

Optogenetic Control of Microbial Consortia Populations for Chemical Production

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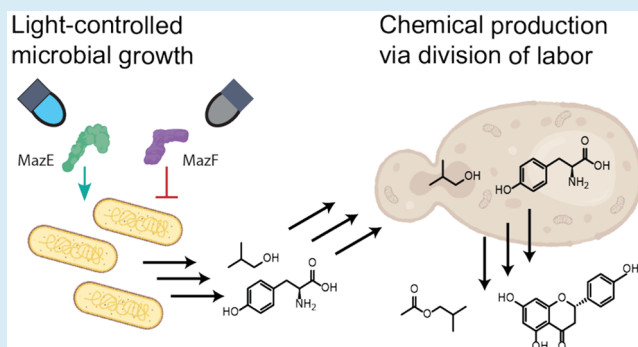
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ABSTRACT: Microbial co-culture fermentations can improve chemical production from complex biosynthetic pathways over monocultures by distributing enzymes across multiple strains, thereby reducing metabolic burden, overcoming endogenous regulatory mechanisms, or exploiting natural traits of different microbial species. However, stabilizing and optimizing microbial subpopulations for maximal chemical production remains a major obstacle in the field. In this study, we demonstrate that optogenetics is an effective strategy to dynamically control populations in microbial co-cultures. Using a new optogenetic circuit we call OptoTA, we regulate an endogenous toxin-antitoxin system, enabling tunability of *Escherichia coli* growth using only blue light. With this system we can control the population composition of co-cultures of *E. coli* and *Saccharomyces cerevisiae*. When introducing in each strain different metabolic modules of biosynthetic pathways for isobutyl acetate and naringenin, we found that the productivity of co-cultures increases by adjusting the population ratios with specific light duty cycles. This study shows the feasibility of using optogenetics to control microbial consortia populations and the advantages of using light to control their chemical production.

KEYWORDS: optogenetics, co-culture fermentations, metabolic engineering, microbial consortia, dynamic control



Metabolic engineering aims to rewire the metabolism of microorganisms to convert simple feedstocks into products of interest.¹ Dynamic control,² pathway compartmentalization,³ and adaptive laboratory evolution⁴ have helped to further improve microbial chemical production in engineered strains. However, overburdening an organism with genetic modifications can lead to growth defects and loss of productivity.⁵ Additionally, maximizing metabolic flux through a pathway of interest often requires bypassing endogenous regulatory mechanisms,⁶ functionally expressing heterologous proteins (including enzymes and transporters),⁷ or avoiding intermediate loss to competing pathways by deleting endogenous genes.⁸ When such interventions to enhance a particular step of a pathway undermine others, necessary compromises may lead to pathway inefficiencies. For example, when an enzyme important for one step of the pathway also competes in another step to make a byproduct, it may not help to overexpress or delete the enzyme.⁹ Another frequent challenge is the ability to functionally express some heterologous enzymes in organisms that otherwise have important advantages to produce a specific chemical. For example, the difficulty of expressing some eukaryotic enzymes in *E. coli* undermines the extraordinary ability of this organism to produce many precursors.¹⁰ These often-encountered challenges limit strain titers, yields, and productivities.

A solution that has been proposed to overcome these challenges is to separate and distribute metabolic roles among different members of a microbial consortium.¹¹ Natural microbial consortia have been used, in some cases for millennia, to produce a wide variety of products, notably in the food and bulk chemical industries.¹² Metabolic engineers have thus spent significant effort in engineering synthetic microbial consortia for chemical production. Co-culturing strains or organisms with specialized roles can improve productivity by reducing the total resources or genetic modifications required of each member. Furthermore, biosynthetic pathways can be split into submodules and be optimized based on the unique advantages of different species while avoiding endogenous regulation and competing pathways. Engineered microbial consortia have been applied to divide and optimize biosynthetic pathways within separate strains or species, leading to more efficient production of compounds such as muconic acid (by overcoming secretion of

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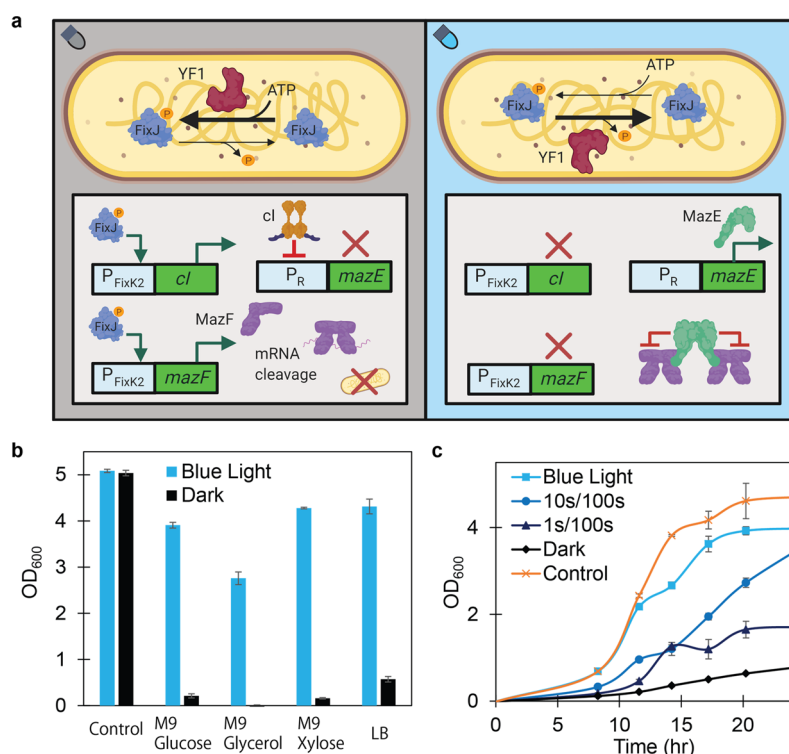


Figure 1. OptoTA enables blue light-dependent bacterial growth. (a) OptoTA uses the pDusk and pDawn systems to control growth of *E. coli* using the *mazEF* toxin-antitoxin system. Growth is suppressed in the dark (left panel) via pDusk expression of *mazF* leading to mRNA degradation. Growth is enabled in blue light (right panel) through pDawn expression of *mazE*, which inhibits MazF, and ceased expression of *mazF*. *E. coli* cells and protein shapes were created using Biorender.com. (b) Growth of *E. coli* strain containing OptoTA (EMAL144) in M9 medium with glucose, glycerol, or xylose, or LB in blue light or darkness, compared to a control strain lacking OptoTA (EMAL221) grown in M9 medium with glucose. (c) Growth time course of EMAL144 in M9 medium with glucose under different light conditions: full light (square), 10 s ON/90 s OFF (circle), 1 s ON/99 s OFF (triangle), and full darkness (diamond), compared to EMAL221 (Control, X) grown in ambient light. All data are shown as mean values; error bars represent the s.d. of $n = 3$ biologically independent samples.

an intermediate), resveratrol (by avoiding downregulation of an intermediate pathway step), and rosmarinic acid (by suboptimization of pathway modules).^{13–15} They have also been utilized in consolidated bioprocessing approaches to more efficiently break down complex feedstocks, such as lignocellulosic biomass, into biofuels such as ethanol, butanol, and isobutanol,^{16–18} while avoiding the increases in production time, process complexity, and sterilization costs introduced by two-stage bioprocesses.¹⁹ Such studies demonstrate the benefits of combining the strengths of specialized strains or species to perform complex tasks.

Despite this progress made in engineering microbial consortia, maintaining population stability has remained an obstacle to their more widespread use and application in commercial processes. There is a natural propensity for the fastest growing member of a co-culture to eventually outcompete the others and establish a monoculture. The most common approach to address this problem is to adjust the initial inoculation sizes of each member to ensure they are all maintained throughout the fermentation.^{18,20} However, variations at inoculation (such as deviations in preculture age or measurement errors), amplified throughout fermentation, can lead to batch-to-batch inconsistencies. Furthermore, this strategy does not provide the opportunity to adjust microbial populations once the fermentation is initialized, which could help offset these variations. Clever solutions have been found to ensure all members of engineered co-cultures are maintained during fermentations by engineered neutrality or

symbiosis: for example, by making strains reliant on different carbon or nutrient sources,¹³ modifying them to be co-dependent so that one species cannot grow without the other(s),²¹ or using quorum sensing to regulate growth via interpopulation communication.²² While these strategies have been largely successful at stabilizing members of engineered consortia, they still require careful tuning of parameters such as inoculation size and media components, which cannot be easily altered mid-fermentation. Furthermore, the population distributions established by these strategies are determined by the properties of each system and cannot be dynamically controlled to find, achieve, and maintain varying optimal population ratios to maximize chemical production.

We propose that optogenetic control of microbial growth offers a promising solution for optimizing co-culture fermentations. Light provides a tunable and reversible method of regulating gene expression, and has previously been applied to dynamically control and optimize monoculture fermentations,^{23–26} including in lab-scale bioreactors at high cell densities. It has also been used to modulate growth rates of species such as *Escherichia coli*²⁷ and *Saccharomyces cerevisiae*²⁸ by controlling expression of essential metabolic valves such as amino acid biosynthesis or glycolysis. Unlike initial inoculation size and ratio, light can be easily turned on and off at any time and in any duty cycle, allowing for real-time adjustments to perturbations during a fermentation. Additionally, optogenetic growth valves can be designed to be independent of process conditions or other consortium members, such that each

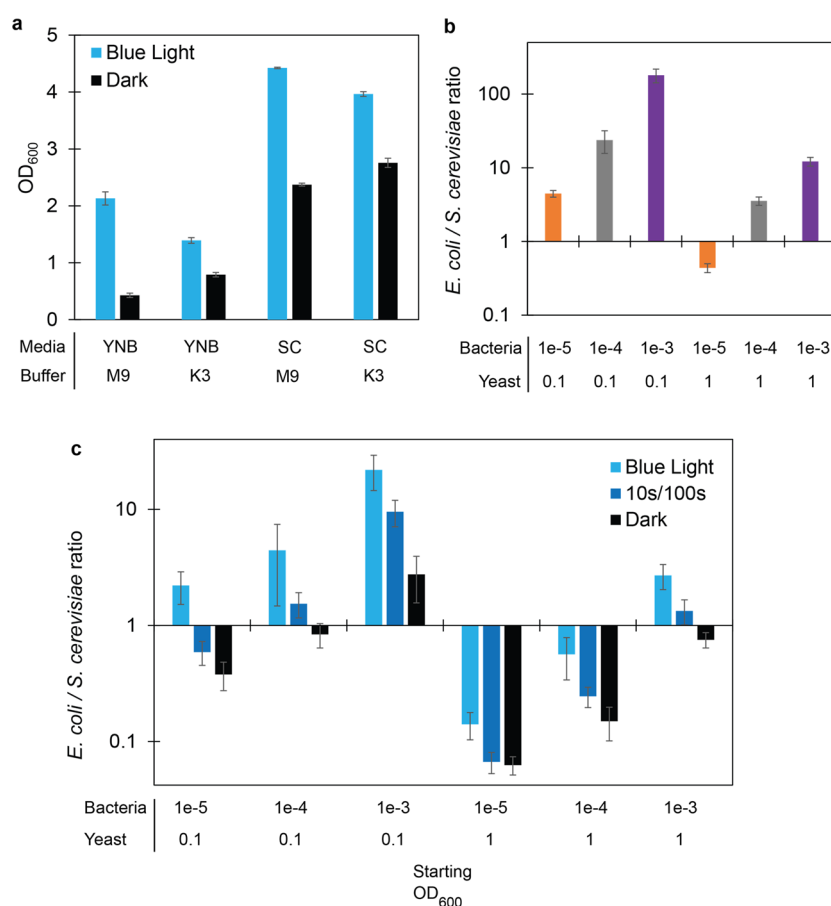


Figure 2. Characterization of bacteria–yeast growth in consortium. (a) Growth measurements of *E. coli* strain containing OptoTA (EMAL144) in YNB + M9 salts (YNB-C), YNB + K3 salts, SC + M9 salts, or SC + K3 salts media under full light or darkness. (b) Final single cell ratios of wild-type *E. coli*/*S. cerevisiae* grown for 24-h in co-cultures initiated with different inoculation sizes (shown as OD₆₀₀ values in table under the graph) in YNB-C medium. Cells were counted with flow cytometry using *E. coli* (EMAL221) and yeast (yMAL272) strains containing mCherry and BFP, respectively. (c) Final single cell *E. coli*/*S. cerevisiae* ratios of *E. coli* containing OptoTA (EMAL144) and wild-type yeast (yMAL272) grown for 24-h in co-cultures initiated from different inoculation sizes (shown as OD₆₀₀ values in table under the graph) in YNB-C medium and exposed to full light, 10 s ON/90 s OFF, or full darkness. Cells were counted with flow cytometry using mCherry and BFP to label EMAL144 and yMAL272, respectively. All data are shown as mean values; error bars represent the s.d. of $n = 3$ biologically independent samples.

organism is controlled orthogonally without specific restrictions from nutrients, carbon sources, or symbiotic relationships (natural or engineered). Such advantages of optogenetics could make it possible to establish optimal populations in co-culture fermentations for chemical production, irrespective of fluctuations in inoculations and intermicrobial interactions.

An effective way to dynamically regulate microbial growth is using gene circuits that selectively repress essential functions or activate toxins. Strategies to control proliferation of engineered strains, often in the context of biocontainment, have included “kill switches” that activate toxic genes²⁹ and “safeguards” that render strains dependent on expression of essential genes.^{30,31} Of these options, the MazEF toxin-antitoxin (TA) system seemed especially amenable to optogenetic control. The MazEF system consists of a stable toxin, MazF, an endoribonuclease that degrades mRNA, and an unstable antitoxin, MazE,³² which binds to MazF homodimers to inhibit them.³³ Under conditions of cellular stress, the unstable MazE is degraded by the ClpP protease,³² leading to mRNA cleavage by MazF and either programmed cell death³² or reversible growth inhibition.³⁴ As an endoribonuclease, MazF toxicity is decoupled from any specific metabolic pathway and more self-contained than nutrient-dependent strategies such as

auxotrophy. Furthermore, it can be neutralized through expression of *mazE*, enabling titratability of its toxicity. Previous studies have shown inhibition and recovery of growth in *E. coli* using chemical induction of *mazF* and *mazE*,^{35,36} respectively, so we reasoned that we could similarly implement optogenetic control of these genes for effective light regulation of *E. coli* growth.

In this study, we apply optogenetics to control the population dynamics of co-cultures of the model organisms *E. coli* and *S. cerevisiae* for chemical production. We develop optogenetic circuits to manipulate bacterial growth rate by regulating expression of the MazEF TA system using blue light, eliminating the need to engineer symbiosis or carbon source specialization between the species. We then harness the ability of *E. coli* to produce simple precursor molecules (such as amino acids^{37,38}) at high yields and titers, which are then assimilated by *S. cerevisiae* to synthesize downstream products while avoiding endogenous upstream regulatory mechanisms and competition for intermediate metabolites. Using a division of labor approach to feed *E. coli*-derived intermediates into *S. cerevisiae* for conversion into final products, we produce isobutyl acetate and naringenin in light-controlled co-culture fermentations and show that specific light schedules can be

used to establish subpopulations with increased production. Our findings demonstrate the potential of using optogenetics as a new method for controlling microbial consortia populations and optimizing the productivity of co-culture fermentations for chemical production.

RESULTS AND DISCUSSION

Development of a Light-Dependent Growth Control Circuit for *Escherichia coli*. To establish optogenetic control over *E. coli*, we applied the pDusk and pDawn systems³⁹ to control the MazEF TA system using darkness and blue light. Briefly, pDusk uses the synthetic two-component YF1-FixJ system to activate gene expression in darkness, while pDawn reverses the signal of pDusk using the cI repressor to activate expression in blue light (SI Note). Using these systems, we developed a new optogenetic circuit, which we call OptoTA, to control bacterial growth with light. OptoTA expresses *mazF* using pDusk (P_{FixK2}) and *mazE* using pDawn (P_R), such that MazE-mediated inhibition of MazF enables growth in blue light, while repression of *mazE* and expression of *mazF* prevents growth in darkness (Figure 1a). When grown in M9 media containing glucose as a carbon source, a $\Delta mazEF$ strain containing OptoTA shows 19-fold higher growth under full blue light compared to darkness, while reaching 77% of the cell density of a control strain that is not light-dependent (Figure 1b), suggesting that MazE-mediated repression of MazF is either incomplete or that MazEF production carries some burden. The difference between light and dark (27-fold) is even higher in xylose. Additionally, growth in darkness is lower ($OD_{600} < 0.25$ after 24 h at 30 °C) under different carbon sources such as glycerol and xylose than in glucose, but higher ($OD_{600} = 0.57$ after 12 h) in the richer lysogeny broth (LB), consistent with previous observations that MazF-mediated toxicity is media-dependent.³⁵ These results show that OptoTA can regulate *E. coli* growth in several types of media commonly used for biotechnological applications.

To determine whether light dosage can be used to tune growth rate using OptoTA, we performed growth curve experiments under different light duty cycles, chosen based on the rapid time scales of YF1 phosphorylation of FixJ (<30 s)⁴⁰ and activation times of pDusk and pDawn (<10 min).³⁹ Intermediate duty cycles (10 s or 1 s of light per 100 s) result in intermediate growth profiles between full light and darkness, showing that growth rate is titratable by varying light exposure (Figure 1c). A 10% light dose (10 s ON/90 s OFF) of light recovers 72% of the growth rate observed in full light (0.17 vs 0.23 h⁻¹), though less light exposure results in a ~1-h increase in lag phase. This strain containing OptoTA, however, shows a 20% decrease in growth rate under full light compared to a wild-type control (0.23 vs 0.29 h⁻¹). Nevertheless, the ability of OptoTA to tune bacterial growth provides the capability needed to dynamically control populations in co-cultures with other species.

Optogenetic Control of a Consortium of *E. coli* and *S. cerevisiae*. The optogenetic regulation of *E. coli* growth attainable with OptoTA is sufficient to control populations in co-cultures with other organisms with lower growth rates that would otherwise be outcompeted in media favorable to *E. coli* (which is the case for common culturing conditions),^{41,42} such as the model yeast *S. cerevisiae*. However, *S. cerevisiae* does not grow on M9 medium (commonly used in *E. coli* cultures), while *E. coli* does not grow on synthetic complete (SC) medium (commonly used in *S. cerevisiae* cultures) (Supple-

mentary Figure S1a). To find a defined minimal medium that would support both *E. coli* and *S. cerevisiae* cultivation, while still favoring *E. coli*, we tested combinations of commonly used bacterial buffers—M9 and K3⁴³—with two commonly used yeast media—yeast nitrogen base (YNB) and SC. A strain of *E. coli* containing OptoTA shows differences in growth between blue light and darkness in each of these hybrid media, though growth in darkness is significantly increased in buffered SC media ($OD_{600} > 2$ after 14 h at 30 °C), suggesting that the toxicity of MazF is attenuated by supplementation of nutrients such as amino acids, probably due to reduced degradation of MazE by Lon protease, which occurs under conditions of nutrient starvation (Figure 2a).^{34,35} YNB media supplemented with glucose and M9 buffer shows the best optogenetic control over *E. coli* growth (5-fold difference in OD_{600}) while still supporting yeast growth, albeit with a longer lag phase (Supplementary Figure S1b). We therefore continued our experiments using this media formulation, which we named YNB-Consortia (YNB-C).

To characterize the growth dynamics of *E. coli* and *S. cerevisiae* in a consortium, we sought to examine how light control can create divergent population ratios from the same initial inoculation. To do so, we inoculated co-cultures to different initial cell densities for each species in YNB-C medium and grew them under blue light or darkness. Because OD_{600} cannot be used to distinguish population sizes in co-cultures due to the differing optical properties of each species, we quantified cell counts using flow cytometry with counting beads, distinguishing bacterial and yeast populations by light scattering properties and expression of distinct fluorophores—mCherry for *E. coli* and BFP for *S. cerevisiae* (Supplementary Figure S2; see Methods). When using a strain of *E. coli* that is not light-regulated, the population ratios after 12 h of co-culture at 30 °C greatly favor *E. coli* in most inoculation ratios (Figure 2b). A final bacteria-to-yeast ratio <1 is only achieved when inoculating a very small amount of *E. coli* ($OD_{600} = 1 \times 10^{-5}$, or $\sim 1 \times 10^4$ cells/mL) and a much larger amount of *S. cerevisiae* ($OD_{600} = 1$, or $\sim 1 \times 10^7$ cells/mL), reflecting the need to give yeast a considerable advantage in initial inoculation to compensate for its slower growth in YNB-C medium (Figure 2b). Such large disparities in inoculation size could reduce the efficiency of co-culture fermentations, in which the relative abundances of biosynthetic submodules must be carefully balanced to maximize productivity. In contrast, when controlling *E. coli* growth with light using OptoTA, it becomes possible to reach bacteria-to-yeast ratios of <1 even at much lower yeast ($OD_{600} = 0.1$) or higher bacterial ($OD_{600} = 1 \times 10^{-3}$) inoculations (Figure 2c). Such inoculations are more balanced and more closely represent ratios previously used in *E. coli*–*S. cerevisiae* co-cultures (cell ratios ~1:100–100:1).^{14,44} Our goal was not necessarily to achieve specific target population ratios, but instead to demonstrate that it is possible to obtain several final population ratios from the same initial inoculation, or to reach similar ratios from different initial inoculations, simply by changing light duty cycle. Thus, the light control enabled by OptoTA provides an additional degree of freedom that augments adjustment of inoculation sizes to control microbial populations in co-culture fermentations.

Production of Isobutyl Acetate Using an *E. coli*–*S. cerevisiae* Consortium. Precise control over microbial co-cultures holds promise for applications in metabolic engineering, in which biosynthetic pathways can be divided into

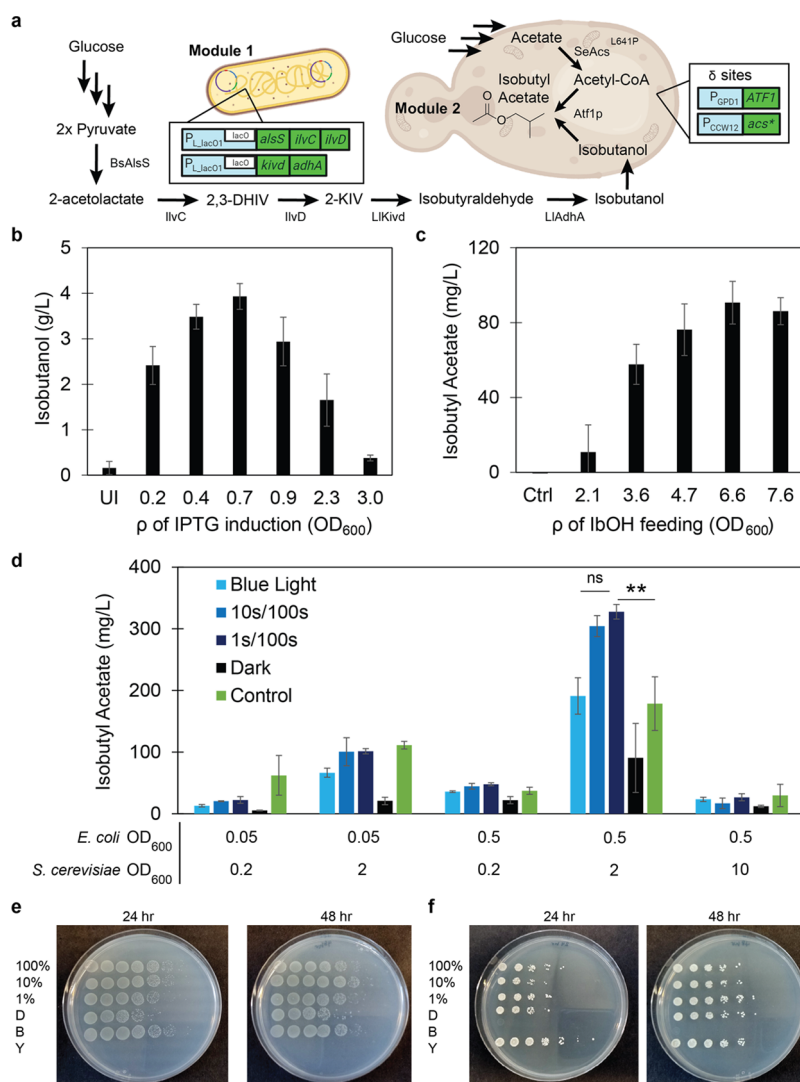


Figure 3. Isobutyl acetate production using bacteria–yeast co-cultures. (a) Isobutyl acetate biosynthetic pathway split into two modules. Module 1 (*E. coli*) produces isobutanol from glucose and is comprised of *alsS* from *Bacillus subtilis*, *ilvC*, *ilvD*, *kivd* from *Lactococcus lactis*, and *adhA* from *L. lactis*. Module 2 (*S. cerevisiae*) converts isobutanol and acetyl-CoA into isobutyl acetate and is comprised of *ATF1* and feedback-insensitive mutant *acs** from *S. enterica* (L641P). The acetate to make acetyl-CoA is derived from pyruvate produced from glycolysis, which is decarboxylated by *Pdc1p* and then oxidized by *Ald6p* (Supplementary Figure S3a). *E. coli* and *S. cerevisiae* cells were created using Biorender.com. (b) Isobutanol production from monocultures of *E. coli* strain containing Module 1 (EMAL274), grown in M9 medium + 2% glucose under full light, then induced with IPTG at different cell densities and fermented under 10 s ON/90 s OFF light for 48 h. UI denotes cultures not induced with IPTG. (c) Isobutyl acetate production from monocultures of a yeast strain containing Module 2 (yMAL362), grown in SC medium + 2% glucose, then resuspended in fresh media + 4% glucose + 1 g/L of isobutanol at different cell densities and fermented for 48 h. Control cultures (Ctrl) were not resuspended in media containing isobutanol. (d) Isobutyl acetate production from co-cultures of an *E. coli* strain containing OptoTA and Module 1 (EMAL274) and a yeast strain containing Module 2 (yMAL362), starting from different inoculation sizes (shown in table below graph) and grown for 48-h under different light conditions: full light, 10 s ON/90 s OFF, 1 s ON/99 s OFF, and full darkness. Production from co-cultures using a light-insensitive strain of *E. coli* (EMAL203), instead of EMAL274, grown in the dark is shown as control. (e,f) Relative populations of bacteria and yeast after 24 and 48 h of co-culture fermentations, shown as serial dilutions on agar plates compatible for bacterial growth: (e) EMAL274 on M9 + kanamycin + carbenicillin plates, or yeast growth: (f) yMAL362 on SC plates. Each plate contains serial dilutions from co-cultures grown in different light conditions 100% = continuous blue light; 10% = 10 s ON/90 s OFF; 1% = 1 s ON/99 s OFF; D = darkness; B = *E. coli* only control; Y = *S. cerevisiae* only control. Statistics are derived using a two-sided *t*-test (***P* < 0.01); ns = not significant. All data are shown as mean values; error bars represent the s.d. of *n* = 4 biologically independent samples.

separate modules among different species to make products of interest. To test this, we devised a division of labor strategy to produce isobutyl acetate, a chemical solvent, fragrance, and potential biofuel that is derived from isobutanol and acetyl-CoA.⁴⁵ We divided the biosynthetic pathway for isobutyl acetate into two modules: an *E. coli* module that produces isobutanol, followed by an *S. cerevisiae* module that combines isobutanol with acetyl-CoA to produce isobutyl acetate (Figure

3a). Isobutanol and acetyl-CoA are both derived from pyruvate in opposing pathways, so there is an inherent competition in the biosynthesis of these two precursors that could be resolved using two specialized strains in co-culture (Supplementary Figure S3a). We engineered a light-controlled isobutanol-producing strain of *E. coli* by transforming a $\Delta mazEF$ strain with plasmids containing OptoTA and the isobutanol biosynthetic pathway.²⁶ This strain (EMAL274) secretes up

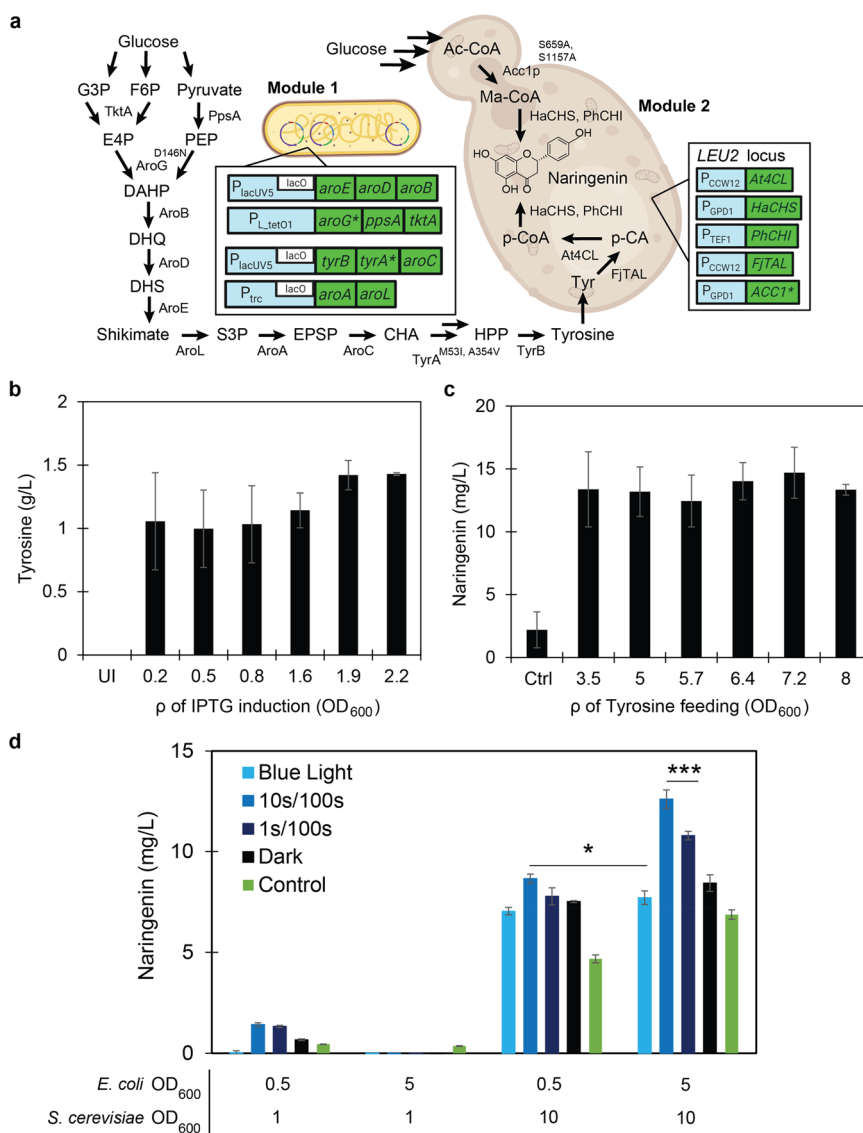


Figure 4. Naringenin production using a bacteria–yeast consortium. (a) Naringenin biosynthetic pathway split into two modules. Module 1 (*E. coli*) produces tyrosine from glucose and is comprised of *tktA*, *ppsA*, *aroG*^{D146N}, *aroB*, *aroD*, *aroE*, *aroL*, *aroA*, *aroC*, *tyrA*^{M53I, A354V}, and *tyrB*. Module 2 (*S. cerevisiae*) converts tyrosine into naringenin and is comprised of FjTAL, At4CL2, HaCHS, PhCHI, and ACC1^{S650A, S1157A}. * denotes feedback-insensitive mutant enzymes. *E. coli* and *S. cerevisiae* cells were created using Biorender.com. (b) Tyrosine production from monocultures of an *E. coli* strain containing Module 1 (EMAL374) grown in M9 medium + 2% glucose under full light, then induced with IPTG at different cell densities and fermented under 10 s ON/90 s OFF light for 48 h. UI denotes cultures not induced with IPTG. (c) Naringenin production from monocultures of a yeast strain containing Module 2 (yMAL485) grown in SC medium + 2% glucose, then resuspended in fresh SC + 4% glucose + 1 g/L of tyrosine at different cell densities and fermented for 48 h. Control cultures (Ctrl) were not resuspended in media containing tyrosine. (d) Naringenin production from co-cultures of an *E. coli* strain containing OptoTA and Module 1 (EMAL374) and a yeast strain containing Module 2 (yMAL485), starting from different inoculation sizes (shown in table below graph) and grown for 48-h under different light conditions: full light, 10 s ON/90 s OFF, 1 s ON/99 s OFF, and full darkness. Production from co-cultures using a light-insensitive strain of *E. coli* (EMAL60), instead of EMAL374, grown in the dark is shown as control. **P* < 0.05, ****P* < 0.001. Statistics are derived using a two-sided *t*-test. All data are shown as mean values; error bars represent the s.d. of *n* = 4 biologically independent samples.

to 3.9 ± 0.3 g/L isobutanol into the culture supernatant when grown in M9 media, with production peaking when IPTG induction occurs at intermediate cell densities ($0.2 \leq \rho \leq 0.9$, where ρ represents the cell density at induction, measured as OD₆₀₀) (Figure 3b). We next engineered a strain of *S. cerevisiae* with increased acetyl-CoA production that could assimilate isobutanol from the culture supernatant and convert it into isobutyl acetate. To achieve this, we integrated multiple copies of a feedback-insensitive acetyl-CoA synthetase⁴⁶ from *Salmonella enterica* (*acs* L641P) and the yeast alcohol acetyltransferase (*ATF1*) into the δ -sites of CEN.PK2–1C.

The resulting strain (yMAL362) produces up to 90 ± 10 mg/L isobutyl acetate when grown to various cell densities and then resuspended in SC media containing 1 g/L isobutanol, with production saturating around $\rho \geq 6.6$ (Figure 3c; see Methods). These results show that our bacterial strain can produce and export isobutanol while our yeast strain can import it and convert it to isobutyl acetate, offering the potential for each module to be optimized independently to balance demand for the acetyl-CoA precursor while completing a complex production pathway.

To determine if optogenetics can be used to improve chemical production from microbial consortia, we combined our bacterial and yeast modules to produce isobutyl acetate in light-controlled fermentations. We grew co-cultures of EMAL274 and yMAL362 at 30 °C for 48 h under various light conditions and initial inoculum sizes, using 1 mM IPTG to induce isobutanol production at the time of co-inoculation. We found that isobutyl acetate production depends greatly not only on the inoculation but also on the light duty cycles applied to the co-cultures (Figure 3d). The highest titer of isobutyl acetate (330 ± 10 mg/L) is obtained using intermediate inoculation sizes of bacteria ($OD_{600} = 0.5$) and yeast ($OD_{600} = 2$), as well as a 1% duty cycle of light (1 s ON/100 s). This isobutyl acetate production is 6.9-fold or 12.3-fold higher than if the co-culture is inoculated with less yeast ($OD_{600} = 0.2$, 48 ± 3 mg/L) or more yeast ($OD_{600} = 10$, 27 ± 6 mg/L), respectively (while keeping the inoculation size of bacteria constant), emphasizing the influence of initial inoculation on fermentation productivity. However, within the most productive inoculation, light control of the consortia population during the fermentation can significantly improve production by 71% (from 190 ± 30 mg/L) relative to conditions that would favor bacterial growth (under continuous light), and 3.6-fold higher (from 90 ± 60 mg/L) relative to conditions that would favor yeast growth (under darkness). These results demonstrate the benefit of adjusting microbial populations from productive inoculums during co-culture fermentation, which we achieve with light control of bacterial cell growth using OptoTA.

Light duty cycles in optogenetically controlled co-cultures can also influence the levels of intermediates relayed between strains, shedding light on the system's dynamics. Production of the intermediate, isobutanol, increases with higher light exposure at lower bacterial inoculations, but is maximized by intermediate light exposures at higher inoculations (Supplementary Figure S3b). These results suggest that intermediate light doses help tune bacterial growth to maximize production of isobutanol without outcompeting yeast, thereby leading to improved isobutyl acetate production. This is supported by the observation that under the most productive conditions we found, isobutyl acetate production is 83% higher using a light-regulated bacterial strain relative to using a light-insensitive strain (180 ± 40 mg/L), even though isobutanol production is comparable (Figure 3d; Supplementary Figure S3b). The levels of isobutanol ultimately reflect the bacterial population and reveal that yeast-catalyzed esterification is limiting, probably because of limited acetyl-CoA supply,⁴⁷ which would need to be further improved to increase conversion to isobutyl acetate. Serial dilution analysis of consortia populations shows that bacterial growth is still light-controlled under fermentation conditions, while yeast growth is slightly more efficient under lower light dosage, likely due to less competition from *E. coli* (Figure 3e,f). There is no apparent contribution from phototoxicity, as yeast growth is the same under both full light and darkness (Supplementary Figure S4). Bacteria-only controls solely produce isobutanol, while yeast-only controls produce neither isobutanol nor isobutyl acetate, confirming that isobutyl acetate is produced only by combining the two strains in co-cultures (Supplementary Figure S3c). Our findings establish optogenetics as an effective strategy to dynamically regulate bacteria–yeast populations during co-culture fermentations to optimize chemical production.

Production of Naringenin Using an *E. coli*–*S. cerevisiae* Consortium. To demonstrate that light-based control of microbial consortia can be extended to other metabolic pathways, we developed a co-culture production strategy for naringenin, a natural product with potential therapeutic properties.⁴⁸ This long metabolic pathway, which starting from increasing aromatic amino acid biosynthesis comprises 16 different enzymes, can load a single strain with excessive metabolic burden. Thus, taking advantage of *E. coli*'s sound ability to produce aromatic amino acids,³⁸ and aiming to bypass the strong endogenous regulation of aromatic amino acid biosynthesis in yeast,⁶ we split the naringenin production pathway into an *E. coli* module that produces and exports tyrosine followed by an *S. cerevisiae* module that intakes tyrosine and combines it with malonyl-CoA to produce naringenin (Figure 4a). To engineer a light-responsive *E. coli* strain to produce tyrosine, we transformed a $\Delta mazEF$ strain with plasmids encoding for OptoTA and enzymes to enhance tyrosine biosynthesis.⁴⁹ This strain (EMAL374) exports up to 1.4 ± 0.1 g/L tyrosine into the supernatant when grown in M9 media, with production peaking when inducing with IPTG at relatively high cell densities ($\rho \geq 1.9$) (Figure 4b). Next, to produce a *S. cerevisiae* strain that produces naringenin from imported tyrosine, we integrated the naringenin biosynthetic pathway,^{50,51} as well as feedback-insensitive acetyl-CoA carboxylase,⁵² into CEN.PK2–1C (see Methods). Naringenin production from this strain (yMAL485) in SC media shows no dependence on the cell density at which tyrosine is fed, reaching 12–14 mg/L from 1 g/L tyrosine (Figure 4c). These results suggest that, as with isobutanol and isobutyl acetate, the tyrosine and naringenin modules can be combined to complete a complex biosynthetic pathway while avoiding endogenous regulatory mechanisms.

We next tested whether co-cultures of our tyrosine and naringenin modules can be controlled with light to improve naringenin production. Fermentations initiated from inoculums of various sizes and proportions and conducted under different light conditions revealed that, as with isobutyl acetate, naringenin production depends on both variables. The highest naringenin titers (12.6 ± 0.5 mg/L) are obtained at intermediate levels of light exposure, this time using a 10% (10 s ON/100 s) duty cycle and the largest inoculum sizes tested (Figure 4d). This titer is not only 84% higher than what a light-insensitive control strain of *E. coli* produces (6.8 ± 0.2 mg/L), but it is also 64% higher than what is obtained with the light-sensitive strain under full light (7.7 ± 0.3 mg/L) and 50% higher than what is produced by the same strain under darkness (8.4 ± 0.4 mg/L). This suggests that the populations of *E. coli* and yeast obtained in co-cultures of the light-sensitive strain exposed to intermediate levels of light are more favorable for naringenin production, and that these populations can be achieved by the optogenetic regulation afforded by OptoTA. The similar or lower levels of production of the negative controls (conducted in the dark with a light-insensitive strain) compared to the full light controls indicate that differences in naringenin titers under various light duty cycles reflect real improvements in production due to optogenetic growth control, and are not due to naringenin photobleaching in full light conditions, although light sensitivity of the product could be influencing our final titers.⁵³ Production is still influenced by initial inoculums, with the most productive yielding 46% higher production than that of a co-culture initiated with a 10-fold lower *E. coli* inoculum and kept under the same light

conditions (8.7 ± 0.2 mg/L), indicating that higher levels of the first module are beneficial but must be carefully regulated. Additionally, production is completely abolished when the *S. cerevisiae* inoculum is reduced 10-fold, emphasizing the importance of having an abundance of the second yeast module. Nevertheless, these results confirm that the additional degree of freedom afforded by optogenetic controls of co-cultures is applicable to multiple biosynthetic pathways.

CONCLUSIONS

In this study, we establish tunable control of microbial consortia using light, which addresses the longstanding challenge of dynamically regulating population dynamics for optimal co-culture fermentation performance. Our OptoTA circuit is not tethered to the properties of a specific metabolic pathway, allowing it to function independently of strain modifications (such as gene overexpression or deletions) catered to specific biosynthetic pathways, and requiring only that the endogenous *mazEF* genes are deleted. We demonstrate this advantage by using OptoTA to control co-culture production of two chemicals derived from very different metabolic pathways: isobutyl acetate (central carbon metabolism, branched-chain alcohol biosynthesis) and naringenin (aromatic amino acid biosynthesis). Previous studies using monocultures of more extensively engineered strains and optimized fermentations have reported yields higher than what we obtained.^{54,55} However, while such additional genetic interventions could be implemented in our strains to further increase production, the goal of our study is to provide a proof of principle that light can be used as a flexible agent to dynamically control co-culture populations during fermentations to optimize production of diverse chemicals.

Optogenetics provides a unique advantage as using light allows for continuous control of population dynamics, even once the fermentation has started and the inoculum can no longer be adjusted. In this sense, light regulation of microbial growth provides a new degree of freedom to optimize chemical production. This is demonstrated in the ability to increase titers of both isobutyl acetate and naringenin by manipulating both light condition and inoculation sizes of bacteria and yeast (Figures 3d, 4d), with light schedules being capable of boosting production by as much as 3.6-fold in co-cultures initiated with the most productive inoculums (Figure 3d). The light duty cycle in some fermentations may in fact determine production above inoculation size, as seen by the higher naringenin production in 10% light dose (with 0.5:10 *E. coli*:*S. cerevisiae* inoculums) compared to production in 100% light in co-culture fermentations initiated with 10-fold higher bacterial inoculums (Figure 4d). While experimentation is still necessary to determine the optimal population composition for maximal chemical production, optogenetic controls allow for increased throughput of testing, population adjustment in response to potential perturbations, and further optimization once a productive inoculation regime has been found. Such advantages make light a powerful new variable to dynamically control consortia populations for fermentations and metabolic engineering applications.

Dividing metabolic pathways into submodules within different strains allows for optimization of individual modules without genetically or metabolically overburdening a single strain. By using *E. coli* to produce isobutanol and tyrosine at high levels, we avoid extensive genetic modification of *S. cerevisiae*, which endogenously regulates production of

both molecules.^{6,56} In doing so, we can focus engineering of the yeast module on overproducing other precursors—acetyl-CoA for isobutyl acetate, malonyl-CoA for naringenin, which compete for pyruvate with the upstream modules—as well as downstream biosynthesis of the final products. Production could likely be improved by increasing the copy number of bottleneck enzymes,⁵⁷ deleting competing endogenous enzymes,^{54,58} increasing transport of intermediates between modules,⁵⁹ and developing a media formulation that better balances the needs of both species.⁶⁰ More granular experimentation with different inoculation sizes and ratios, which can then be dynamically differentiated using varying light doses, would likely further increase production. Additionally, optimization of process variables, such as fermentation length and controlling pH and oxygenation, should reveal productive inoculation sizes that are lower than those used in this study. Nevertheless, our results conclusively demonstrate that light can be used to control and optimize chemical production from microbial consortia.

OptoTA functions by regulating the expression levels of the *mazEF* toxin-antitoxin system, which is endogenous to *E. coli* (Figure 1a). MazEF-like paralogs have been discovered in other bacterial species,⁶¹ raising the possibility that OptoTA could be directly used in other bacterial species to impart optogenetic growth control, while likely exhibiting less cross-talk with endogenous systems. Alternatively, the architecture of OptoTA could feasibly be applied to control other toxin-antitoxin systems, which are ubiquitous in nature,⁶² enabling optogenetic regulation of cell growth in diverse bacterial genera such as *Corynebacterium*, *Bacillus*, *Pseudomonas*, or *Lactococcus* using different toxin-antitoxin pairs. Building redundancies by simultaneously using multiple TA systems (including with different mechanisms of action) in a single strain could help attain more stringent growth control and attenuate the variability observed in different media (Figures 1b and 2a). Light-dependent growth of other commonly used model organisms, each with its own advantages, could make different types of light-controlled microbial consortia viable for a variety of biotechnological applications.

OptoTA uses single-input (blue light) control over consortia in which the growth rates of two species are heavily stratified, such as the *E. coli*–*S. cerevisiae* co-culture we use in this study. This strategy is extendable to situations in which fermentation conditions (pH, temperature, oxygenation, media) are disadvantageous to one organism. It could potentially also be used for co-cultures featuring two different strains of the same species but containing genetic modifications that diverge their growth rates. Fermentations for chemical production in lab-scale bioreactors have been optogenetically controlled using pDawn.²⁶ However, at larger scales, in which light penetration may become limiting,⁶³ YF1 mutants with increased light sensitivity⁶⁴ could be incorporated to achieve the required light stimulation. OptoTA establishes light control over the faster growing strain irrespective of process conditions or genetic background, allowing for flexible implementation of co-cultures for a wide variety of conditions and applications.

However, orthogonal optogenetic growth regulators that respond to stimuli other than blue light could be developed and used alongside OptoTA to effectively engineer and control more complex microbial co-cultures. Additional optogenetic systems that respond to darkness²⁸ could be employed to allow opposing growth in co-cultured species in the presence or absence of light. Alternatively, optogenetic systems such as

those based on phytochromes and cyanobacteriochromes,^{65,66} which respond to green, red, and far-red light, could enable decoupled control of multiple species using different wavelengths. Such polychromatic multi-input growth controls could be used to dynamically regulate population of consortia with more complex functions, including those that feature strains or species with similar growth profiles or contain more than two members.

The improved control of optogenetics over co-culture fermentations raises a unique optimization problem: how to adjust the timing and duration of light pulses to optimize populations to maximize production from co-cultures. Previous studies have investigated *in silico* approaches to analyze (and potentially engineer) interpopulation dynamics in microbial communities,^{67,68} as well as computer-assisted control of optogenetic inputs to stabilize gene expression profiles toward desired set points.⁶⁹ Moving forward, kinetic models could be designed based on growth data (species, light control) and pathway information (precursor supply, metabolic branch points) to predict highly productive population regimes for different products *a priori*, as well as to implement control strategies to keep a co-culture within these desired regimes *via* well-timed light pulses. Similar to this study, populations could be distinguished using fluorescent markers, or other quickly measurable outputs such as pH, oxygenation, and temperature, to provide live feedback to the optogenetic controller. Such “cybergenetic” approaches could substantially increase the throughput of process optimization while alleviating the burden of user control over implementing complex light schedules.⁷⁰

Optogenetic regulation of populations in microbial communities constitutes a new paradigm that could impact the fields of metabolic engineering, fermentation technology, and beyond. The unique advantages that light provides can make it possible to identify and maintain optimal microbial populations throughout fermentation for maximal chemical production. Optogenetics could thus help resolve the long-standing challenge of population control in co-culture fermentations and help realize the full potential of improving biosynthesis of complex chemical products by microbial division of labor. This study also offers a blueprint to establish optogenetic controls of microbial communities in different settings such as in the areas of bioremediation,⁷¹ agriculture and food technology,^{72,73} and microbiome research.⁷⁴

METHODS

Plasmid and Strain Construction. Plasmids were cloned into *E. coli* strain DH5 α made chemically competent using the Inoue method as previously described.⁷⁵ Transformants were inoculated on LB agar plates with appropriate antibiotics: 100 μ g/mL ampicillin, 100 μ g/mL carbenicillin, 50 μ g/mL kanamycin, or 34 μ g/mL chloramphenicol. Epoch DNA Miniprep, Omega E.Z.N.A. Gel Extraction, and Omega E.Z.N.A. Cycle Pure kits were used to extract and purify plasmids and DNA fragments. Genes were amplified from bacterial or yeast genomic DNA or lab plasmids. Backbones and inserts were either digested using restriction enzymes purchased from NEB or PCR amplified using CloneAmp HiFi PCR premix from Takara Bio. Primers were ordered from Integrated DNA Technologies (Coralville, IA). Gibson isothermal assembly was performed based on previously described protocols.⁷⁶ For *S. cerevisiae* constructs, we cloned promoter-gene-terminator sequences into standardized vector

series (pJLA vectors) as previously described⁷⁷ (Supplementary Figure S5). All plasmids were verified using Sanger sequencing from Genewiz. We avoid using tandem repeats to prevent recombination after transformation, and thus do not observe instability of strains or plasmids. A detailed description of all the plasmids used in this study can be found in Supplementary Table S1.

Plasmids pDusk and pDawn were obtained as a gift from Andreas Möglich (Addgene #43795 and #43796, respectively).³⁹ The sequence for the *acs* (L641P) gene from *Salmonella enterica* was codon-optimized for expression in *S. cerevisiae* and synthesized by Bio Basic's gene synthesis service.

Bacterial and Yeast Transformations. Deletion of endogenous *mazEF* in *E. coli* was performed using the Datsenko–Wanner method.⁷⁸ Cells were made electro-competent and electroporated as described previously.⁷⁹ FRT-flanked resistance markers were cured using FLP recombinase from pCP20.⁸⁰ Gene deletions were genotyped by sequencing PCR products amplified from purified genomic DNA using primers flanking the region of deletion.

Yeast transformations were carried out using standard lithium acetate protocols as previously described. Gene constructs derived from pYZ162⁶³ or pYZ23²⁸ vectors were linearized with PmeI and genomically integrated into the *LEU2* locus or δ -sites (YARCdelta5), respectively. Zeocin (purchased from Thermo Fisher Scientific) was used at a concentration of 1200 μ g/mL to select for δ -integration. Detailed descriptions of all bacterial and yeast strains used in this study can be found in Supplementary Tables S2 and S3, respectively.

Microbial Cell Culture Growth and Measurements. Single colonies from agar plates were inoculated into liquid media and grown in 96-well (USA Scientific Item #CC7672–7596) or 24-well (USA Scientific Item #CC7672–7524) plates at 30 °C and shaken at 200 rpm (19 mm orbital diameter). For *E. coli* experiments, unless otherwise stated, we used M9 minimal salts buffer containing 2% glucose, 0.2% w/v casamino acids (Bio Basic), K3 trace metal mixture,⁴³ and appropriate antibiotics using the previously specified concentrations (hereafter referred to as M9 medium). For *S. cerevisiae* experiments, we used yeast nitrogen base (YNB) or synthetic complete (SC: YNB supplemented with amino acids) dropout media containing 2% glucose.⁸¹ YNB media was supplemented with 0.3 mM histidine, 1.67 mM leucine, 0.2 mM uracil, and 0.4 mM tryptophan for strains with these auxotrophies. For experiments involving characterization of *E. coli* and *S. cerevisiae* growth in consortium in defined media, we used YNB or SC media containing 2% glucose, either M9 minimal salts buffer or K3 salts (31) buffer, and appropriate antibiotics. For chemical production experiments, we used a previously published²¹ medium formulation: 13.3 g/L KH₂PO₄, 4 g/L (NH₄)₂HPO₄, 1.7 g/L citric acid, 5 g/L yeast extract, K3 trace metal mixture, 4% glucose, and appropriate antibiotics (hereafter referred to as ESC medium).

To stimulate cells with blue (465 nm) light, we used LED panels (HQRP New Square 12" Grow Light Blue LED 14W) placed above the culture plates such that light intensity was between 80 and 110 μ mol/m²/s as measured using a Quantum meter (Apogee Instruments, Model MQ-510). To control light duty cycles, LED panels were regulated with a Nearpow Multifunctional Infinite Loop Programmable Plug-in Digital Timer Switch.

To measure cell concentration, optical density measurements were taken at 600 nm (OD_{600}), using media (exposed to the same light and incubation conditions as the bacteria/yeast cultures) as blank. Measurements were taken using a TECAN plate reader (infinite M200PRO) or Eppendorf spectrophotometer (BioSpectrometer basic) with a microvolume measuring cell (Eppendorf μ Cuvette G1.0), using samples diluted to a range of OD_{600} between 0.1 and 1.0.

Flow Cytometry. To measure cell counts during co-culture experiments, in which OD_{600} measurements can no longer be correlated with cell densities of individual species, we used flow cytometry to distinguish populations of different species. Events were counted using fluorescent counting beads (Spherotech AccuCount Particles, ACFP-50-5) as a stopping gate, until 100 counting beads were detected. GFP fluorescence (for detecting counting beads) was quantified by flow cytometry using a BD LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) and BD FACS Diva 8.0.2 software with a 488 nm laser and 525/50 nm bandpass filter. Fluorescence from mCherry (for detecting *E. coli*) was quantified with a 561 nm laser and 610/20 nm bandpass filter. BFP fluorescence (for detecting *S. cerevisiae*) was quantified with a 405 nm laser and 450/50 nm bandpass filter.

The gating used in our analyses was defined to exclude particles that are either too small or too large to be single living *E. coli* and *S. cerevisiae* cells or counting beads, based on the side scatter (SSC-A) vs forward scatter (FSC-A) plots as well as side scatter area (SSC-A) vs width (SSC-W) plots (Supplementary Figure S2a–d). Within the single cell populations, events with the correct fluorescence (mCherry for *E. coli* cells and TagBFP for *S. cerevisiae* cells) were gated and considered for analysis (Supplementary Figure S2e). Events that indicated yeast in size but emitted both mCherry and TagBFP fluorescence were considered as events in which *E. coli* and yeast had stuck together; these counts were added to both *E. coli* and *S. cerevisiae* populations. Single cell counts obtained using counting beads correlated linearly with OD_{600} values for both *E. coli* and *S. cerevisiae* samples (Supplementary Figure S2f,g).

Construction and Characterization of Light-Dependent Bacterial and Yeast Strains. OptoTA was characterized in *E. coli* strains derived from MO001 (MG1655 $lacI^q \Delta lacZYA::P_{T5_mCherry}$).⁸² The endogenous copies of *mazE* and *mazF* were simultaneously knocked out of MO001 and the kanamycin resistance marker removed through FLP-FRT recombination to make EMAL142. A plasmid containing pDawn controlling *mazE* and pDusk controlling *mazF* (OptoTA, pMAL511) was used to transform electrocompetent strain EMAL142 to make EMAL144. As a control, an empty plasmid containing pDusk was used to transform strain EMAL142 to make EMAL221.

To evaluate light dependence of *E. coli* growth in different media (Figure 1b), we inoculated 1 mL overnight cultures of EMAL221 into M9 medium + kanamycin + 2% glucose, as well as EMAL144 into M9 medium + kanamycin + 2% glucose, glycerol, or xylose, or LB + kanamycin, under constant blue light. The next day, we back-diluted the cultures into the same media to $OD_{600} = 0.01$ in 1 mL into 24-well plates and grew the cultures for 24 h (or 12 h for LB) under blue light or in the dark, after which point OD_{600} measurements were taken.

To evaluate kinetics of *E. coli* growth using OptoTA under different light conditions (Figure 1c), we inoculated 1 mL overnight cultures of EMAL144 and EMAL221 into M9

medium + kanamycin + 2% glucose media under blue light. The next day, we back-diluted the cultures to $OD_{600} = 0.01$ in 1 mL into 24-well plates and grew the cultures until steady state was reached. Light conditions tested for EMAL144 were full blue light; pulses of 10 s ON/90 s OFF, 1 s ON/99 s OFF; and full darkness (plates wrapped in aluminum foil). OD_{600} measurements were taken every 3–5 h.

Characterization of *E. coli* and *S. cerevisiae* Co-cultures. To test the viability of *E. coli* and *S. cerevisiae* on SC and M9 2% agar plates (Supplementary Figure S1a), we inoculated 1 mL overnight cultures of EMAL144 into M9 medium + kanamycin + 2% glucose under constant blue light and CEN.PK2–1C into SC + 2% glucose. The next day, we streaked both strains out onto an M9 + kanamycin + 2% glucose agar plate, as well as an SC + 2% glucose agar plate, and incubated both plates under blue light at 30 °C for 36 h.

To screen for media formulations in which both *E. coli* and *S. cerevisiae* could grow and in which OptoTA would still be effective (Figure 2a), we inoculated 1 mL overnight cultures of EMAL144 into M9 medium + kanamycin + 2% glucose under constant blue light. The next day, we back-diluted the cultures to $OD_{600} = 0.01$ in 1 mL into 24-well plates into the following media: YNB + M9 salts buffer (YNB-C); YNB + K3 salts buffer; SC + M9 salts buffer; and SC + K3 salts buffer. All media contained 2% glucose and kanamycin. We then grew the cultures for 14 h under blue light or in the dark, after which OD_{600} measurements were taken.

To characterize the growth of *S. cerevisiae* in YNB-C media (Supplementary Figure S1b), we inoculated 1 mL overnight cultures of CEN.PK2–1C into YNB + 2% glucose or YNB-C + 2% glucose. The next day, we back-diluted the cultures into the same media to $OD_{600} = 0.1$ in 1 mL into 24-well plates and grew the cultures until steady state.

To characterize potential toxic effects of light toward *S. cerevisiae* (Supplementary Figure S4), we inoculated 1 mL overnight cultures of CEN.PK2–1C into SC + 2% glucose. The next day, we back-diluted the cultures into the same media to $OD_{600} = 0.01$ in 1 mL into 24-well plates under blue light and darkness and grew the cultures until steady state.

To evaluate light-dependence of strains grown in consortium (Figure 2c), we integrated constitutively expressed TagBFP (pMAL653) into the *LEU2* locus of CEN.PK2–1C to create yMAL272. We inoculated overnight cultures of EMAL221, EMAL144 (both of which express mCherry when induced with IPTG), and yMAL272 separately in YNB-C + 2% glucose + kanamycin media (under full blue light for EMAL144). The next day, we used these overnight cultures to inoculate 150 μ L of fresh media (same medium as the overnights) + 1 mM IPTG to induce mCherry expression, in 96-well plates to calculated OD_{600} values of 10^{-5} – 10^{-3} for bacteria and 0.1–1 for yeast. The co-cultures were then grown at 30 °C under different blue light conditions, including full light; 10 s ON/90 s OFF pulses; and full darkness (plates wrapped in aluminum foil). After 12 h, 5 μ L of each culture was diluted into 95 μ L of ice-cold PBS containing the manufacturer's recommended concentration (500 μ L of beads per 10 mL) of fluorescent counting beads for flow cytometry analysis.

Development of Isobutanol-Producing *E. coli* and Isobutyl Acetate-Producing *S. cerevisiae*. To make a light-sensitive strain of *E. coli* that produces isobutanol, we transformed electrocompetent EMAL142 with pMAL511 (containing OptoTA) as well as pMAL534 containing a five-gene isobutanol biosynthesis pathway:²⁶ acetolactate synthase

from *Bacillus subtilis* (*alsS*), acetohydroxyacid reductoisomerase (*ilvC*), dihydroxyacid dehydratase (*ilvD*), 2-ketoacid decarboxylase from *Lactococcus lactis* (*kivd*), and alcohol dehydrogenase from *L. lactis* (*adhA*). The resulting strain, EMAL274, was plated onto LB + kanamycin + ampicillin agar under full light. As a light-insensitive control, electrocompetent MG1655 was transformed with pMAL534 to create EMAL203, which was plated onto LB + ampicillin agar. Eight colonies were then used to inoculate 1 mL of M9 medium + 2% glucose + carbenicillin media in 24-well plates, grown overnight (under blue light and + kanamycin for EMAL274). The next day, each culture was back-diluted into the same media to an OD₆₀₀ of 0.01 and grown for 12 h at 30 °C at 200 rpm (under blue light for EMAL274). At this point, 50 μ L of 40% glucose was added to each culture, as well as 10.6 μ L of 100 mM IPTG (final concentration of 1 mM). The plates were then sealed with Nunc Sealing Tape (Thermo Scientific) to prevent evaporation of isobutanol. The strains were fermented for 48 h under ambient light for EMAL203 or 10% (10 s ON/90 s OFF) light for EMAL274, after which samples were prepared for GC-FID analysis as described below. The highest producing colony was selected for subsequent optimization.

To find the optimal cell density at which isobutanol production is induced with IPTG (Figure 3b), we back-diluted 1 mL overnight cultures of EMAL203 and EMAL274 to different OD₆₀₀ values (ranging from 0.005 to 0.3) in 1 mL cultures of M9 medium + 2% glucose + carbenicillin (under blue light and + kanamycin for EMAL274). The different dilutions were grown for 12 h, reaching different OD₆₀₀ values, at which point the same fermentation procedure described above was then followed.

To construct a *S. cerevisiae* strain that converts isobutanol to isobutyl acetate, we integrated multiple copies of endogenous alcohol acetyltransferase (*ATF1*) and feedback-insensitive acetyl-CoA synthetase from *Salmonella enterica* (*acs*^{L641P})⁴⁶ into δ -sites (YARCdelta5) of CEN.PK2–1C (linearized pMAL788), creating yMAL362. Transformants were plated on YPD agar overnight; the next day, colonies were replica plated onto YPD agar supplemented with 1200 ng/ μ L Zeocin. Eight colonies were then used to inoculate 1 mL overnight cultures of SC + 2% glucose media in 24-well plates. The next day, each culture was back-diluted to an OD₆₀₀ of 0.1 in fresh medium and grown for 24 h. The cultures were then centrifuged in a Sorvall Legend XTR (Thermo Fisher) at 2000 rpm for 10 min and resuspended in fresh SC + 4% glucose + 1 g/L isobutanol media, in plates that were sealed with Nunc Sealing Tape. The cultures were fermented for 48 h, after which samples were prepared for GC-FID analysis as described below. The highest producing colony was selected for subsequent analysis.

To find the optimal cell density at which to feed isobutanol to maximize isobutyl acetate production in this strain (Figure 3c), we back-diluted 1 mL overnight cultures of yMAL362 to different OD₆₀₀ values (ranging from 0.01 to 0.3) in 1 mL cultures of SC + 2% glucose. The different dilutions were grown for 12 h, reaching different OD₆₀₀ values, at which point the same fermentation procedure described above was followed.

To produce isobutyl acetate using an *E. coli*–*S. cerevisiae* consortium (Figure 3d), we inoculated 50 mL overnight cultures of EMAL203 or EMAL274 into M9 medium + 2% glucose + carbenicillin media (under blue light and + kanamycin for EMAL274), as well as yMAL362 into SC +

2% glucose media. These cultures were centrifuged at 3700 rpm for 10 min, resuspended in ESC + 4% glucose + carbenicillin media (+ kanamycin for EMAL274), and back-diluted to the following OD₆₀₀ values: 0.05 or 0.5 for EMAL203 and EMAL274; 0.2, 2, or 10 for yMAL362. The plates were then sealed, and the co-cultures were fermented for 48 h under the following light conditions: full blue light, 10 s ON/90 s OFF, 1 s ON/99 s OFF, or full darkness (plates wrapped in aluminum foil). Co-cultures containing EMAL203 were grown in the dark, while monocultures of EMAL274 or yMAL362 were grown under full blue light. After 48 h, samples were centrifuged and collected for GC-FID analysis as described below.

Development of Tyrosine-Producing *E. coli* and Naringenin-Producing *S. cerevisiae*. To make a light-sensitive strain of *E. coli* that produces tyrosine, we transformed electrocompetent EMAL142 with pMAL511 (containing OptoTA), and plasmids pS4 and pY3, containing 11 genes combined that convert glucose into tyrosine:⁴⁹ transketolase (*tktA*); phosphoenolpyruvate synthase (*ppsA*); feedback-insensitive DAHP synthase (*aroG*^{D146N}); DHQ synthase (*aroB*); DHQ dehydratase (*aroD*); shikimate dehydrogenase (*aroE*); shikimate kinase II (*aroL*); EPSP synthase (*aroA*); chorismate synthase (*aroC*); feedback-insensitive chorismate mutase/prephenate dehydrogenase (*tyrA*^{M53I, A354V}); and tyrosine aminotransferase (*tyrB*). The resulting strain, EMAL374, was plated onto LB + kanamycin + ampicillin + chloramphenicol agar plates and incubated under full light. As a light-insensitive control, electrocompetent MG1655 was transformed with pS4 and pY3 to create EMAL60, which was plated onto LB + ampicillin + chloramphenicol agar plates. Eight colonies were then used to inoculate 1 mL of M9 medium + 2% glucose + carbenicillin + chloramphenicol media and grown overnight (under blue light and + kanamycin for EMAL374). The next day, the same procedures were followed as for isobutanol to identify the highest producing colony; also sealing the plates, but with a single hole poked to allow for limited aeration. After 48 h of fermentation under ambient light for EMAL60 or 10% light for EMAL374, samples were prepared for HPLC analysis as described below. To find the optimal cell density at which tyrosine production is induced with IPTG (Figure 4b) we followed the same procedure described above for isobutanol production, except using both carbenicillin and chloramphenicol for selection (with blue light and kanamycin added for EMAL374).

To construct a *S. cerevisiae* strain that converts tyrosine into naringenin, we integrated tyrosine ammonia-lyase from *Flavobacterium johnsoniae* (FjTAL),⁵⁰ 4-coumarate-CoA ligase 2 from *Arabidopsis thaliana* (At4CL2),⁵¹ chalcone synthase from *Hypericum androsaemum* (HaCHS),⁵¹ chalcone isomerase from *Petunia hybrida* (PhCHI),⁵¹ and feedback-insensitive acetyl-CoA carboxylase (ACC1^{S650A, S1157A})⁵² into the *LEU2* locus of CEN.PK2–1C (linearized pMAL959), creating yMAL485. Transformants were plated on SC – Leu + 2% glucose agar. Eight colonies were then used to inoculate 1 mL overnight cultures of SC + 2% glucose media. The next day, the same procedures were followed as for isobutyl acetate, using 1 g/L tyrosine in the media and sealed plates with a single hole poked on the tape. After 48 h of fermentation, samples were prepared for HPLC-MS analysis as described below. The highest producing colony was selected for subsequent analysis.

To find the optimal density at which to feed tyrosine to maximize naringenin production (Figure 4c), we followed the same procedure as above for isobutyl acetate production by feeding tyrosine instead of isobutanol.

To produce naringenin using an *E. coli*–*S. cerevisiae* consortium, we inoculated 50 mL overnight cultures of EMAL60 or EMAL374 into M9 medium + 2% glucose + carbenicillin + chloramphenicol media (under blue light and + kanamycin for EMAL374), as well as yMAL485 into SC + 2% glucose media. The next day, these cultures were centrifuged at 3700 rpm for 10 min, resuspended in ESC + 4% glucose + carbenicillin + chloramphenicol media (+ kanamycin for EMAL374), and back-diluted in 1 mL to the following OD₆₀₀ values: 0.5 or 5 for EMAL60 and EMAL374; and 1 or 10 for yMAL485. The plates were then sealed with a single hole poked to allow for limited aeration, and the co-cultures were fermented for 48 h under the following light conditions: full blue light, 10 s ON/90 s OFF, 1 s ON/99 s OFF, or full darkness (plates wrapped in aluminum foil). Co-cultures containing EMAL60 were grown in the dark. After 48 h, samples were centrifuged and collected for HPLC-MS analysis as described below.

Fermentation Sample Preparation and Analytical Methods. For isobutanol and isobutyl acetate, cultures were centrifuged in a Sorvall Legend XTR at 2000 rpm for 10 min at 4 °C. Then, 500 μ L of cell-free supernatant was mixed with 1 mL of ethyl acetate in a 2 mL microcentrifuge tube and vortexed for 20 min, then centrifuged at 17 000 RCF in an Eppendorf 5424 microcentrifuge for 15 min at 4 °C. Finally, 400 μ L of the organic phase was transferred to an autosampler vial for analysis. For tyrosine, cultures were centrifuged in a Sorvall Legend XTR at 2000 rpm for 10 min at 4 °C. Then, 800 μ L of supernatant was transferred to a 1.5 mL microcentrifuge tube and centrifuged at 17 000 RCF for 45 min at 4 °C. Finally, 250 μ L of cell-free supernatant was transferred to an autosampler vial for analysis. For naringenin, 500 μ L of cell culture was mixed with 500 μ L of ethanol in a 1.5 mL microcentrifuge tube and vortexed for 20 min, then centrifuged at 17 000 RCF for 45 min at 4 °C. Finally, 250 μ L of cell-free supernatant was transferred to an autosampler vial for analysis.

Concentrations of isobutanol and isobutyl acetate were quantified using an Agilent 7890B GC System equipped with a flame ionization detector (FID). Cultures were centrifuged in a Sorvall Legend XTR at 2000 rpm for 10 min at 4 °C. Then, 500 μ L of cell-free supernatant was mixed with 1 mL of ethyl acetate in a 2 mL microcentrifuge tube and vortexed for 20 min, then centrifuged at 17 000 RCF in an Eppendorf 5424 microcentrifuge for 15 min at 4 °C. Then, 500 μ L of the organic phase was transferred to an autosampler vial for analysis. Samples were injected and subjected to a split (0.5 μ L injection volume; 1:20 split), using a constant helium flow of 1.5 mL/min. Samples were separated using a DB-Wax column (30 m length, 0.25 mm diameter, 0.5 μ m film) and a gradient as follows: Initial oven temperature was held at 70 °C for 3 min, temperature was then ramped at a rate of 20 °C/min to 230 °C and held at that temperature for 5 min. Samples were quantified using Flame-ionization detection (300 °C, H₂ flow 30 mL/min, Air flow 400 mL/min, Makeup flow 25 mL/min), and compared to commercial standards (Sigma-Aldrich for isobutanol; Alfa Aesar for isobutyl acetate).

Concentrations of tyrosine and naringenin were quantified with high-performance liquid chromatography–mass spec-

trometry (HPLC-MS), using an Agilent 1260 Infinity and 6120 instrument (Agilent Technologies, Santa Clara, CA, USA). Samples were analyzed using Alltech Alltima C18 column (250 $\times$ 4.6 mm, 5 μ m particle size) using 0.1% formic acid in acetonitrile (Solvent A), 0.1% formic acid in water (Solvent B) at 35 °C, a flow rate of 0.3 mL/min, and the following gradient method: start at 10% Solvent A; from 0 to 10 min, linear increase of Solvent A from 10% to 40%; from 10 to 15 min, linear increase of Solvent A from 40% to 60%; from 15 to 15.5 min, linear increase of Solvent A from 60% to 100%; hold at 100% Solvent A from 15.5 to 21 min; linear decrease of Solvent A from 100% to 10% A from 21 to 21.5 min; hold at 10% Solvent A from 21.5 to 28 min. Tyrosine was monitored with a diode array detector (DAD) using a detection wavelength of 270 nm. Naringenin was monitored using the mass spectrometer under selective ion monitoring (SIM) using the following settings: *m/z* = 271.1, Fragmentor = 100, Gain = 1.00, Dwell = 590 ms, %Rel Dwell = 100. To determine analyte concentrations, peak areas were integrated and compared to those of standard solutions for quantification (Sigma-Aldrich for tyrosine; Alfa Aesar for naringenin).

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acssynbio.1c00182>.

Supplementary Note on how pDusk and pDawn function; characterization of bacterial and yeast growth in standard and hybrid media; flow cytometry gating strategy; isobutanol production using a bacteria–yeast consortium; yeast growth under blue light and darkness; plasmid assembly diagram; tables of plasmids and bacterial and yeast strains (PDF)

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Author Contributions

M.A.L. and J.L.A. conceived this project and designed the experiments. M.A.L. and H.K. constructed the strains and plasmids. M.A.L., H.K., R.L.M., and S.M.H. executed the experiments. M.A.L. and J.L.A. analyzed the data and wrote the paper.

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Notes

The authors declare the following competing financial interest(s): The authors have applied for a patent for some of the technologies described in this study.

The authors declare that all data supporting the findings of this study are available within the paper (and its [Supporting Information](#) files), but original data that supports the findings are available from the corresponding author upon reasonable request.

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