control of *pgi* expression regulates carbon flux toward either the Embden-Meyerhof-Parnas (EMP) pathway or the oxidative pentose-phosphate pathway (oxPP). Green light, by inducing expression of *pgi*, enables similar fluxes between the two pathways, while red light, by repressing *pgi*, inhibits flux toward the EMP pathway, and shunts carbon toward the oxPP pathway. **(b)** Control of *tpiA* expression regulates flux between glycolysis and methylglyoxal (MGO) biosynthesis. Green light induces expression of *tpiA*, which converts dihydroxyacetone phosphate (DHAP) to D-glyceraldehyde 3-phosphate (GAP) directing flux toward glycolysis, but under red light the majority of DHAP is instead converted to MGO. Additional abbreviations: glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), pyruvate (PYR), and 6-phosphogluconate (6PG).

high tunability and reversibility of light inputs made it possible to regulate the growth and production of lactic acid or branched-chain alcohols (isobutanol and 2-methyl-1-butanol) by balancing redox cofactors ($NAD^+$/NADH) using periodic light pulses throughout fermentation, which resulted in the production of 10.9 ± 0.4 g/L of branched-chain alcohols in lab-scale bioreactors. Similar capabilities have been developed in *Escherichia coli* using a light-controlled LAC operon, which improved isobutanol and mevalonate production in light-controlled fermentations by 27% and 24%, respectively, relative to optimal IPTG induction [19]. Additional systems that use light to control microbial fermentations are now proliferating, including some that use photoactivated recombinases [10], split RNA polymerases [20], and CRISPR-Cas9 [8] described in more detail in a recent review [14].

A major concern in scaling up optogenetically controlled fermentations is the potential for light penetration to become limiting in large industrial bioreactors operating at high cell densities. This challenge has not yet been observed in lab-scale bioreactors of up to at least 5L, which have been successfully controlled with light even at cell densities of up to at least 50 $OD_{600}$ [6,21]. Nevertheless, in preparation for further scaleup, optogenetic circuits have been developed that invert or amplify the transcriptional response to light. OptoINVRT circuits have been developed in yeast by harnessing the galactose or quinic acid regulons, in which VP16-EL*222* is used to induce expression of their corresponding repressors, *GAL80* or QS, in blue light [22,23] (Fig. 1c). Placing metabolic pathways under the control of these repressors effectively turns light into an inhibiting agent used during the growth phase, when low cell densities do not hinder light penetration, and darkness into the inducing agent for the production phase, at which point the opaqueness of high cell densities become irrelevant. The bacterial optogenetic lac operon (OptoLAC) is also an inverter circuit, in which the two-component pDawn system [24] is used to induce expression of the lacI repressor with blue light (Fig. 1d), thereby inducing pathways under its control with darkness [19]. Similarly, the blue light repression tool (BLRT) [7] uses EL*222* to directly repress GOIs in blue light and allow their preferential expression in the dark (Fig. 1d).

Optogenetic amplifier (OptoAMP) circuits have also been developed in yeast using the same galactose and quinic acid regulons and light hypersensitive mutants of EL*222* [21,23]. In this case, VP16-EL*222* is used to control the strong transcriptional activators *GAL4* or QF2, which in turn activate the pathways of interest in blue light (Fig. 1c). These amplifiers can achieve 23-fold stronger induction than VP16-EL*222* alone and fully respond to light duties cycles as low as 1% of maximal illumination in lab-scale bioreactors at cell densities approaching 50 $OD_{600}$[21]. Challenges of light penetration can also be addressed using photobioreactors designed for algae and cyanobacteria cultivation. As light requirements for optogenetic systems are substantially lower than for the metabolic needs of photosynthetic organisms, these bioreactors may be sufficient to scaleup light-controlled fermentations [25]. It is encouraging that the first applications of optogenetics in metabolic engineering were for dynamic control of fermentations for chemical production, including in bioreactors, as these early demonstrations set a solid foundation for applying optogenetics in other areas of metabolic engineering.

## Optogenetics to study and optimize metabolic pathways

A powerful method to study metabolism is through metabolic flux analysis (MFA), which could be greatly augmented with optogenetics. MFA uses mass spectrometry to measure concentrations of relevant metabolites to determine the fluxes, inefficiencies, bottlenecks, and energetics of metabolic pathways. This information serves as a guide to construct and improve production strains. MFA also provides a basic understanding of metabolic network dynamics and how the expression of certain genes affects flux across metabolic modules, which may be incorporated into genome-scale models [26,27]. Optogenetics can augment MFA by providing the ability to control with high specificity the levels of expression of metabolic enzymes and measuring the effects on metabolic fluxes. This takes advantage of not only light's tunability but also its orthogonality, which avoids the side effects of chemical inducers or gene deletions and facilitates the probing of essential enzymes that cannot be deleted.

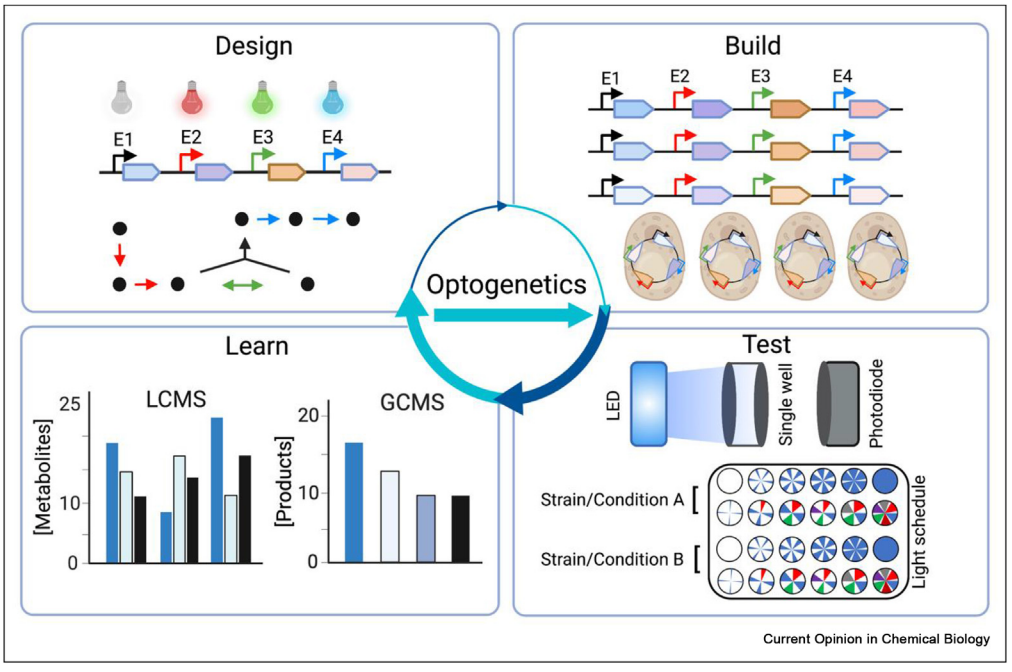
These capabilities were first demonstrated using CcaS/CcaR to control the expression levels of *pgi*, thereby varying the flux ratios between two glycolytic pathways: the Embden-Meyerhof-Parnas (EMP) and oxidative pentose-phosphate (oxPP) pathways (Fig. 2a) [28]. This achieved control over EMP:oxPP metabolic flux ratios ranging from 50:49 to 0.5:99 by exposing cells to green or red light, respectively, which is an improvement from the dynamic range previously obtained with IPTG induction [29]. This research can advance our understanding on how metabolic flux distribution between these two pathways affects the biosynthesis of highly reduced products, such as mevalonate, fatty acids, and isoprenoids, which require both the reducing power provided by oxPP and the acetyl-coA provided by EMP. In a separate study, CcaS/CcaR was used to vary the flux distributions between glycolysis and methylglyoxal (MGO) (Fig. 2b) [30]. Repressing *tpiA* with red light increases MGO production four-fold, but also reduces growth rate by ~33%. These pioneering studies demonstrate that optogenetics can be used to control major metabolic pathways for MFA studies, achieving finely timed variations of metabolic fluxes and higher dynamic ranges than traditional methods.

In future applications, optogenetics will likely have a significant impact on metabolic pathway optimization and even challenge current paradigms for strain development. Prevailing strategies to optimize pathways and strains use iterative Design−Build−Test−Learn (DBTL) cycles typically in combination with multivariate modular metabolic engineering (MMME) (Fig. 3) [31−33]. However, the large design space needed to thoroughly screen even relatively short pathways often requires the costly and time consuming construction of very large libraries [33,34]. While these projects are facilitated by proliferating biofoundries [34], these facilities are still difficult to access for most academic and small company labs.

The unique advantages of optogenetics could substantially simplify MMME implementation while augmenting its potential, leading us to rethink the DBTL model. Instead of modulating enzymatic activities through laborious and costly construction of combinatorial libraries, a subset library of optogenetically controlled enzymes or modules could be built. Although smaller, these libraries could sample a broad distribution of enzyme expression levels by systematically varying the schedules and wavelengths of light [6,11,35,36]. Strains and optogenetic schedules could be screened in multi-well plate formats using genetically encoded biosensors, colorimetric assays, or optical density to monitor cell growth. Optogenetically controlled strains were recently combined with genetically encoded biosensors to enable high throughput screening using fluorescence-activated cell sorting (FACS), demonstrating the feasibility of this approach [37]. Overall, adding optogenetic components to the DBTL framework would allow multiple Test and Learn cycles before a new Design and Build cycle is required, thus expediting and reducing the cost of strain development (Fig. 3).

The feasibility of this concept was recently demonstrated. Dual optogenetic modulation of linalool versus geraniol synthesis in yeast using simultaneous amplification and inversion of light inputs was used to tailor the relative production of these monoterpenes [23]. Lessons learned from optogenetic DBTL cycles could be applied to production strains for conventional fermentations using constitutive promoters benchmarked against different light doses. Alternatively, strains optimized through these cycles could be used directly in optogenetically controlled fermentations in which the optimization of light schedules becomes an empowering feature of the scaleup process [6]. In a recent proof-of-principle study, OptoAMP circuits enabled fermentations of three temporal phases (growth, induction, and production), in which lactic acid, isobutanol, or naringenin production was improved by optimizing the light schedules in each phase, which were different for each metabolic pathway [21]. Optogenetics could further

**Figure 3**




The potential impact of optogenetic MMME on the DBTL cycle. Optogenetics could significantly simplify MMME by reducing the number of constructs that need to be designed and constructed, which would then be screened over different light schedules of different wavelengths to explore a vast space of metabolic conditions. This would dramatically shift the focus of the DBTL cycle to multiple iterative Test-Learn cycles before new Design−Build steps are conducted if necessary.

augment MMME by allowing dynamic control over gene expression during the fermentation screens, adding multiple new dimensions in which to optimize pathways and strains.

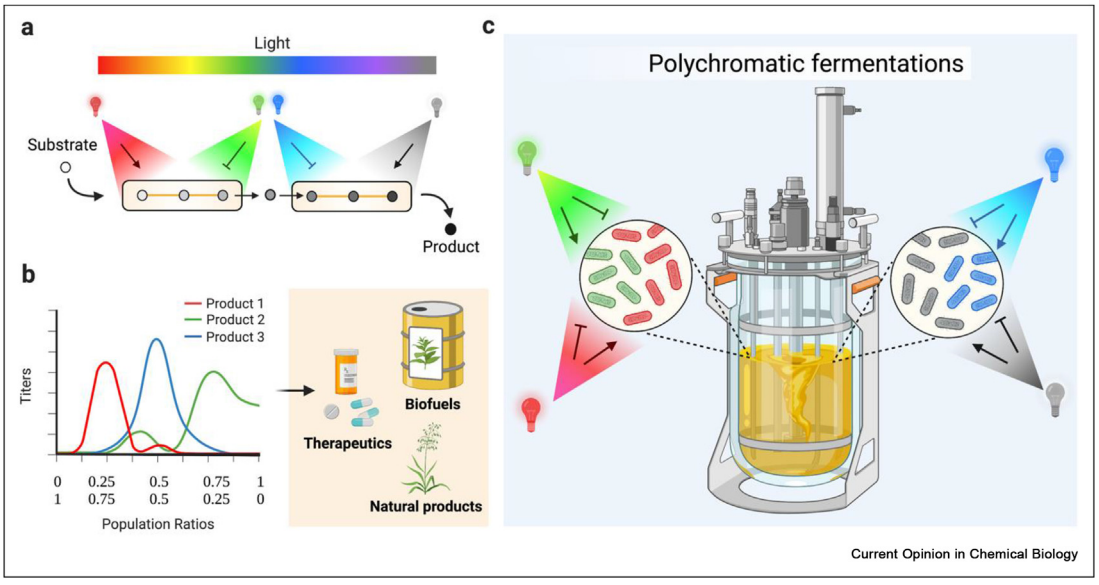
## Optogenetic control of co-culture fermentations

There is enormous potential in using microbial co-cultures to improve chemical production. Distributing engineered biosynthetic pathways across multiple microbial strains offers several advantages which have been thoroughly reviewed [32,38,39]. These include reduced metabolic burden, crosstalk, and toxicity, as well as the ability to optimize pathways in individual modules and take advantage of special capabilities of different microbial species. However, the full potential of co-culture fermentations has not been realized because of difficulties in stabilizing microbial consortia and optimizing their populations for maximal chemical production. Current strategies to stabilize consortia typically involve manipulating inoculation ratios, growth media, and culture conditions [32,40]. More elegant solutions have been devised, for example, by engineering co-dependence between *E. coli* and *S. cerevisiae* for the production of oxygenated taxanes and other terpenoids, in which bacteria assimilate the only carbon source provided, while yeast detoxify the media of acetic acid produced by the bacteria [41]. Even though such sophisticated engineered symbioses are effective at stabilizing consortia (preventing the depletion of any member from the culture), they are still not effective at optimizing population compositions for maximal chemical production.

Optogenetics can not only stabilize microbial consortia but also has the potential to dynamically control their subpopulations to maintain optimal compositions throughout fermentations (Fig. 4). A proof-of-principle study showed that co-cultures of *E. coli* and *S. cerevisiae* can be controlled by using blue light to regulate the growth rate of *E. coli* alone, as bacteria grow significantly faster than yeast [42]. This was achieved by controlling the toxin/anti-toxin pair mazF/mazE with an optogenetic circuit, called OptoTA [42], derived from pDawn/pDusk [24]. Co-culture fermentations with optogenetically controlled populations achieved significantly higher levels of isobutyl acetate and naringenin production in a wider range of inoculums than uncontrolled consortia.

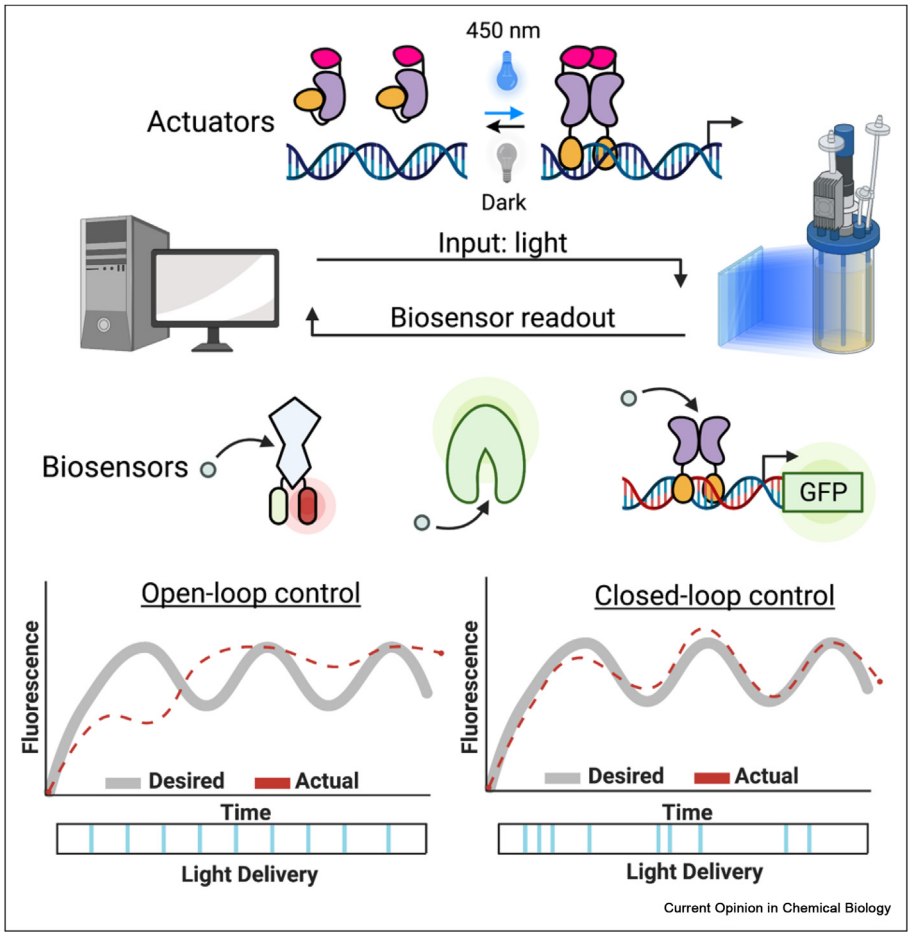
Additional strategies have been developed to control the growth rates of yeast and bacteria with light [6,7,35,43,44], which could be adapted to control microbial consortia. One prominent example used blue (Fig. 1d) and near-infrared light-activated systems (BLAT and NRAT) in *E. coli* to control ribonucleotide reductase NrdA/NrdB and division regulatory proteins FtsZ, FtsA, and SulA, thereby controlling cell cycle progression and cell division [7]. While this strategy was used to improve the production of acetoin and poly(-lactate-co-3-hydroxybutrate) with monocultures, it could also find applications in controling microbial

**Figure 4**




Polychromatic control of microbial co-culture fermentations to produce valuable products. (**a**) Biosynthetic pathways divided into metabolic modules engineered across different strains grown in consortia could be optimized using polychromatic optogenetic controls of co-culture fermentations. (**b**) The population composition of microbial co-cultures could be optimized for maximal chemical production using light to control the relative growth rates of different strains. (**c**) Polychromatic controls could be used to control microbial consortia composition in bioreactors, in which different wavelengths induce or repress the growth rate of different strains in co-cultures.

**Figure 5**




Basic molecular components for metabolic cybergenetics. Genetically encoded biosensors respond to the metabolic state or pathway of interest in the cell by transmitting a fluorescence signal by a variety of mechanisms [25]. This signal can be detected and fed via a computer to a controller which could then inform the operation of LEDs illuminating the bioreactor, modifying light intensity and/or periodicity to achieve or maintain desired setpoints. This form of closed-loop control, wherein the actuator activation can be changed in real-time, would facilitate the operation, optimization, automation, and reproducibility of fermentations.

consortia populations. Mixed populations of *S. cerevisiae* have also been controlled by light-induced cell differentiation, in which VP16-EL222 was used to induce Cre-recombinase to rearrange gene expression cassettes. With this strategy, mixed populations of defined composition could be maintained for as long as 48 h [45]. Even though these mixed cultures were not used for chemical production, this approach could be applied in co-culture fermentations in which cell differentiation implies the expression of different metabolic modules. In principle, any other current or future optogenetic system able to control cell growth or differentiation could be used to stabilize and optimize microbial consortia for chemical production with light.

## Metabolic cybergenetics

Cybergenetics is a growing field that seeks to apply computer-assisted control to biological systems using control theory. This involves implementing actuators that enable the control of measurable physiological outputs, such as optical density or fluorescent protein expression. These outputs are used as feedback to the actuators to achieve a desired set point. The ease with which light inputs can interface with computers makes optogenetics an ideal strategy to implement biological actuators. Real-time optogenetic control of fluorescent protein production and cell growth has been established in both *S. cerevisiae* and *E. coli* [46–48]. Using CcaS/CcaR, it was possible to establish model predictive control (MPC) over protein production in *E. coli*, which dramatically outperformed open-loop or proportional-integral (PI) control systems [47]. Conversely, for CcaS/CcaR control of bacterial growth through methionine synthesis regulation, simple PI controllers were sufficient to maintain a desired setpoint of optical density [47].

A recent opinion article from our group focused on how these capabilities could be extended to launch a new

field in metabolic cybergenetics [25]. Optogenetic actuators could be paired with genetically encoded biosensors that report on relevant metabolic states or activities of the cell. By interfacing biosensor readout detectors and LED actuators through computers during microbial fermentations, it would be possible to maintain a predetermined metabolic setpoint that maximizes chemical production (Fig. 5). Indeed, several biosensors have been developed for different metabolic pathways and products of interest, as well as for key cofactors and metabolites [49–52]. Although metabolic cybergenetics has not yet been demonstrated, a biosensor for isobutanol production was recently coupled with optogenetic controls of isobutanol biosynthesis in yeast, showing that both genetic programs can functionally coexist in the same strain without significant burden [37]. This raises many opportunities to combine existing and future biosensors with the growing number of optogenetic controls for metabolic pathways to drive the development of metabolic cybergenetics.

While there is great potential in metabolic cybergenetics, formidable challenges will need to be overcome to realize it. Perhaps the greatest challenge is coping with the significant delays that exist in both optogenetic actuators and biosensors, particularly those based on transcriptional processes, which is the majority. The development of optogenetic circuits (OptoINVRT7 and OptoQ-INVRT4) with improved kinetics, which reduces time delays from several hours to ~30 min or less is very promising. However, post-translational optogenetic controls, such as light-induced protein phase transitions for metabolic pathway clustering [53] or optical binders [54,55], which act on a much faster timescale, could hold the key to implementing effective actuators for metabolic cybergenetics. Much less progress has been made for biosensors, given that their traditional use in high-throughput screens does not require fast-acting sensing. An additional challenge for biosensors is the need for them to be predictive throughout fermentation, as opposed to only during certain phases of cell growth, although some progress has been made in this direction [56]. While many improvements are clearly needed, the rapid progress in the fields of biosensors, cybergenetics, and metabolic optogenetics bode well for the future of metabolic cybergenetics [49,57].

In addition to molecular and cellular improvements, there is growing availability of instrumentation that could assist in boosting the development of metabolic optogenetics and cybergenetics. Low-cost microwell plate readers and optogenetic microwell plates equipped with multi-color LEDs [58,59] have been combined to build optical plate readers for high-throughput optogenetic and cybergenetic experiments [60]. Additionally, mini-bioreactors such as the eVOLVER [61] and Chi.Bio [62] systems with several controls and detectors have become available, which could be applied to metabolic cybergenetics. Successful implementation of metabolic cybergenetics in industrial fermentations would bring to biomanufacturing all the benefits of process control, such as automation, optimization, and batch-to-batch reproducibility, which have been essential to the success of the chemical industry.

## Conclusions

Optogenetics has many advantages for controlling microbial metabolic pathways, given light is readily reversible and almost infinitely tunable across intensity, periodicity, wavelength, and time. Furthermore, light exhibits low toxicity and is orthogonal to the physiology of non-photosynthetic microbial hosts [63].

These unique capabilities give optogenetics enormous potential to impact many areas of metabolic engineering. The potential applications range from light-controlled fermentations of monocultures or co-cultures for chemical production, to pathway evaluation and optimization through light-controlled MFA and MMME, respectively. Even the most ambitious bioprocess control, automation, and optimization of fermentations, obtained through metabolic cybergenetics, are now within the realm of possibility. Microbial optogenetics continues to advance, with constant developments of new and improved tools, discoveries of new proteins responsive to different wavelengths of light, and demonstrations of multichromatic controls over microbial physiologies [12,64–66]. As more metabolic engineers realize the power of this technology, we expect the number of applications of metabolic optogenetics in the field will grow to further explore this bright new frontier.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:
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** of outstanding interest


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This was the first application of optogenetics for dynamic control of microbial chemical production. Direct (OptoEXP) and inverter (OptoINVRT) circuits were developed in *S. cerevisiae*, using VP16-EL222, to control cell growth (in light) and chemical production (in darkness), achieving record-breaking levels of branched chain alcohol production.

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This paper demonstrated a novel application of optogenetics for microbial chemical production by manipulating cell division. Using multichromatic optogenetic systems it was shown that shortening or prolonging cell division improved chemical production of acetoin and poly(lactate-co-3-hydroxybutyrate), respectively.

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This study reports the development of an optogenetic CRISPRi system, which was used to repress central carbon metabolism in *E. coli* to improve the production of muconic acid. Multiplex sgRNA targeting was shown to be additive and useful for large-scale CRISPRi induction for microbial chemical production.

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