OptiYeast: Optimizing Production in Yeast by Ethanol Regulation and Optogenetic Gain- and Loss-of-Function

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Abstract

Our global community deserves access to healthcare and nutrition currently available to only the most fortunate among us. Thanks to synthetic biology applications exploiting the yeast chassis, valuable compounds such as anti-malarial drugs and specialty chemicals can now be produced inexpensively. Using Golden Gate assembly designed for BioBrick compatibility, we have developed two tools to improve yeast expression of non-native pathways. First, we engineered an ethanol control system that reduces yeast's endogenous stress response and diverts more cellular resources towards product synthesis. Second, we constructed a light-induced system for instantaneous gain- and loss-of-function at the protein level. These tools will allow engineers to optimize heterologous pathways by monitoring toxic intermediates or regulating flux in a controllable, time-dependent manner. We hope our ideas will shape the future of industrial cell-based manufacturing.

Introduction

Ethanol Control

- Yeast is a **cost-effective** chassis for producing high value compounds.
- Yields can be maximized by **reducing ethanol stress** during fermentation
- We propose an ethanol control system in yeast (*figure 1a,b*).
- The design depends on the human cytochrome **p450 CYP2E1** gene, whose encoded protein converts ethanol to acetaldehyde (*figure 1b*).
- **CYP2E1** will break down ethanol produced during fermentation, thereby reducing ethanol stress.
- The system is controlled by ethanol induced promoter so CYP2E1 is only expressed when ethanol is in the system.

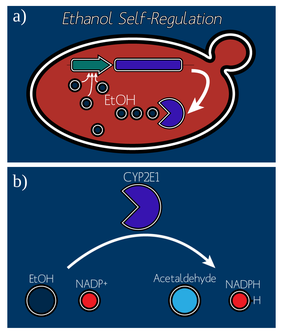


Figure 1. a) A scheme of ethanol control system in cell. Purple represents the CYP2E1 gene and protein. The green arrow represents ethanol induced promoter. b) Chemical mechanism of CYP2E1 to convert ethanol to acetaldehyde.

Optogenetic Protein Control

- Effective yeast industrial applications depend on **accurate control** and **understanding** of the relevant biomolecular pathways.
- Current standard of pathway control depends on use of chemical agents, which is **slow and imprecise**.
- **Optogentic control** is fast, spatially precise, and additive neutral, allowing individual steps of pathways to be modulated instantaneously.
- We propose the use of **TULIPs** system, which provides tunable control at the **protein level**.
- TULIPs consists of two domains: LOVpep, which loosens and exposes a binding epitope upon exposure to 470nm (blue) light; and ePDZb, which binds to the exposed epitope (*figure 2*).

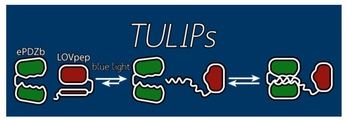


Figure 2. Schematic of the mechanism of TULIPs. ePDZb and LOVpep bind upon exposure to blue light.

Ethanol Control

Ethanol Control Theory

- To model our idea, we fitted the system to a basic control system, in which expression of CYP2E1 **decreases ethanol concentration in the system**, which in turn **affects promoter activity** (*figure 3*).
- We also fitted the model to actual fermentation data to predict ethanol content in the system within a 48 hour period.

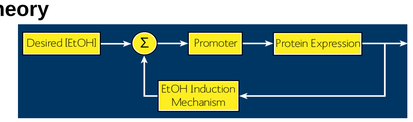
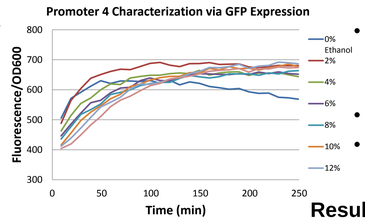


Figure 3. A simplified view of the ethanol control system. The promoter controls CYP2E1 protein expression, which then affects ethanol induction, which is fed back to the system to determine promoter activity.

Characterization of an Ethanol-inducible Promoter Library

Figure 4. An exemplary promoter characterization curve. The output was GFP expression, which was normalized against growth (OD600). The experiment was done over 250 minutes



- Promoter of CYP2E1 needs to be ethanol dependent.
  - CYP2E1 is only turned on when **ethanol is present**, which is when the protein is needed.
- We developed 27 ethanol-sensitive promoters.
- All promoters were tested by using them to express GFP under various ethanol levels (*figure 4*).

Results

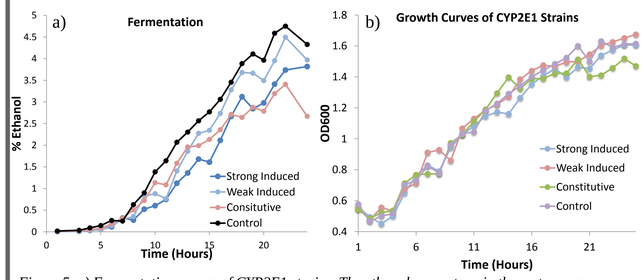


Figure 5. a) Fermentation curves of CYP2E1 strains. The ethanol percentage in the system was measured over a 24 hour period. Lower percentage means the strain is better able to break down ethanol in the system. b) Growth curves of the same strains from a). No difference in growth was observed.

- We successfully introduced three CYP2E1 constructs into yeast.
  - Constitutive: constantly express CYP2E1.
  - Strong: highly induced by ethanol.
  - Weak: weakly induced by ethanol.
  - Control: no CYP2E1 expression.
  - CYP2E1 strains reduced ethanol more than the control (*figure 5a*).
- CYP2E1 expression **does not interfere** with yeast cell growth (*figure 5b*).

Optogenetic Protein Control

TULIPs and Strategies

- We designed two strategies to implement TULIPs:
- Strategy 1: **Splitting a functional protein into two domains**. Each is attached to either LOVpep or ePDZb. Upon binding, the split sections form a whole functional protein (*figure 6 left*).
- Strategy 2: Tethering LOVpep to a subcellularly localized protein and ePDZb to a **protein of interest**. Upon excitation the ePDZb domain with protein attaches to the localized protein through LOVpep (*figure 6 right*).

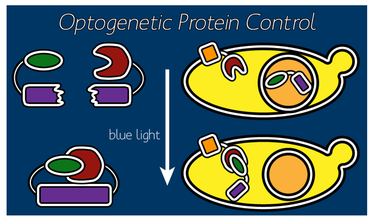


Figure 6. Diagram of the two different optogenetic strategies. The left portion shows split protein (strategy 1), while the right side shows relocalization of protein (strategy 2).

Application to Cell Cycle Arrest

- We propose applying TULIPs to **cell cycle arrest** as a **proof of concept** of both strategies.
- We applied strategy 1 to proteins known to cause cell cycle arrest. Activation causes cell cycle arrest.
- Strategy 2 was applied to proteins essential to cell cycle progression.
- We made **nine constructs** for strategy 1 and **seven constructs** for strategy 2.
- Expression of both fusion proteins and correct sub-cellular localization of tethers were confirmed (*figure 7*).

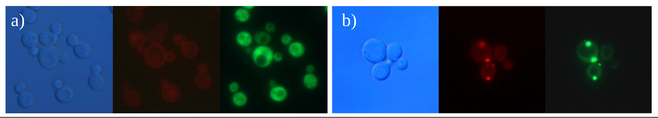


Figure 7. Representative microscopy of yeast with our constructs (done without blue light excitation). a) yeast with Mid2/LOVpep/mCherry (middle) and Cdc15/ePDZb/GFP(right). b) yeast with the C and N terminal halves of Whi3 fused to LOVpep/mCherry (middle) and ePDZb/GFP (right), respectively.

Yeast Golden Gate and Parts Course

Yeast Golden Gate

- Yeast Golden Gate (**YGG**; **RFC88**) is a **new standard** for assembly of basic yeast Transcriptional Units (TUs).
- RFC88 uses **type IIS** restriction enzyme **BsaI** to generate standardized and user-defined signature overhangs.
- Through this standard, parts can be assembled without "scars" (*figure 8*).
- Assembly of a TU may be carried out in a **one-pot reaction** with both BsaI and T4 DNA ligase.
- RFC88 parts can also be **converted** to BioBrick (BB) standard parts using our new pSB1C3 **acceptor vectors.**
- Acceptor vectors have RFP inserts (flanked by BB sequences) that can be **cut out** and **replaced** with RFC88 parts (*figure 9*).

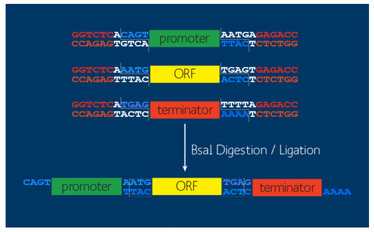


Figure 8. A schematic of the Golden Gate assembly. Each TU consists of a promoter, an ORF, and a terminator. Note that the start and stop codons are encoded in the overhangs, ensuring virtually seamless assembly of the TU.

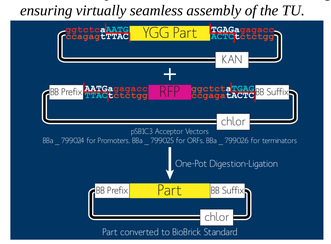


Figure 9. Converting from RFC88 to BB. E. coli *transformed with the converted parts will appear on a chloramphenicol plate as white colonies (transformants with insert-negative plasmids will appear as red colonies).*

Parts Course

- We developed a **standardized synthetic biology lab course.**
- The course provides a guide for synthetic biology curriculum in schools where such curriculum is not well-established.
- The course can be deployed in any introductory biology lab facility.
- We wrote a **lab manual** and a **course curriculum** that focus on the "language" of synthetic biology.
- The manual focuses on using RFC88.
- We refined the course content over the summer by testing the course internally at Hopkins and with a Baltimore high school student.
- We also implemented the course at Dalton High School (NYC) (*figure 10*).
- Together, we produced **more than 900 parts** for yeast expression.

Figure 10. The parts course was implemented at Dalton High School (above) and tested with a student from Baltimore Polytechnic High School (below).

Future Research

- We plan to test the optogenetic system with exposure to blue light, using a custom blue LED construct (*figure 11*).
- We plan to compare ethanol reduction by CYP2E1 to that achieved by endogenous yeast pathways (e.g. *ADH1* overexpression).

Figure 11. The custom blue LED construct.