“A molecular rheostat maintains ATP levels to drive a synthetic biochemistry system.”

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* Rheostat that regulates ATP levels by controlling flow down either an ATP generating or non ATP generating pathway according to the free phosphate concentration
* Implemented for production of isobutanol from glucose
* Maintains adequate ATP concentration even in the presence of ATPase contamination
* Used in design of continuously operating, self-sustaining synthetic biochemistry systems
* Need high yield, high productive for economic competitiveness of low-value chemicals such as biofuels
* Constraints imposed by having to maintain cell viability
* We want to build cell free pathways that can economically sustain high flux for long periods of time without the metabolic regulatory systems that exist in cells – requires new design principles
* Must consider generation, regulation, and recycling of high-energy cofactors such as ATP, NADH, and NADPH.
* High energy cofactors generated in catabolic or breakdown phase (glycolysis), used and regenerated in anabolic or biosynthetic phase
* Demand perfect stoichiometry – if 2 atp generated in breakdown, 2 atp used in biosynthetic
* What is a rheostat?
  + A contrivance for adjusting or regulating the strength of electrical currents, operating usually by the intercalation of resistance which can be varied at will

**A stoichiometric isobutanol pathway**

* The 2-ketoacid isobutanol biosynthetic phase employs two equivalents of pyruvate and two equivalents of NADPH, but no ATP
  + Canonical pathway produces 2 equivalents of NADPH and ATP
* They designed a 14 enzyme pathway to make the cofactor use stoichiometric
* They use COPASI to model and identify likely key steps in the pathway
  + An open source software app for creating and solving mathematical models of biological processes such as metabolic network, cell-signaling pathways, regulatory networks, infectious diseases, etc
* Found that Hex and Pfk were critical for achieving flux through the system
* Can focus on detecting only phosphate
  + Can we assume that phosphate concentration is indicative of ATP concentration? Yes if that’s the only thing being phosphorylated in the artificial cell
* Based on high or low P concentrations, certain pathways are preferred

**Design of an ATP rheostat**

* Results showed that ATP depletion is major problem for the long-term sustainability of the stoichiometric reaction system
* Design something that restores and regulates ATP levels as an intrinsic feature of the system
* Molecular rheostat has two comp. pathways that transform G3P into 3PG
* One generates atp, other doesn’t
* mGapDH-PGK branch produces an additional equivalent of ATP compared to the GapN-only branch
* rheostat responds to the depletion of ATP and acts to restore ATP by using the phosphorylating mGapDH branch
* The molecular rheostat reaches a steady state at any time the flux through mGapDH and PGK is greater than the ATPase activity being introduced
  + Steady state can be achieved with wide range of ATPase activities

**Engineering mGapDH**

* They introduced basic residues to help create (30 fold difference) affinity for Nadp+ over NAD+

**Isobutanol production with the molecular rheostat**

* Rheostat reaction held and ATP steady state concentration at round 600 µM for 48 h

**Enzyme stability limits production**

* The reaction completely stopped after 72 hours
* A precipitate began to form after 24 hrs
  + Conincided w decrease in isobutanol production
  + Enzymes were denaturing over time, bc of increasing isobutanol conc
* Complete inactivation of GapN and KivD to isobutanol
* Identified a handful of enzymes that are high priority targets for improving their stability to increase the overall sustainability of the system

**Discussion**

* We can quickly design, implement, and further optimize systems in cell-free synthetic biochemistry environments
* Can quickly identify and isolate
* Synthetic biochemistry systems should run longer, be more flexible
* Rheostat is like metabolite proofreading
* Molecular purge valve – regulates production and consumption of nadp+/nadph
* Major practical limitation for commercial feasibility remains the procurement of enzymes that are sufficiently stable to warrant the added initial investment required to produce them