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Light-Controlled Fermentations for Microbial Chemical and Protein Production

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Abstract

Microbial cell factories offer a sustainable alternative for producing chemicals and recombinant proteins from renewable feedstocks. However, overburdening a microorganism with genetic modifications can reduce host fitness and productivity. This problem can be overcome by using dynamic control: inducible expression of enzymes and pathways, typically using chemical- or nutrient-based additives, to balance cellular growth and production. Optogenetics offers a non-invasive, highly tunable, and reversible method of dynamically regulating gene expression. Here, we describe how to set up light-controlled fermentations of engineered *Escherichia coli* and *Saccharomyces cerevisiae* for the production of chemicals or recombinant proteins. We discuss how to apply light at selected times and dosages to decouple microbial growth and production for improved fermentation control and productivity, as well as the key optimization considerations for best results. Additionally, we describe how to implement light controls for lab-scale bioreactor experiments. These protocols facilitate the adoption of optogenetic controls in engineered microorganisms for improved fermentation performance.

Introduction

Optogenetics, the control of biological processes with light-responsive proteins, offers a new strategy to dynamically control microbial fermentations for chemical and protein production[1,2]. The burden of engineered metabolic pathways and the toxicity of some intermediates and products often impairs cell growth[3]. Such stresses can lead to poor biomass accumulation and reduced productivity[3]. This challenge can be addressed by temporally dividing fermentations into a growth and production phase, which devote metabolic resources to biomass accumulation or product synthesis respectively[4]. We recently showed that the transition from growth to production in this two-phase fermentation can be induced with changes in illumination conditions[5,6,7]. The high tunability, reversibility, and orthogonality of light inputs[8] offer unique advantages to light-controlled fermentations that are difficult or impossible to replicate with chemical

inducers used in dynamical control of conventional two-phase fermentations[4,9,10,11].

The blue-light responsive EL222 protein derived from *Erythrobacter litoralis* has been used to develop several optogenetic circuits for metabolic engineering in *Saccharomyces cerevisiae*[5,7,12,13]. EL222 contains a light-oxygen-voltage sensor (LOV) domain that undergoes a conformational shift upon blue light activation (465 nm), which allows it to bind to its cognate DNA sequence (C120)[13]. Fusing EL222 to the viral VP16 activation domain (VP16-EL222) results in a blue-light responsive transcription factor that can reversibly activate gene expression in *S. cerevisiae*[7] and other organisms[14] from the synthetic promoter P_{C120}. Several circuits based on EL222 have been developed and used for chemical production in *S. cerevisiae,* such as the basic light-activated OptoEXP system[7], in which the gene of interest is directly expressed from P_{C120} (**Figure 1A**). However, concerns of light penetration at the high cell densities typically encountered in the production phase of fermentations motivated us to develop inverted circuits that are induced in the dark, such as the OptoINVRT and OptoQ-INVRT circuits (**Figure 1B**)[5,7,13]. These systems harness the galactose (GAL) or quinic acid (Q) regulons from *S. cerevisiae* and *N. crassa*, respectively, controlling their corresponding repressors (GAL80 and QS) with VP16-EL222, to repress gene expression in the light and strongly induce it in the dark. Combining OptoEXP and OptoINVRT circuits results in bidirectional control of gene expression, enabling two-phase fermentations in which the growth phase is induced with blue light, and the production phase with darkness (**Figure 2A**)[5,7].

Using light instead of darkness to induce gene expression during the production phase would greatly expand the capabilities of optogenetic controls but would also require overcoming the light penetration limitations of the high cell densities typically encountered in this phase of fermentation. To this end, we have developed circuits, known as OptoAMP and OptoQ-AMP, that amplify the transcriptional response to blue light stimulation. These circuits use wild-type or hypersensitive mutants of VP16-EL222 to control production of the transcriptional activators Gal4p or QF2 of the GAL or Q regulons, respectively, achieving enhanced sensitivity and stronger gene expression with light[12,13] (**Figure 1C**). OptoAMP circuits can achieve complete and homogeneous light induction in 5 L bioreactors at an optical density (measured at 600 nm; OD_{600}) values of at least 40 with only ~0.35% of illumination (5% light dose on only ~7% of the bulk surface). This demonstrates a higher degree of sensitivity compared to OptoEXP, which requires close to 100% illumination[12]. The ability to effectively induce gene expression with light at high cell densities opens new opportunities for dynamical control of fermentations. This includes operating fermentations in more than two temporal phases, such as three-phase fermentations, in which growth, induction, and production phases are established with unique light schedules to optimize chemical production (**Figure 2B**)[12].

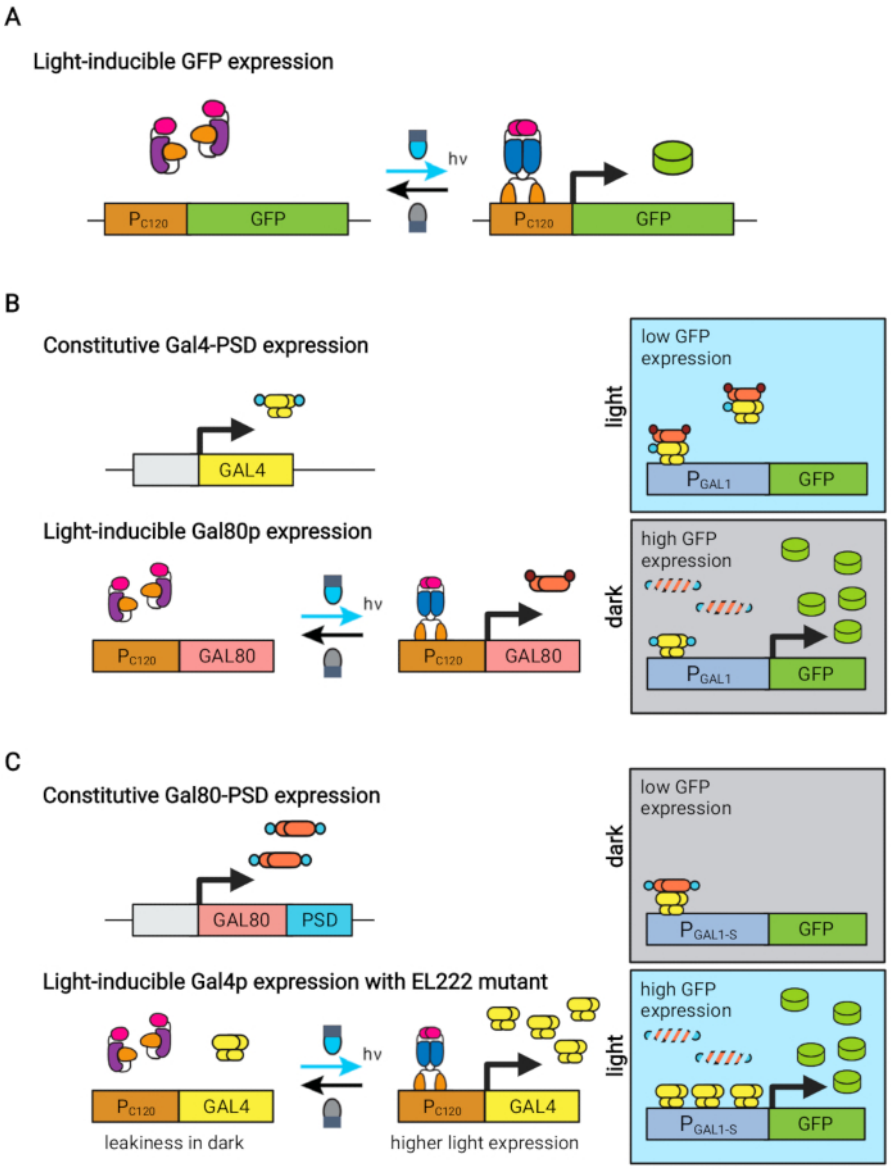

Figure 1: Optogenetic circuits for dynamic control of *S. cerevisiae*. The OptoEXP, OptoINVRT, and OptoAMP circuits are based on the light-sensitive VP16-EL222 system. (**A**) In the OptoEXP circuit, exposure to blue light causes a conformational change and dimerization of VP16-EL222, which exposes a DNA-binding domain and allows for transcription from P_{C120}. The figure has been modified from Zhao et al.[7]. (**B**) OptoINVRT circuits harnesses the GAL (shown) or Q regulons to induce expression in the dark. In GAL-based circuits, VP16-EL222 and *GAL4* are constitutively expressed, while P_{C120} drives expression of the *GAL80* repressor (in Q-based circuits, *GAL4* and *GAL80* are replaced by QF2 and QS, respectively, and a synthetic QUAS-containing promoter is used instead of a GAL promoter). In light, Gal80p prevents activation of the gene of interest from P_{GAL1}. In the dark, *GAL80* is not expressed and rapidly degraded by fusing it to a constitutive degron domain (small brown domain), which allows for activation of P_{GAL1} by Gal4p. The figure has been

modified from Zhao et al.[5]. (**C**) OptoAMP circuits also use VP16-EL222 to control the GAL (shown) or Q regulons. In these circuits, the *GAL80* repressor (or QS) is constitutively expressed and fused to a photo-sensitive degron (small blue domain) ensuring tight repression in the dark. P_{C120} and a hypersensitive VP16-EL222 mutant control expression of *GAL4* (or QF2) with light, which strongly activates P_{GAL1} (or a QUAS-containing promoter) in the light. GAL-derived circuits can use engineered forms of P_{GAL1}, such as P_{GAL1-M} or P_{GAL1-S}, which have increased activity, as well as wild-type promoters controlled by the GAL regulon (P_{GAL1}, P_{GAL10}, P_{GAL2}, P_{GAL7}). The figure has been modified from Zhao et al.[12]. Please click here to view a larger version of this figure.

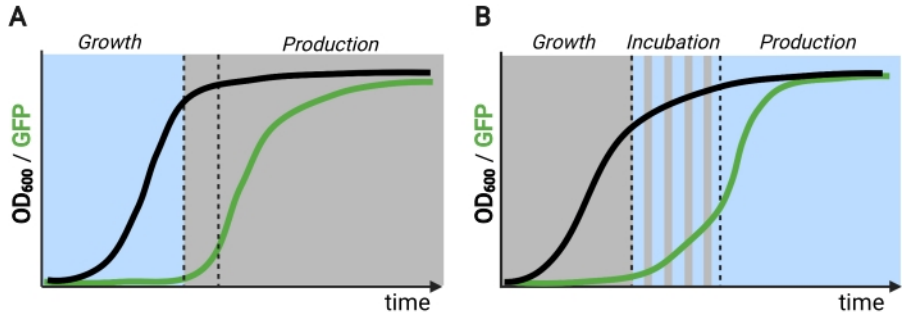

Figure 2: Two- and three-phase fermentations through time. (**A**) Two-phase fermentations operated with inverted circuits consist of a light-driven growth phase and a dark production phase. In the growth phase, biomass accumulates as the production pathway stays repressed. Upon reaching the desired OD_{600}, cells are shifted to the dark to metabolically adjust before being resuspended in fresh media for the production phase. (**B**) In a three-phase process, the growth, incubation, and production phases are defined by unique light schedules, which may consist of a dark growth period, pulsed incubation, and fully illuminated production phase. Figure created with Biorender. Please click here to view a larger version of this figure.

Optogenetic circuits have also been developed for dynamical control of chemical and protein production in *E. coli*. OptoLAC circuits control the bacterial LacI repressor using the light-responsive pDawn circuit, which is based on the YF1/FixJ two-component system[6] (**Figure 3**). Similar to OptoINVRT[5], OptoLAC circuits are designed to repress gene expression in the light and induce it in the dark. Expression levels using OptoLAC circuits can match or exceed those achieved with standard isopropyl β-d-1-thiogalactopyranoside (IPTG) induction, thus maintaining the strength of chemical induction while offering enhanced tunability and reversibility[6]. Therefore, OptoLAC circuits enable effective optogenetic control for metabolic engineering in *E. coli*.

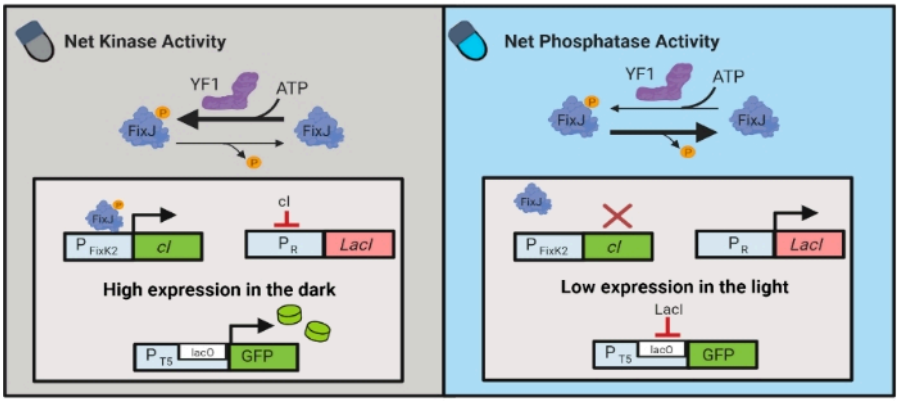

Figure 3: OptoLAC circuits for dynamic control of *E. coli*. The OptoLAC circuits adapt the pDawn system and lac operon to achieve activation in the dark and repression in the light. In the dark, YF1 phosphorylates FixJ, which then activates the P_{FixK2} promoter to express the *cI* repressor. The *cI* repressor prevents expression of the *lacI* repressor from the P_R promoter, which permits transcription of the gene of interest from a *lacO*-containing promoter. Conversely, blue light reduces YF1 net kinase activity, reversing FixJ phosphorylation and thus *cI* expression, which derepresses expression of *lacI* and prevents expression from the *lacO*-containing promoter. The figure has been modified from Lalwani et al.[6]. Please click here to view a larger version of this figure.

We describe here the basic protocols for light-controlled fermentations of *S. cerevisiae* and *E. coli* for chemical or protein production. For both yeast and bacteria, we first focus on fermentations with a light-driven growth phase and a darkness-induced production phase enabled by OptoINVRT and OptoLAC circuits. Subsequently, we describe a protocol for a three-phase (growth, induction, production) light-controlled fermentation enabled by OptoAMP circuits. Furthermore, we describe how to scale up optogenetically controlled fermentations from microplates to lab-scale bioreactors. With this protocol, we aim to provide a complete and easily reproducible guide for performing light-controlled fermentations for chemical or protein production.

Protocol

1. Light-controlled chemical production using the *S. cerevisiae* OptoINVRT7 circuit

1. Strain construction
    1. Obtain a strain with *his3* auxotrophy, as this marker is necessary for most existing OptoINVRT plasmids[5]. If seeking optogenetic regulation of a gene that is native to *S. cerevisiae*, construct a strain in which any endogenous copy of the gene is deleted.
    2. Linearize the plasmid containing the OptoINVRT7 circuit, such as EZ-L439[5], and integrate it into the *his3*-locus of the auxotrophic strain using

standard lithium-acetate transformation methods[15]. If using the EZ-L439 plasmid, which contains the components to repress P_{GAL1} in the light and activate it in the dark, linearize at the PmeI restriction site.

3. Following the transformation, centrifuge the cells at 150 x *g* for 1 min and gently resuspend in 200 µL of fresh histidine-dropout synthetic complete medium (SC-His) medium.

4. Plate the entire cell volume onto SC-His agar plates and incubate at 30 °C for 2-3 days until colonies appear.

5. Prepare competent cells from this strain using standard lithium acetate transformation protocols, and transform them with a plasmid containing the gene(s) to be controlled optogenetically downstream of either the P_{GAL1-M} or P_{GAL1-S} promoter[5].

   NOTE: Using a plasmid that integrates at δ-sites (YARCdelta5) and selects with Zeocin allows for stable multicopy integration[7,16,17,18].

6. After transformation, centrifuge the culture at 150 x *g* for 1 min and gently resuspend in 200 µL of fresh SC-dropout medium.

   NOTE: The P_{GAL1-M} promoter is a synthetic version of the P_{GAL1} promoter with the Mig1p repression sites deleted, while P_{GAL1-S} is an engineered version of P_{GAL1-M}, which has extra Gal4p activator binding sites. The regular P_{GAL1} promoter can be used to control the expression; however, the expression strength will be lower than from these engineered promoters.

7. Plate the entire cell volume on a yeast extract peptone dextrose (YPD) agar plate if integrating into δ-sites, or an SC-dropout plate if transforming with a plasmid containing a selection marker. Incubate at 30 °C for 16 h under constant blue light to keep the optogenetically controlled gene repressed.

   NOTE: For some strains, colonies might grow faster when incubated in blue light pulses (e.g., 1 s on/79 s off, 5 s on/75 s off, 10 s on/70 s off, etc.) rather than in constant illumination, which must be determined experimentally for each strain, if necessary.

8. Use any 465 nm light source and place an LED panel ~40 cm above the plate such that the light intensity is ~80-110 µmol/m^2/s. Measure the intensity using a quantum meter (see **Table of Materials**).

9. If integrating into δ-sites, create a replica of the plate onto YPD plates containing a range of Zeocin concentrations between 400 µg/mL and 1,200 µg/mL to select for a variety of integration copy numbers[5,7,12,16,17,18]. Incubate the replica plates at 30 °C under constant or pulsed blue light for 2-3 days until the colonies appear.

2. Preliminary screening for the best colonies

   1. Select eight colonies from each plate and use them to inoculate 1 mL of SC-His medium supplemented with 2% glucose in individual wells of a 24-well plate. Grow in 24-well plates under the cells overnight (16-20 h) at 30 °C with 200 rpm (19 mm orbital diameter) shaking under constant blue light illumination.

   2. The next morning, dilute each culture in 1 mL of a fresh SC-His medium with 2% glucose to OD_{600} values ranging from 0.01-0.3 and grow in 24-well

plates under constant or pulsed light at 30 °C with 200 rpm shaking until they reach cell densities between 2 and 9 OD_{600} values (**Figure 4A**). The amount of time needed for this growth phase will depend on the strain.

3. Incubate the plates in dark for 4 h at 30 °C with 200 rpm shaking by turning off the light panel and wrapping the plates in aluminum foil.
   NOTE: This step allows the cells to metabolically transition to the production phase before resuspension in the production media.

4. To start the production phase, centrifuge the cultures in the 24-well plate at 234 x *g* for 5 min and resuspend cells in 1 mL of fresh SC-dropout medium with 2% glucose. Seal the plates to prevent evaporation of the desired product by using sterile microplate sealing tape.

5. Ferment the sealed plates in the dark for 48 h at 30 °C with shaking at 200 rpm. Ensure that the plates are wrapped in aluminum foil to prevent any exposure to light.
   NOTE: Wrapping the plates in foil does not limit oxygen or gas availability in fermentations; however, the sterile sealing tape does limit gas transfer. Small holes can be poked in the tape to introduce oxygen, if necessary.

3. Harvesting and analysis

   1. To harvest the fermentations, centrifuge the plates for 5 min at 234 x *g* and transfer 800 µL of the supernatant into 1.5 mL microcentrifuge tubes.

   2. Depending on the chemical of interest, analyze using high-performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), or another analytical method using the sample preparation technique best suited for the instrument used.

2. Light-controlled protein production using the *E. coli* OptoLAC system

1. Strain construction

   1. Co-transform electrocompetent BL21 DE3 Δ*lacI* Δ*lacI*-DE3 with a plasmid containing the OptoLAC1B or OptoLAC2B circuit[6] and a plasmid that expresses the recombinant protein of interest from the P_{T7} promoter[19].

   2. After transforming, recover the cells for 1 h in 1 mL of super optimal broth with catabolite repression (SOC; 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) at 37 °C with rotation or shaking.
      NOTE: The plasmid containing the protein of interest must be compatible with the OptoLAC plasmid (i.e., different resistance marker and origin of replication) and must not contain a copy of *lacI*.

   3. Centrifuge the cells at 4845 x *g* for 3 min and concentrate the pellet in 200 µL of lysogeny broth (LB) media. Plate the entire concentrated cells onto an LB agar plate with appropriate antibiotics and grow at 37 °C overnight under constant blue light to keep protein expression repressed.

2. Initial screening to verify expression

   1. Take three single colonies and use them to inoculate 1 mL of LB media with appropriate antibiotics in individual wells of a 24-well plate. Grow overnight (16-20 h) at 37 °C with 200 rpm shaking under constant blue light illumination (**Figure 4A**).

2. The next day, use 1.5 µL of culture to measure OD_{600} in a spectrophotometer with a microvolume measurement. Dilute cultures into 1 mL of fresh LB in 24-well plates to OD_{600} values ranging from 0.01-0.1.
3. Grow the cultures at 37 °C with 200 rpm shaking under blue light for 2-3 h. Starting from the second hour, take OD_{600} measurements every 15 min to ensure that the cultures do not overgrow the OD_{600} range of 0.1-1.5.
4. Once the cultures are at the desired OD_{600}, turn off the light panel and wrap the plate in aluminum foil to initiate the production phase. Keep the plate in the dark for 8 h (37 °C), 20 h (30 °C), or 48 h (18 °C), with 200 rpm shaking.
5. Measure and record the final OD_{600} value of each culture.

3. Harvesting and analysis
   1. Transfer 800 µL of each culture into a 1.5 mL microcentrifuge tube and centrifuge for 5 min at 17,000 x *g*.
   2. Resuspend the cell pellet in 200 µL of resuspension buffer (Tris 50 mM, pH 8.0, NaCl 300 mM).
      NOTE: The concentration of NaCl can be adjusted based on the recombinant protein being analyzed.
   3. Add 50 µL of 6x sodium dodecyl sulfate (SDS) sample buffer (Tris 375 mM, pH 6.8, SDS 9%, glycerol 50%, bromophenol blue 0.03%, DTT 9%). Incubate at 100 °C for 10 min with shaking at 700 rpm in a thermomixer.
   4. Load ~3-20 µL of the culture in a 12% SDS-PAGE gel. Using the final OD_{600} measurement as a guide, load approximately the same amount of protein for each sample (equivalent to 10 µL of the sample corresponding to a final OD_{600} value of 1). Use a power supply to run electrophoresis at 100 V until the gel is fully resolved.
   5. Stain the gel with Coomassie brilliant blue G-250 solution by heating for 30-40 s in a microwave oven, and then incubating on a platform rotator for at least 15 min.
   6. Rinse with deionized water twice and destain on a platform rotator for at least 30 min (or overnight), adding two cleaning wipes tied into a knot to help absorb the stain. Boil the gel in sufficient amount of water in the microwave oven for 15 min to speed up the destaining process.

3. Three-phase fermentation using the *S. cerevisiae* OptoAMP system

1. Strain construction
   1. Obtain a strain with a *his3* auxotrophic marker, as this marker is necessary in order to use the existing OptoAMP plasmids[5]. If seeking optogenetic regulation of a gene that is native to *S. cerevisiae*, construct a strain in which the endogenous copy of this gene is deleted.
   2. Linearize a plasmid containing the OptoAMP4 circuit, such as EZ-L580[12], and integrate it in the *his3* locus of the auxotrophic strain using standard lithium-acetate transformation methods[15]. If using EZ-L580, linearize the plasmid at the PmeI restriction site.

3. Following the transformation, centrifuge the cells at 150 x *g* for 1 min and gently resuspend in 200 µL of fresh SC-His medium.

4. Plate the entire cell volume on selective media (SC-His-agar) and incubate at 30 °C for 2-3 days until colonies appear.

5. Prepare competent cells from this strain and transform them with a plasmid containing the gene(s) to be controlled optogenetically downstream of the P_{GAL1-S} promoter[12].

   NOTE: Using a plasmid that integrates at δ-sites and selects with Zeocin allows for stable multicopy integration and selection.

6. After transforming, centrifuge the culture at 150 x *g* for 1 min and gently resuspend in 200 µL of fresh SC-dropout medium.

   NOTE: The P_{GAL1-S} promoter is a synthetic version of the P_{GAL1} promoter in which the Mig1p repression sites are deleted and extra Gal4p activator binding sites are added. The regular P_{GAL1} promoter can be used; however, the expression strength will be lower than this engineered promoter.

7. Plate the entire cell volume on a YPD or SC-dropout agar plate and incubate at 30 °C for 16 h in the dark (wrapped in aluminum foil). Incubating in the dark keeps the optogenetically-controlled gene repressed, which allows the cells to direct their metabolic resources toward cell growth rather than chemical production.

8. If integrating into δ-sites, replica plate onto YPD plates containing a range of Zeocin concentrations to select for a variety of integration copy numbers. Incubate the plates at 30 °C in the dark (wrapped in aluminum foil) for 2-3 days until colonies appear.

2. Preliminary screening for the best colonies

   1. Select eight colonies from each plate and use them to inoculate 1 mL of SC-His medium with 2% glucose in individual wells of a 24-well plate. Grow the cells overnight (16-20 h) in the dark at 30 °C with 200 rpm shaking.

   2. The next morning, dilute each culture in 1 mL of fresh SC-His medium with 2% glucose to 0.1 OD_{600} and grow in dark at 30 °C with 200 rpm shaking until they reach an OD_{600} of 3. Wrap the plates in aluminum foil to prevent exposure to light. The amount of time needed for this growth phase will depend on the strain.

   3. To start the induction phase, incubate the plates under pulsed light (for example, 5 s on/95 s off) for 12 h at 30 °C with 200 rpm shaking. Use any 465 nm light source and place an LED panel above the plate such that the light intensity is ~80-110 µmol/m^2/s for optimum results (**Figure 4A**).

      NOTE: The optimal light pulse duration for this incubation will vary based on the chemical being produced. It is recommended to screen a range of light schedules from 0.1% (e.g., 1 s on 999 s off) to 100% (full light).

   4. To start the production phase, centrifuge the cultures at 234 x *g* for 5 min and resuspend in fresh SC-His medium with 2% glucose. Seal the plates to prevent evaporation of the desired product using sterile microplate sealing tape.

5. Ferment the sealed plates in light for 48 h at 30 °C with shaking at 200 rpm. Optimize the light schedule during this step as some chemicals benefit from a pulsed production phase rather than full light.

3. Harvesting and analysis

1. Harvest the fermentations by centrifuging the plates for 5 min at 234 x *g* and transferring 800 µL of the supernatant into 1.5 mL microcentrifuge tubes.

2. Depending on the chemical of interest, analyze using HPLC, GC-MS, or another analytical method using the sample preparation technique best suited for the instrument used.

4. Chemical (mevalonate) production from *E. coli* in a light-controlled bioreactor

1. Initial inoculation and bioreactor setup

1. Inoculate a colony of an *E. coli* strain engineered with light-controlled chemical production into 5 mL of M9 minimal salts (3.37 mM Na_2HPO_4, 2.2 mM KH_2PO_4, 0.855 mM NaCl, 0.935 mM NH_4Cl) supplemented with 0.2% w/v casamino acids, 5% w/v glucose, and trace metal mixture[20] (0.0084 g/L EDTA, 0.0025 g/L $CoCl_2$, 0.015 g/L $MnCl_2$, 0.0015 g/L $CuCl_2$, 0.003 g/L H_3BO_3, 0.0025 g/L Na_2MoO_4, 0.008 g/L $ZnCl_2$, 0.06 g/L Fe (III) citrate, 0.0045 g/L thiamine, 1.3 g/L $MgSO_4$) in a 50 mL conical tube.

2. Grow the culture overnight at 30 °C with 200 rpm shaking under blue light illumination.

3. Set up the bioreactor vessel head plate, making sure that the following ports are installed: insert for thermal probe; dissolved oxygen (DO) probe; gas sparger - connect to the air source through a 0.2 µm filter; impeller; gas condenser - connect to a 0.2 µm filter; cooling line (x2); feed lines (x2) - one for media addition, one for pH control; sampling line - ensure it reaches the bottom of the vessel; empty port; pH probe - calibrate the probe with standards of pH = 4 and pH = 7 prior to installation, either autoclave with the rest of the vessel or sterilize with 95% ethanol and aseptically insert before setup.

4. Fill the vessel with 1 L of filtered water, and attach the head plate and tighten, making sure that the O-ring snugly fits and seals.

5. Cover any openings into the reactor with aluminum foil.

6. Prepare three strips of tubing: one for removing the water, one for inserting the feed, and one for pH control. Cover the ends with aluminum foil and wrap all the tubing in aluminum foil.

NOTE: NH_4OH, which is used for pH adjustment, does not flow smoothly in silicone tubing, which may lead to inaccurate flow rates and over-basification of the culture. To avoid this issue, use biocompatible pump tubing (BPT) for the NH_4OH feed.

7. Autoclave the bioreactor and tubing, using a 30 min liquid cycle.

8. Remove the materials from the autoclave. Once cool enough to handle, connect the impeller, pH and DO probes, air source, condenser inlet and outlet, and cooling inlet and outlet to the control station.

9. Insert the thermal probe and cover the vessel with a heating jacket. Secure the jacket toward the top of the vessel to avoid blocking the culture from light exposure.

10. Connect one of the sterile tubes to the sampling line and secure through the sampling pump. Place the other end such that it flows into an empty container that can hold at least 1 L. Drain the water inside the vessel.
11. Connect another sterile tube to one of the feed lines and secure through one of the feed pumps. Connect the other end of the tube to a bottle of M9 media. Feed the media into the reactor.
12. Connect another sterile tube to one of the feed lines and secure through one of the feed pumps. Connect the other end of the tube to the bottle containing 28%-30% NH_4OH.

    CAUTION: NH_4OH is corrosive. Work in a fume hood while transferring to a feed bottle and make sure the feed bottle is placed in secondary containment.
13. Place three light panels in a triangular formation ~20 cm away from the reactor, checking that the light intensities on the surface of the vessel reach ~80-110 µmol/m^2/s from each side (**Figure 4B**).
14. The next day, turn on the bioreactor system and chiller. Set the reactor temperature setpoint to 37 °C, the pH setpoint to 7.0, and the agitation to 200 rpm. The heating jacket should turn on.
15. Calibrate the DO probe by first waiting until the temperature and DO measurements become constant (this will be the 100% setpoint). Then, disconnect the probe from the system (this will be the 0% setpoint). Repeat until the DO measurement stabilizes at 100% when the probe is connected, and then set the DO setpoint to 20%.

2. Light-controlled chemical production
    1. Inoculate the bioreactor to an initial OD_{600} of 0.001-0.1. Turn on the light panels to initiate growth.
    2. After 3 h, begin taking samples from the sampling line to take OD_{600} measurements to avoid overgrowing the optimal cell density of induction (ρ_S). Once the optimal ρ_S is reached (the optimal value for mevalonate production is 0.17), turn off the light panels, cover the reactor in aluminum foil, and wrap the setup in black cloth to initiate the dark production phase.
    3. Add 50 µL of antifoam, 8 h after switching to darkness. Unscrew the empty port and pipette the antifoam directly into the reactor.
    4. Use the sampling port to periodically take samples for HPLC or GC analysis.
3. Disassembly and analysis
    1. After the experiment is concluded, turn off the system. Carefully unscrew the DO and pH probes and wash them with soap and water. Unscrew the head plate and wash it with soap and water using a brush.
    2. Transfer the culture into an empty container and add bleach to a final concentration of 10% v/v. Place in a fume hood and dispose of after 30 min.
    3. Wash the reactor vessel with soap and water using a brush.
    4. Prepare the samples for analysis based on the product of interest. For mevalonate production, mix 560 µL of culture with 140 µL of 0.5 M HCl and vortex at high speed for 1 min. This converts mevalonate into (±)-mevalonolactone.

CAUTION: HCl is a health hazard. Handle with proper PPE and ensure sample tubes are properly capped prior to vortexing.

5. Centrifuge at 17,000 x *g* for 45 min at 4 °C. Transfer 250 µL of the supernatant to an HPLC vial.

6. For mevalonate, analyze samples using an organic acids ion exchange column. Quantify production using a refractive index detector (RID), comparing peak areas to a standard of (±)-mevalonolactone.

Representative Results

Optogenetic regulation of microbial metabolism has been successfully implemented to produce a variety of products, including biofuels, bulk chemicals, proteins, and natural products[5,6,7,12,13]. Most of these processes are designed for cell growth to occur in the light (when low cell density poses minimal challenges with light penetration), and for production to be induced by darkness once the cells are grown. Various chemicals that have been produced from yeast using this approach, including lactic acid, a valuable polymer precursor and food additive, as well as isobutanol, a next-generation biofuel. For both chemicals, a common challenge stems from the strong drive of *S. cerevisiae* to metabolize glucose toward ethanol production rather than the product of interest and the inability to delete the ethanol fermentation pathway without causing a severe growth defect[7]. A combination of light-activated OptoEXP and light-repressed OptoINVRT circuits have been used to selectively activate with light the gene for pyruvate decarboxylase (*PDC1*) required for ethanol fermentation, and induce the pathway for the desired product in the dark[5] (**Figure 5A,B**). Using this strategy with an optimized cell density of induction (ρ_S), high titers of both desired chemicals can be achieved (**Figure 5C,D**), highlighting the value of bidirectional control offered by optogenetics.

While most yeast-based optogenetic processes have centered on a light-driven growth phase and darkness-induced production phase, the recent development of extra sensitive and strong OptoAMP circuits has opened opportunities for light-driven fermentations as well[12]. These light-driven fermentations are similar to the previously described processes; however, the light schedules are reversed such that production occurs in the light. Furthermore, these circuits allow for the implementation of three-phase processes, which adds more flexibility and control to production compared to the standard two-phase approach. Given the sensitivity and strength of these circuits, these three-phase processes are typically optimized by screening different light schedules in each phase. Optimal light pulses depend on the strain and product of interest. Such circuits have been successfully applied to the production of naringenin, a natural product with therapeutic applications, in addition to lactic acid and isobutanol[12] (**Figure 6**). The increased production of all three chemicals demonstrates the value of optogenetic regulation across a range of pathway complexities as well as the new potential offered by three-phase fermentations.

Beyond these demonstrations in yeast, optogenetics has also been applied to enhance the production of proteins and chemicals in the bacterial workhorse *E. coli*. Fermentations with this host have followed a light-driven growth and darkness-induced production framework using the OptoLAC suite of circuits[6]. When used to produce a yellow fluorescent protein (YFP) or transcription factor FdeR, light-controlled production is comparable or superior to the levels achieved with standard IPTG induction, but with easier tunability for

intermediate levels of production (**Figure 7A**). In addition, OptoLAC circuits have been applied to produce mevalonate, an important terpenoid precursor, both at the microplate and bioreactor levels (**Figure 7B,C**). These selected results give a general overview of the strength, versatility, and tunability of optogenetic regulation for microbial chemical and protein production.

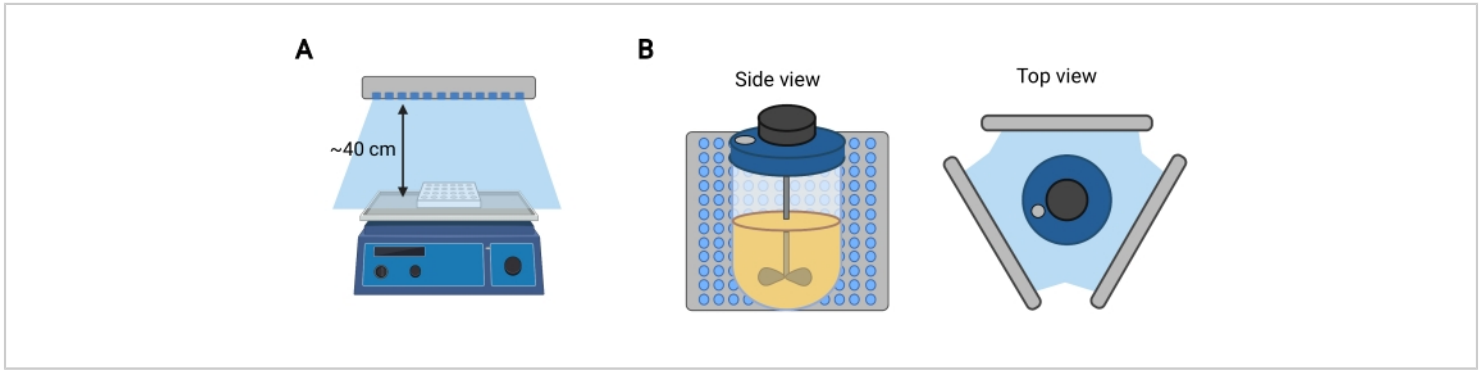

Figure 4: Experimental setup for illuminating plates and bioreactors. (**A**) 24-well plates can be illuminated while shaking by placing a blue LED light panel approximately 40 cm above the shaker. The light intensity should be measured with a quantum meter to ensure it is between ~80-110 µmol/m^2/s. (**B**) To illuminate a bioreactor, place three light panels in a triangular formation around the bioreactor. As with the 24-well plates, the light intensity should be measured and adjusted to reach ~80-110 µmol/m^2/s from all sides. Figure created with Biorender. Please click here to view a larger version of this figure.

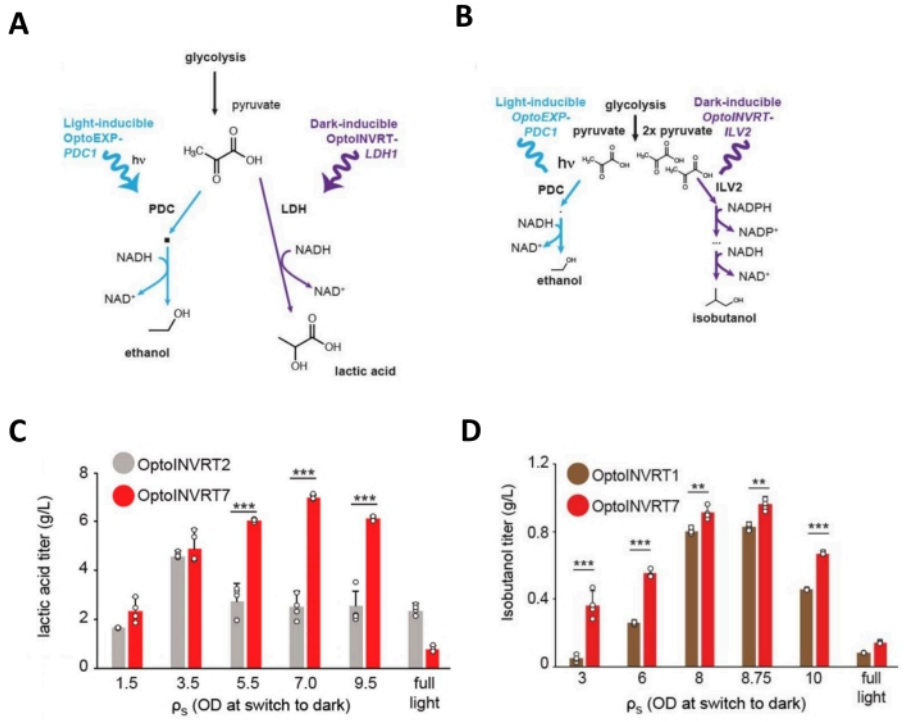

Figure 5: Light-repressed production of chemicals from *S. cerevisiae*. To enhance the production of lactic acid (**A**) or isobutanol (**B**), a combination of light- and dark-inducible circuits have been used to selectively activate specific pathways. In both scenarios, the essential ethanol production pathway is induced in the light by controlling *PDC1* expression with OptoEXP, while the production pathways are activated in the dark using an OptoINVRT circuit. (**C**) Production of lactic acid was tested at a range of ρ_S values with two circuits from the OptoINVRT suite. The OptoINVRT7 version performed best, with an optimal ρ_S value of 7.0. (**D**) The OptoINVRT7 version also maximized isobutanol production compared to the other circuit, with an optimal ρ_S of 8.75.**$p < 0.01$, ***$p < 0.001$. Statistics are derived using a two-sided *t*-test. Data are shown as mean values and error bars represent the standard deviations of four replicates. The figure has been modified from Zhao et al.[5]. Please click here to view a larger version of this figure.

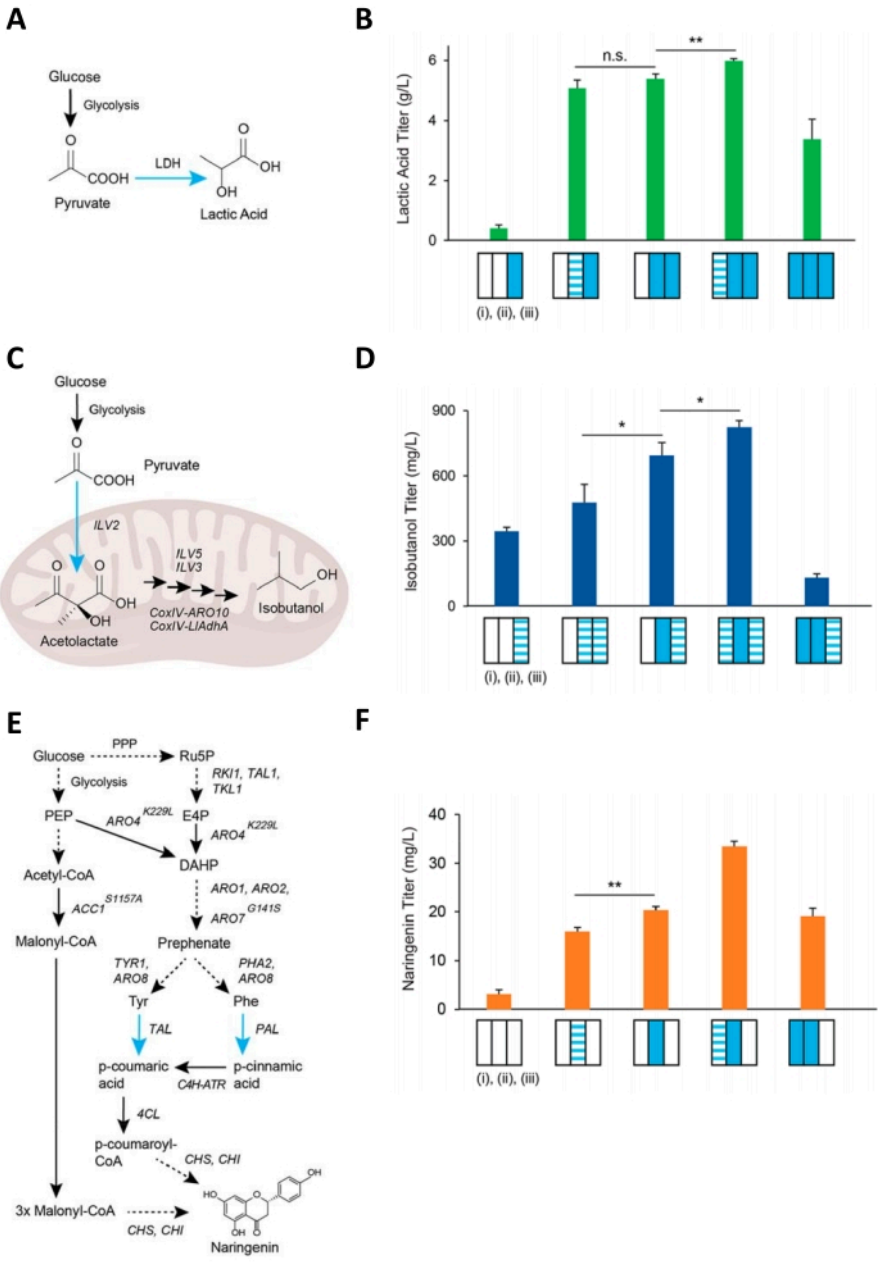

Figure 6: Light-activated production of chemicals in three-phase fermentations of *S. cerevisiae*. Fermentations using the OptoAMP circuits can operate with three dynamic phases: growth (i), induction (ii), and production (iii), each defined by different light duty cycles. (**A**) Biosynthesis of lactic acid is induced in blue light by optogenetic control of *LDH* expression. (**B**) Lactic acid production can be optimized by using a pulsed (1 s on/79 s off) light schedule in the growth phase and full illumination in the induction and production phases. (**C**) Production of isobutanol is induced by optogenetically controlling *ILV2* expression. (**D**) Isobutanol production is optimized using a pulsed growth phase (1 s on/79 s off), fully illuminated induction phase, and pulsed (2 s on/118 s off) production phase. (**E**) Control of the more complex biosynthetic pathway for

naringenin, induced by optogenetically controlling expression of the *TAL* and *PAL* genes. (**F**) Naringenin biosynthesis is best optimized using a pulsed growth (1 s on/79 s off), fully illuminated induction, and dark production phase. *$p < 0.05$, **$p < 0.01$, n.s. = no significance. Statistics are derived using a two-sided *t*-test. Data are shown as mean values and error bars represent the standard deviations of four independent replicates. The figure has been modified from Zhao et al.[12]. Please click here to view a larger version of this figure.

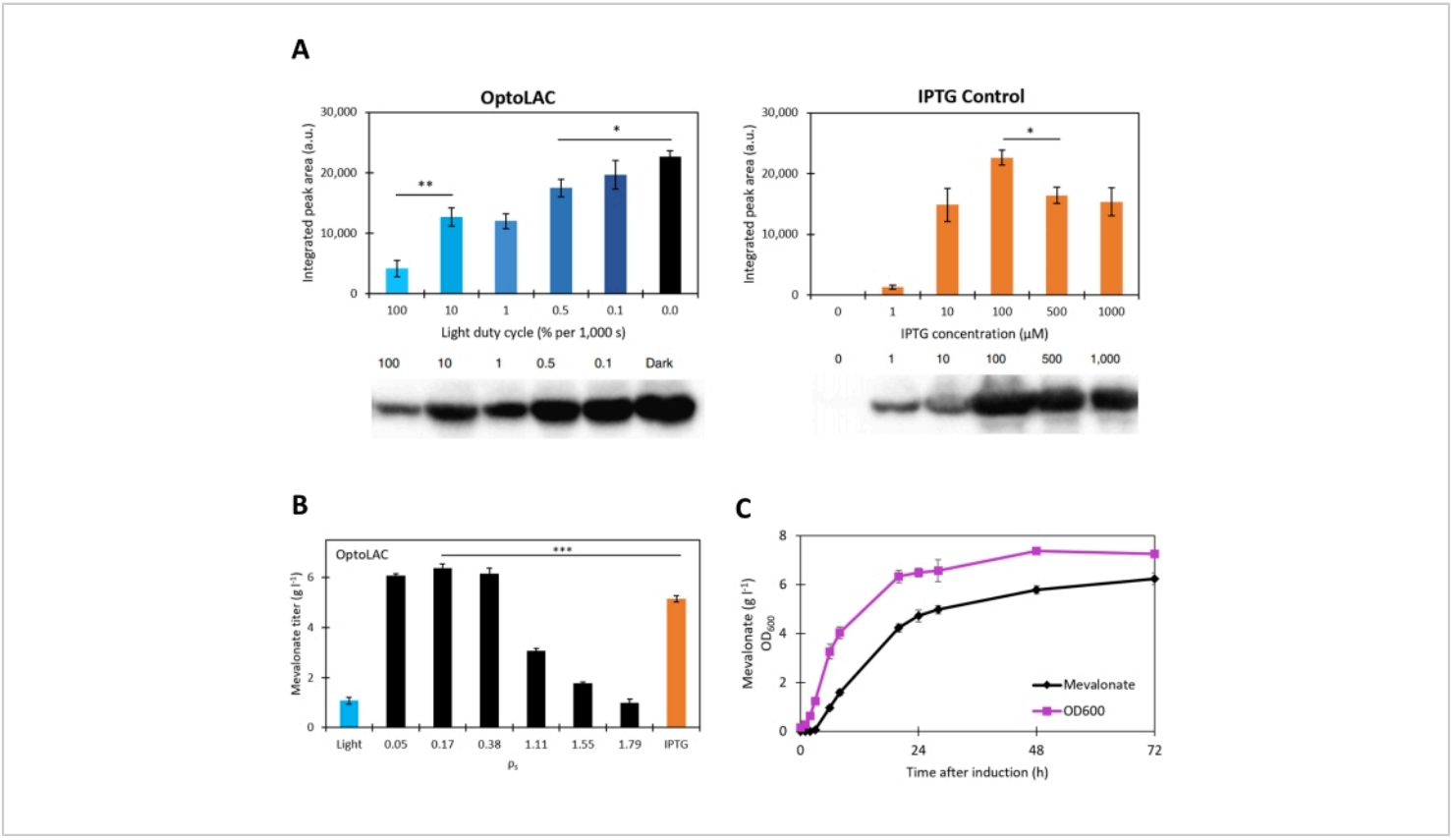

Figure 7: Optogenetic production of recombinant proteins and chemicals in *E. coli*. The OptoLAC system has been used to produce both proteins and chemicals at titers that are comparable to, or higher than those reached using chemical induction with IPTG. (**A**) Optogenetic expression of *FdeR* is both strong and tunable using a variety of light duty cycles, as resolved and quantified with western blot. (**B**) A strain engineered for mevalonate production exceeds titers achieved using IPTG induction at the 24-well scale at the optimal ρ_S value. (**C**) Optogenetic production of mevalonate in a 2 L bioreactor demonstrates the scalability of production beyond microplates. *$p < 0.05$. **$p < 0.01$, ***p < 0.001. Statistics are derived using a two-sided *t*-test. All data are shown as mean values and error bars represent the standard deviations of biologically independent samples. Data for (A) and (C) represent three replicates, while for (B), the number of replicates from left to right= 4, 4, 6, 3, 4, 4, 4, 4. The figure has been modified from Lalwani et al.[6]. Please click here to view a larger version of this figure.

Discussion

Dynamic control has long been applied to improve yields for metabolic engineering and recombinant protein production[4]. Shifts in enzymatic expression are most typically implemented using chemical inducers such as IPTG[21], galactose[22], and tetracycline[23], but have also been mediated using process conditions such as temperature and pH. Optogenetic control of gene expression eliminates the need for changes to fermentation parameters or media composition, making it an easily applicable alternative to traditional induction strategies. The ease with which light can be turned on or off also offers new capabilities like the rapid and reversible tuning of gene dosage. Furthermore, while these protocols focus on blue light-responsive systems, the existence of optogenetic tools that invert existing light responses[24] or respond to other wavelengths of light[25,26,27,28,29] offers exciting potential to orthogonally control multiple pathways for an unprecedented level of control. Such advantages make light a versatile new solution for flexible control over microbial fermentations.

Beyond screening for the best colonies, as discussed in the protocols, other parameters such as the cell density at which cultures are switched from growth to production (ρ_S), lengths of the production and incubation periods, and light duty cycles should also be optimized. The best values of these parameters are product- and strain-dependent and should thus be re-optimized for any new application. For example, pathways involving toxic final products may benefit from higher ρ_S values, which allow for sufficient accumulation of cells before inducing production[6,7], while a weaker but less leaky circuit may favor lower values of ρ_S to maximize total expression time. Likewise, some pathways and recombinant proteins may benefit from intermediate expression levels, which can be achieved with unique light duties. Furthermore, in the case of fermentations that use OptoAMP circuits, the number of temporal phases can be optimized. While the demonstrated protocol describes a three-phase process, fermentations using these circuits can be controlled with a greater number of phases defined by unique light duty schedules and durations. Thus, a range of these parameters should be tested to optimize performance.

Avoiding sources of light contamination presents an important consideration during experimental setup. For processes that require a delay of light stimulation until later stages (e.g., dark to light fermentations), working in a dark room may be advisable to avoid premature activation of optogenetic systems. In these cases, an inert light source can be applied for visibility during the experimental setup (for example, a far-red light source of ~700 nm when working with blue light-activated systems). An advantage of processes that start with light-induced growth (light to dark) is that initial experimental manipulations can be performed under ambient light with ample visibility.

The ease with which a vast number of light duty schedules can be applied to fermentations presents the opportunity to develop higher-throughput methods to balance biosynthetic pathways and elucidate the optimal conditions that maximize fermentation productivity. Instead of balancing metabolic pathways by testing large numbers of combinatorially assembled constructs having each enzyme expressed by promoters of different strengths, pathways could be balanced by varying gene expression levels using different light duty cycles from a much smaller number of constructs. This obviates the need for more cumbersome experimental setups, such as resuspension in different induction media or serial dilutions for chemical inducers. Optogenetic experiments could potentially even be

automated to increase throughput by using *in silico* controllers to deliver specifically timed or localized light pulses to different sample pools[30]. However, high-throughput experiments under different light conditions must be sufficiently separated to avoid cross-contamination of light, which can pose spatial constraints. Additionally, the requirement of light stimulation prevents the use of most plate readers and micro bioreactors for continuous measurements. While not yet broadly available commercially, several apparatuses and algorithms have been recently developed for high-throughput and continuous optogenetic experiments, which help to address these spatial constraints[31,32,33,34]. Thus, despite these limitations, optogenetics offers a huge potential to increase experimental throughput while providing enhanced controllability.

The protocols and video presented here will hopefully lower the barriers for other researchers to adopt optogenetic controls of cellular metabolism and microbial fermentations. Optogenetics is an enabling technology for basic research and biotechnological applications that may benefit from fine-tuned control of gene expressions, such as genetics, molecular and cell biology, metabolism, systems biology, and cybergenetics[35,36,37,38]. Additionally, optogenetic regulation of gene expression has been demonstrated in other microorganisms such as *Bacillus subtilis* and *Pseudomonas aeruginosa*, suggesting that the benefits of light control can be extended to the study and application of diverse species[39,40,41]. These possibilities highlight the future potential of optogenetics for metabolic engineering, protein production, and other biotechnological applications.

Disclosures

The authors have applied for several patents for the optogenetic circuits and methods described in this article.

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