Metabolic Engineering of New Routes to Biofuels

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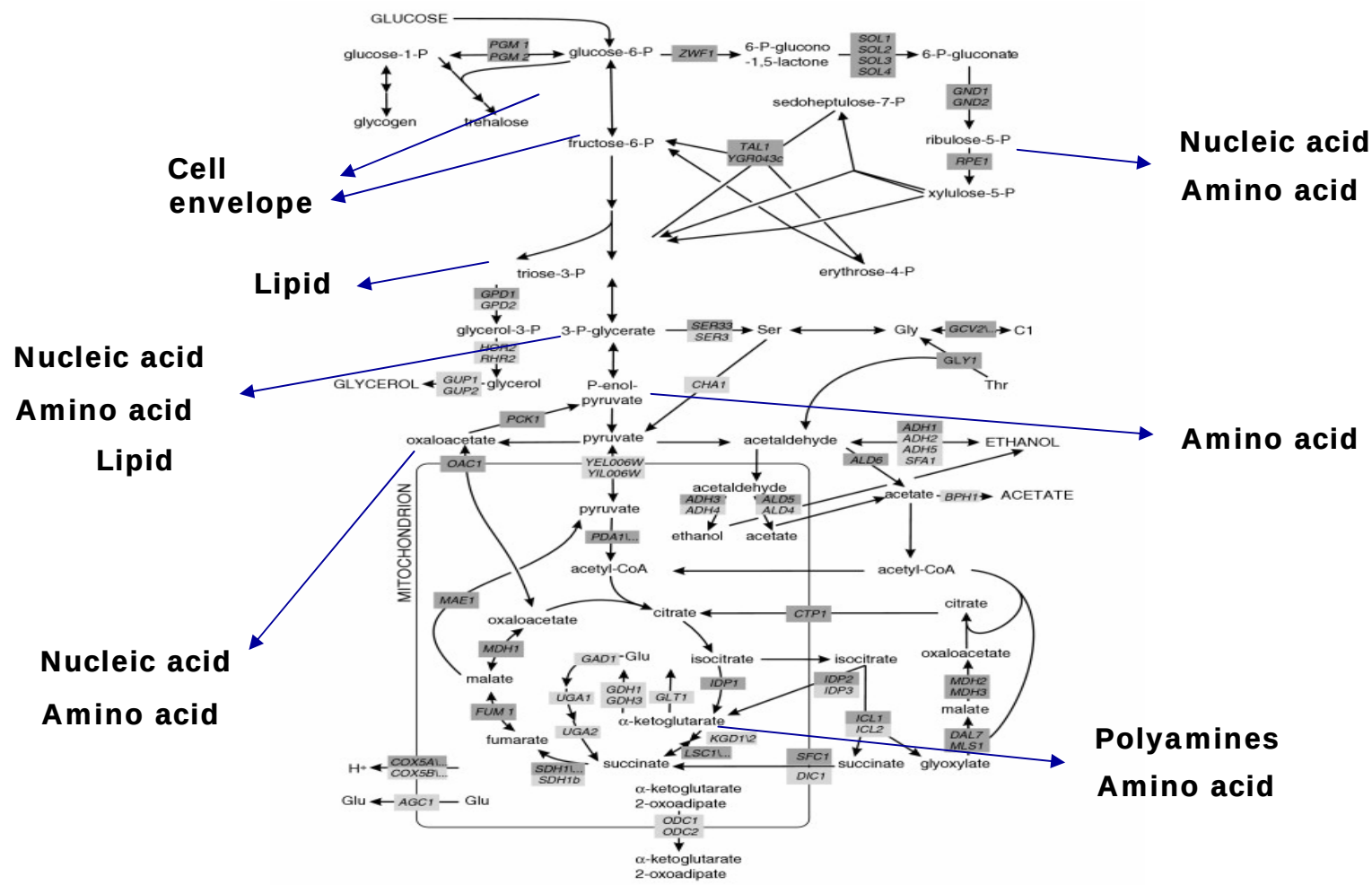
Overview

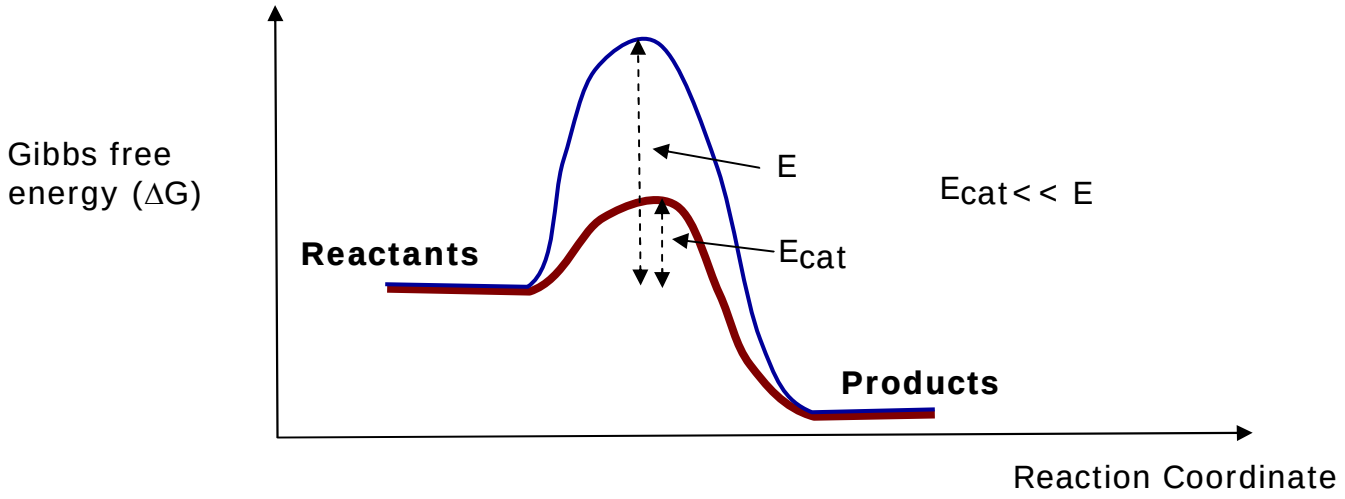
- Metabolism, enzymes, genes
- Bioethanol production in yeast
- Metabolic engineering successes and failures
- Computational systems biology approaches
- Utilisation of xylose as feedstock for yeast fermentation
- Production of butanol in E. Coli
- Future directions for research

Metabolic pathways for energy & biosynthesis



Enzymes

- Enzymes are proteins that catalyse biochemical reactions
- Specific enzymes for specific reactions: alcohol dehydrogenase
- Enzymes reduce the activation energy of the reaction enabling it to run faster



Enzyme Kinetics

- Example: Fermentation by yeast

$$CH_3CH_2OH + NAD^+ \underset{\text{ADH1}}{\overset{\text{ADH2}}{\rightleftharpoons}} CH_3CHO + NADH + H^+$$

(EtOH) (AcAld)

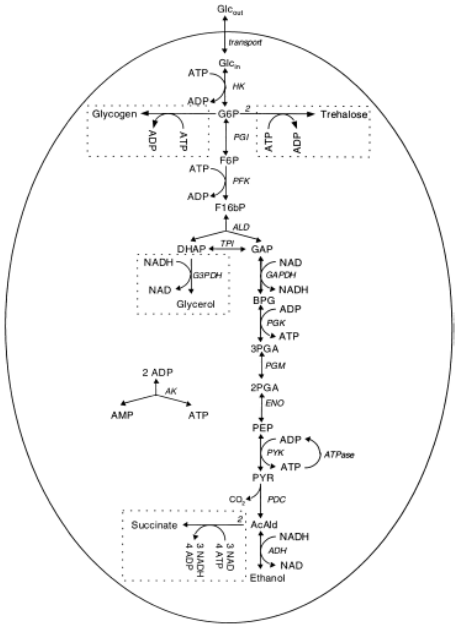
Alcohol dehydrogenase

Byproduct of glycolysis

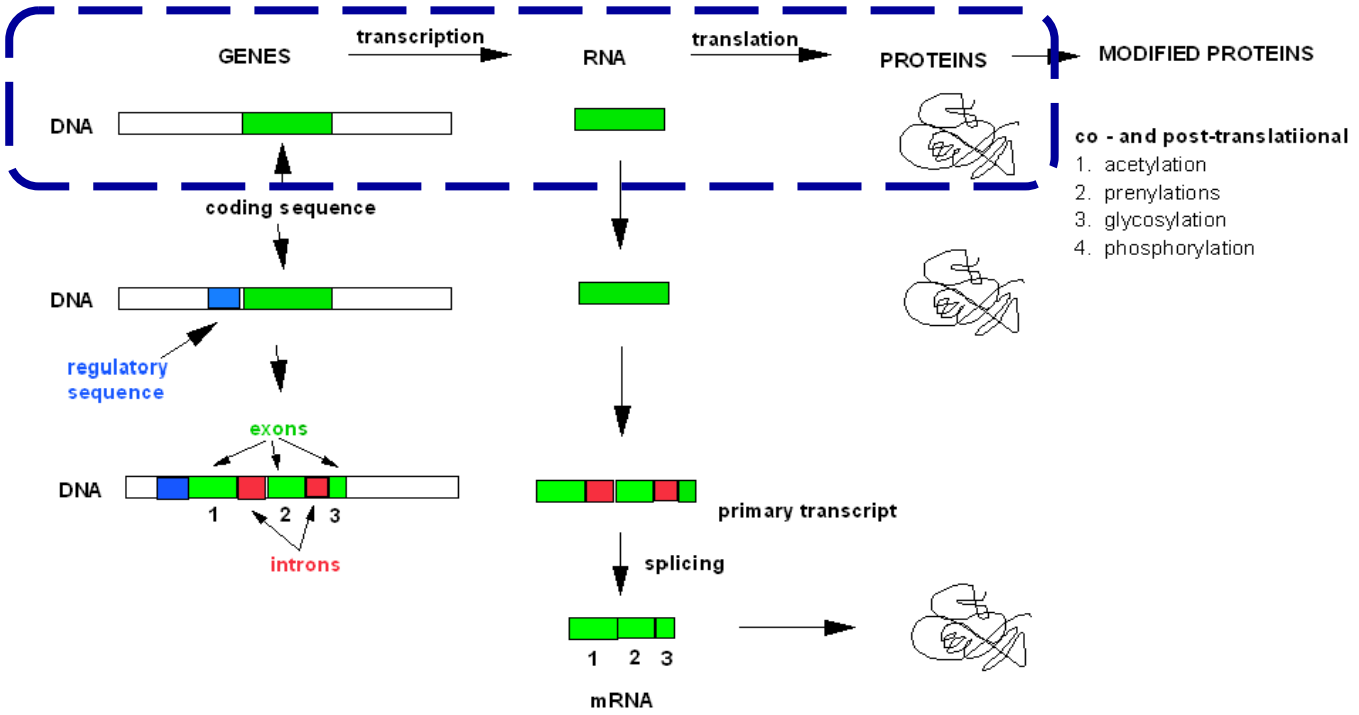
$$v = \frac{\dfrac{k_2 k_3}{k_2\left(1 + \frac{[S]}{K_{ip}} + \frac{[S]}{K_{ip}K_{iL}} + \frac{[S]^2}{K_{ip}^2 K_{iL}}\right) + k_3\left(1 + K_L + \frac{[S]}{K_{ip}}\right)}[E]_0[S]}{1 + a\frac{[S]}{K_{ip}} \quad 1 + b\frac{[S]}{K_{ip}}}$$

$$\frac{k_3 K_{ip} K_L}{1 + b\frac{[S]}{K_{ip}}}$$

$$[S] = \frac{k_2\left(1 + \frac{[S]}{K_{ip}} + \frac{[S]}{K_{ip}K_{iL}} + \frac{[S]^2}{K_{ip}^2 K_{iL}}\right) + k_3\left(1 + K_L + \frac{[S]}{K_{ip}}\right)}{1 + a\frac{[S]}{K_{ip}} \quad 1 + b\frac{[S]}{K_{ip}}}$$



DNA, RNA and Proteins



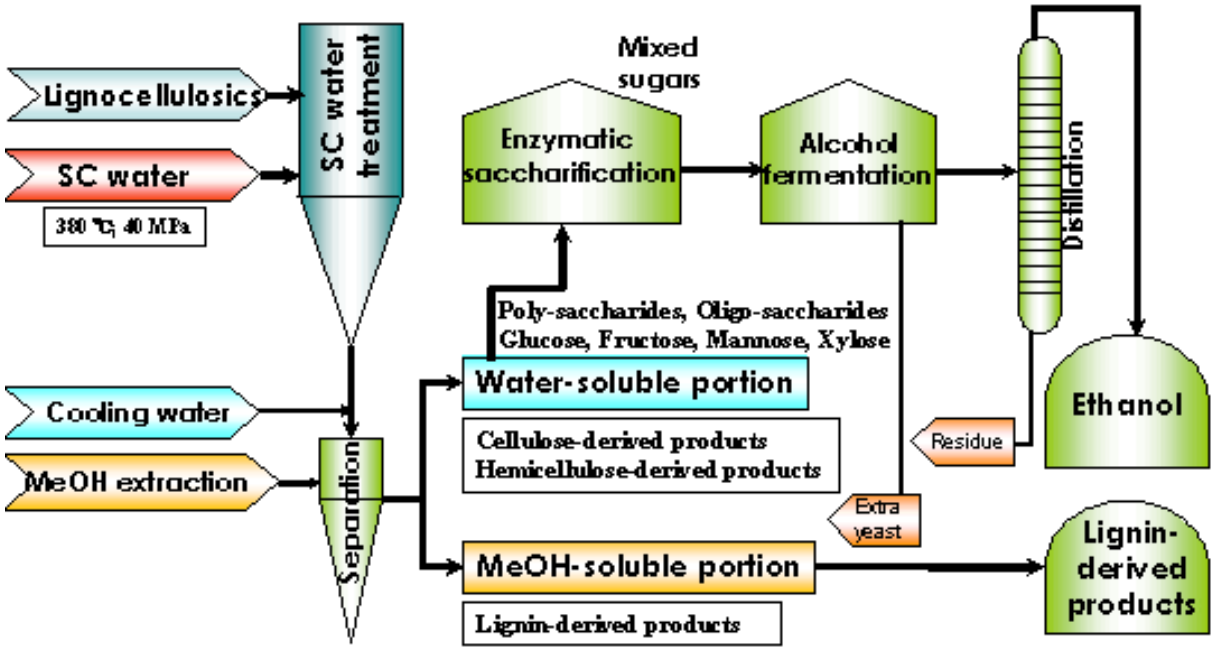
How many genes?: Human = 40,000; yeast = 6,000

About yeast...

- *Saccharomyces cerevisiae*
- Bakers yeast, brewers yeast, budding yeast
- Single celled microorganism (fungi)
- One of the simplest eukaryotes
- Long history and well understood
- Tolerant to high ethanol concentration
- Works at low pH which avoids contamination

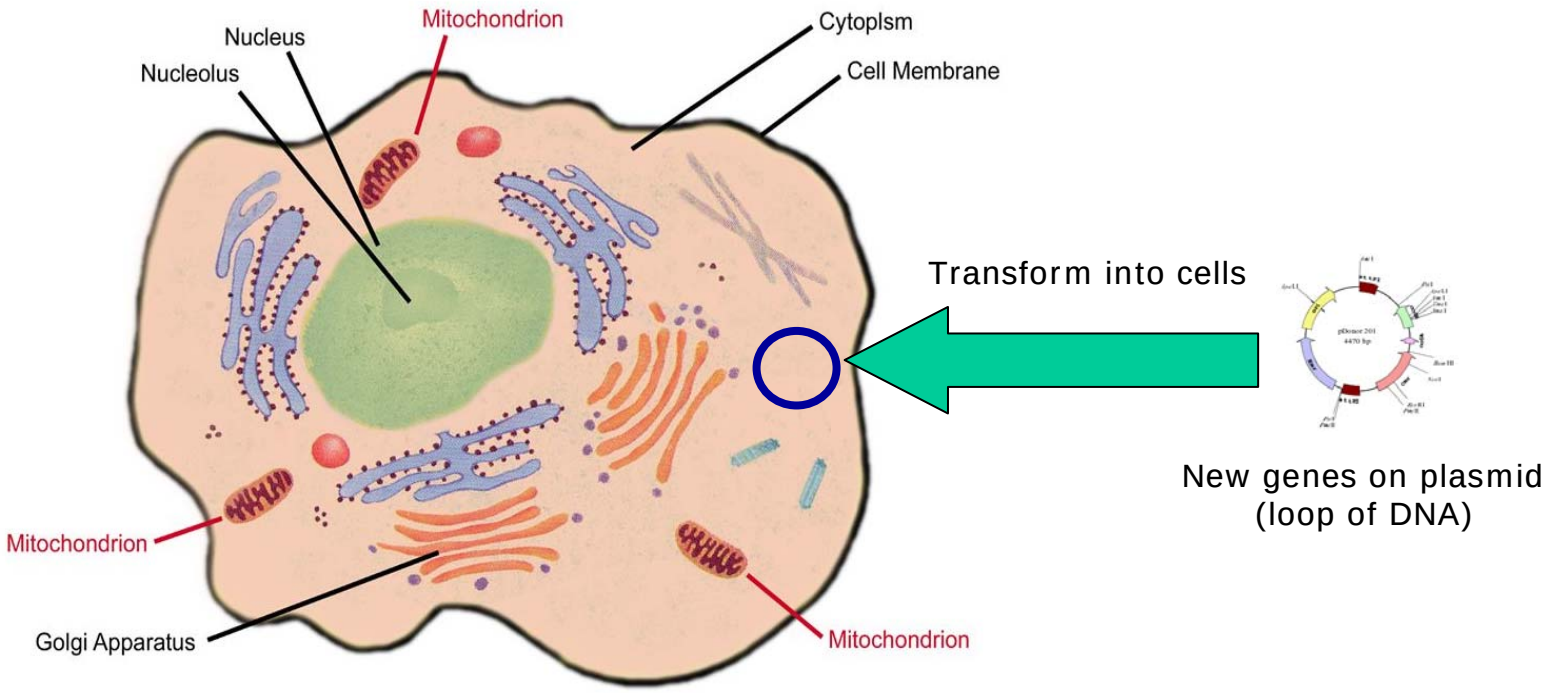
Bioethanol production in yeast

- 22% of all UK greenhouse gas from road transport
- 5-10%: max ethanol in petrol for vehicles to run unmodified



Metabolic Engineering

"Metabolic engineering is the improvement of industrial organisms using modern genetic tools" (James Bailey, ETH)

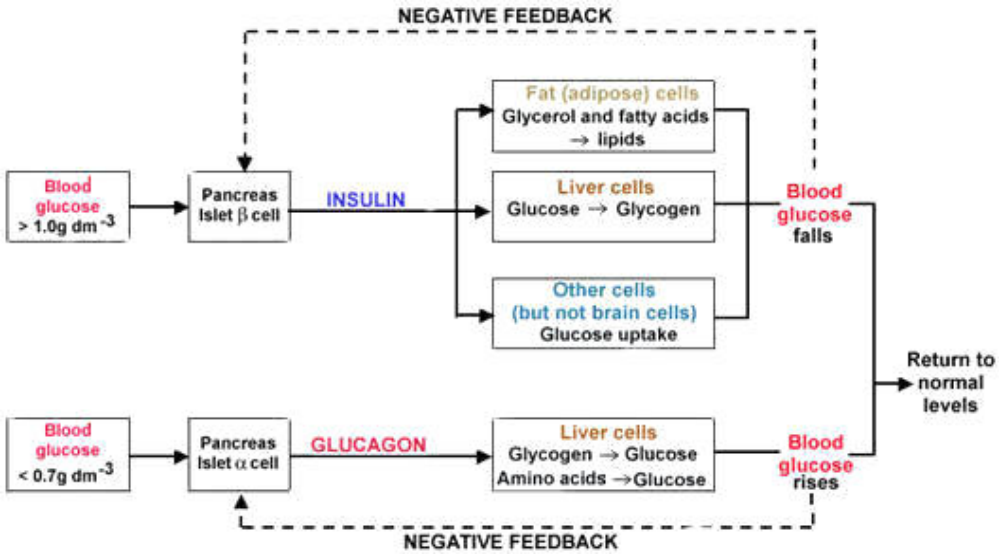


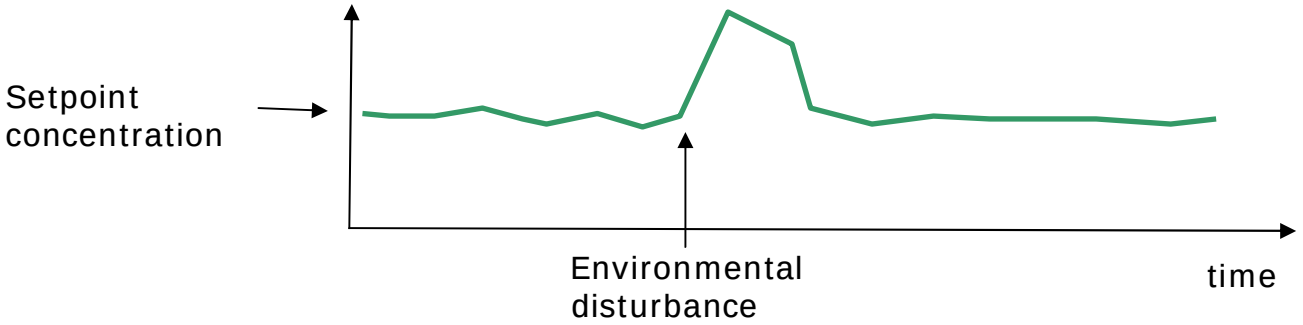
Metabolic Engineering Failures

- Simplistic approaches to find ‘rate limiting’ enzymes have usually failed
  - e.g. overexpression of hexokinase, phosphofructokinase and pyruvate kinase failed to increase the ethanol production rate of yeast

- Reason: cells have evolved to be robust
  - Intrinsic robustness of networks and enzymes
  - Natural redundancy in the metabolic networks
  - Sophisticated control systems for maintaining status quo
  - Regulation of gene expression

Cellular control systems for homeostasis





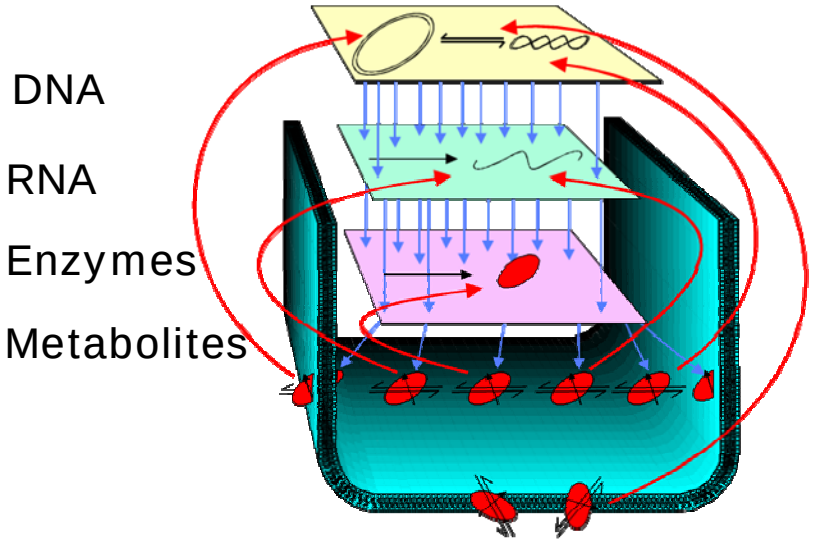
Regulation of genes by signalling pathways

- Glucose repression
  - Triggered by: high levels of glucose
  - Effect: switch off expression of enzymes for other sugars

- AMP kinase signalling
  - Triggered by: low energy levels (ATP:AMP ratio)
  - Effect: increase expression of energy producing enzymes

Computational Metabolic Engineering

- Detailed computational modelling of the cell
- Including all the complex interactions at the ‘system’ level
  - genes, RNA, proteins, metabolites



- Simultaneous and subtle changes in multiple genes required

Metabolic Control Analysis (MCA)

- MCA can be applied to both metabolic and signalling pathways
- MCA = sensitivity analysis
  - how does a small change in parameter X effect model output Y?
- e.g. Flux control coefficients $\mathbf{C_e^J}$ for metabolic networks
  - $\mathbf{C_e^J}$= % change in flux J due to a 1% change in level of enzyme e
  - If 1% increase in enzyme gives 5% increase in flux then $\mathbf{C_e^J}$ = 5
  - If 1% increase in enzyme gives 0.4% increase in flux then $\mathbf{C_e^J}$ = 0.4

Differential Equations for Metabolic Networks

- Chemical equations governing interactions & transformations…

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

(Association, Dissociation, Catalysis)

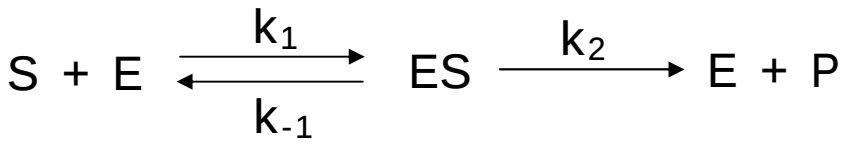
- …are converted into ordinary differential equations (ODEs):

$$\frac{dS}{dt} = -\frac{V_{max} S}{K_m + S}$$

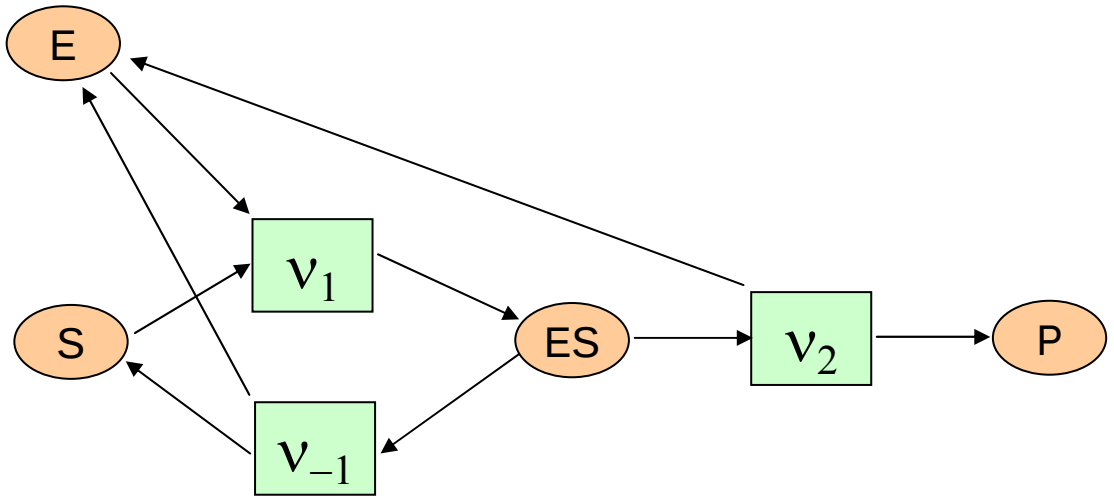
Rate of change of concentration with time

Stoichiometric Matrix

- Back to simple example



$$\frac{d[S]}{dt} = -\nu_1 + \nu_{-1}$$
$$\frac{d[E]}{dt} = -\nu_1 + \nu_{-1} + \nu_2$$
$$\frac{d[ES]}{dt} = \nu_1 - \nu_{-1} - \nu_2$$
$$\frac{d[P]}{dt} = \nu_2$$



$$\begin{bmatrix} \dot{S} \\ \dot{E} \\ \dot{ES} \\ \dot{P} \end{bmatrix} = \begin{bmatrix} -1 & +1 & 0 \\ -1 & +1 & +1 \\ +1 & -1 & -1 \\ 0 & 0 & +1 \end{bmatrix} \begin{bmatrix} \nu_1 \\ \nu_{-1} \\ \nu_2 \end{bmatrix}$$

vector of reaction rates

Stoichiometric matrix

General systems of Differential Equations

- Previous example:

vector of time derivative of species concentrations (system variables)

$$\begin{bmatrix} \dot{S} \\ \dot{E} \\ \dot{ES} \\ \dot{P} \end{bmatrix} = \begin{bmatrix} -1 & +1 & 0 \\ -1 & +1 & +1 \\ +1 & -1 & -1 \\ 0 & 0 & +1 \end{bmatrix} \begin{bmatrix} \nu_1 \\ \nu_{-1} \\ \nu_2 \end{bmatrix}$$

vector of reaction rates

Stoichiometric matrix

- Generally: $\Rightarrow \quad \dot{X} = N\nu$

Each reaction rate is a function of the species concentrations and some parameters

$$\nu_i = \nu_i\left(\overbrace{X_1, X_2, \ldots, X_n}^{\text{variables}}, \overbrace{k_1, k_2, \ldots, k_m}^{\text{parameters}}\right)$$

e.g. Michaelis-Menten $\quad \nu = \dfrac{V_{max}[S]}{K_m + [S]}$

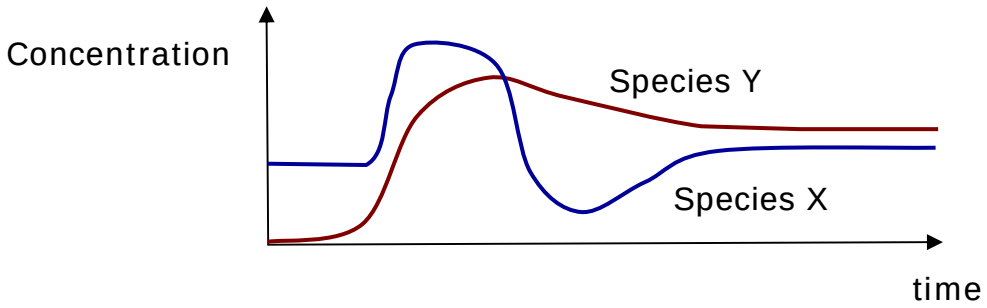
Data required to solve a dynamic (ODE) Model

- ✓ Stoichiometry linking the species and reactions (network structure)
- ✓ Functional form of the reaction rate equations
- ✓ The values of the rate constants in these equations
- ✓ The initial values of all species concentrations
- ✓ The time horizon

Solving (i.e. Integrating) ODE Models

- Use an ODE solver (Matlab, Mathematica, Silicon Cell, etc.)

- Solver output is the concentration profiles over time

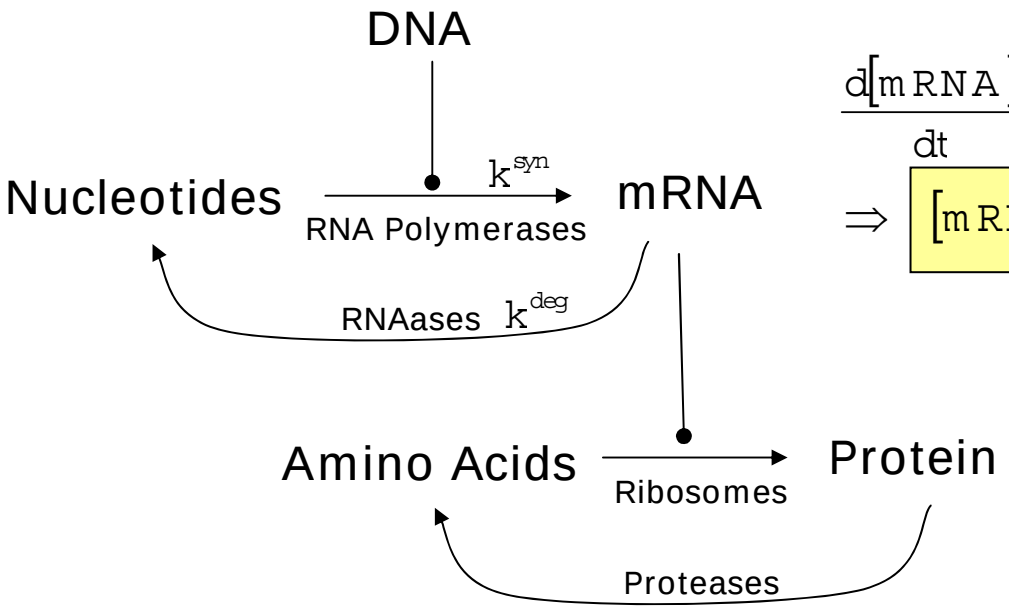


- Compare model output to measured concentration profiles

- If all profiles become flat then the system reaches ‘steady state’

Steady States

- At steady state all the fluxes are in balance
- Total production of each species = total consumption
- Example: Synthesis and degradation of mRNA and proteins



$$\frac{d[mRNA]}{dt} = k^{syn} - k^{deg}[mRNA]$$

$$\Rightarrow \boxed{[mRNA]_{SS} = \frac{k^{syn}}{k^{deg}}}$$

Flux Balance Analysis

- Key idea: look for steady state flux patterns that optimise a given objective function
  - biomass production
  - product yield in metabolically engineered cells

- Use stoichiometric matrix only – flux patterns must satisfy

$$\dot{X} = N\nu = 0$$

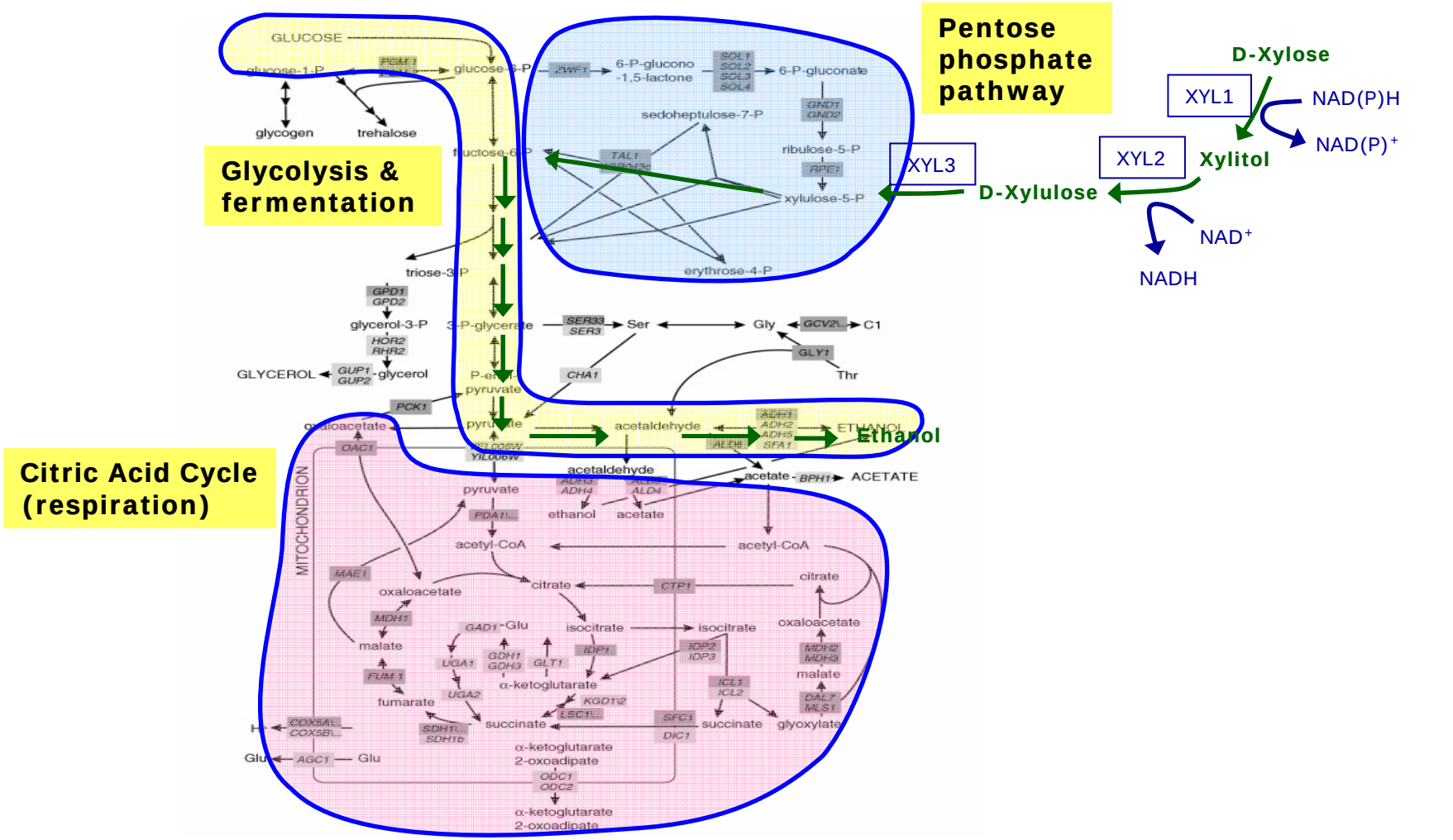
- Ignore kinetics – just have max/min bounds on fluxes

- Find balancing fluxes that maximise flux of product or biomass

Engineering xylose metabolism into yeast

- Xylose is a pentose ('difficult') sugar
- Second most abundant abundant carbohydrate in nature
- Major component of hemicellulose which is ~ 20% plant biomass
- Not a natural substrate for *Saccharomyces cerevisiae…*
- …but there are other native xylose metabolising yeasts
  - e.g. *Pichia Stipitis*

Central Carbon Metabolism



Primary metabolic engineering strategy

- Introduce the following genes (enzymes) from *Pichia stipitis*:
  - D-xylose reductase (XYL1)
  - xylitol dehydrogenase (XYL2)
  - D-xylulokinase (XYL3)

- Growth of this engineered yeast strain is very slow because…

- …Reductive step and oxidative step both require co-factors:
  - NADPH and NAD^+
  - producing $NADP^+$ and NADH respectively

⇒ Excess accumulation of NADH under oxygen limitation

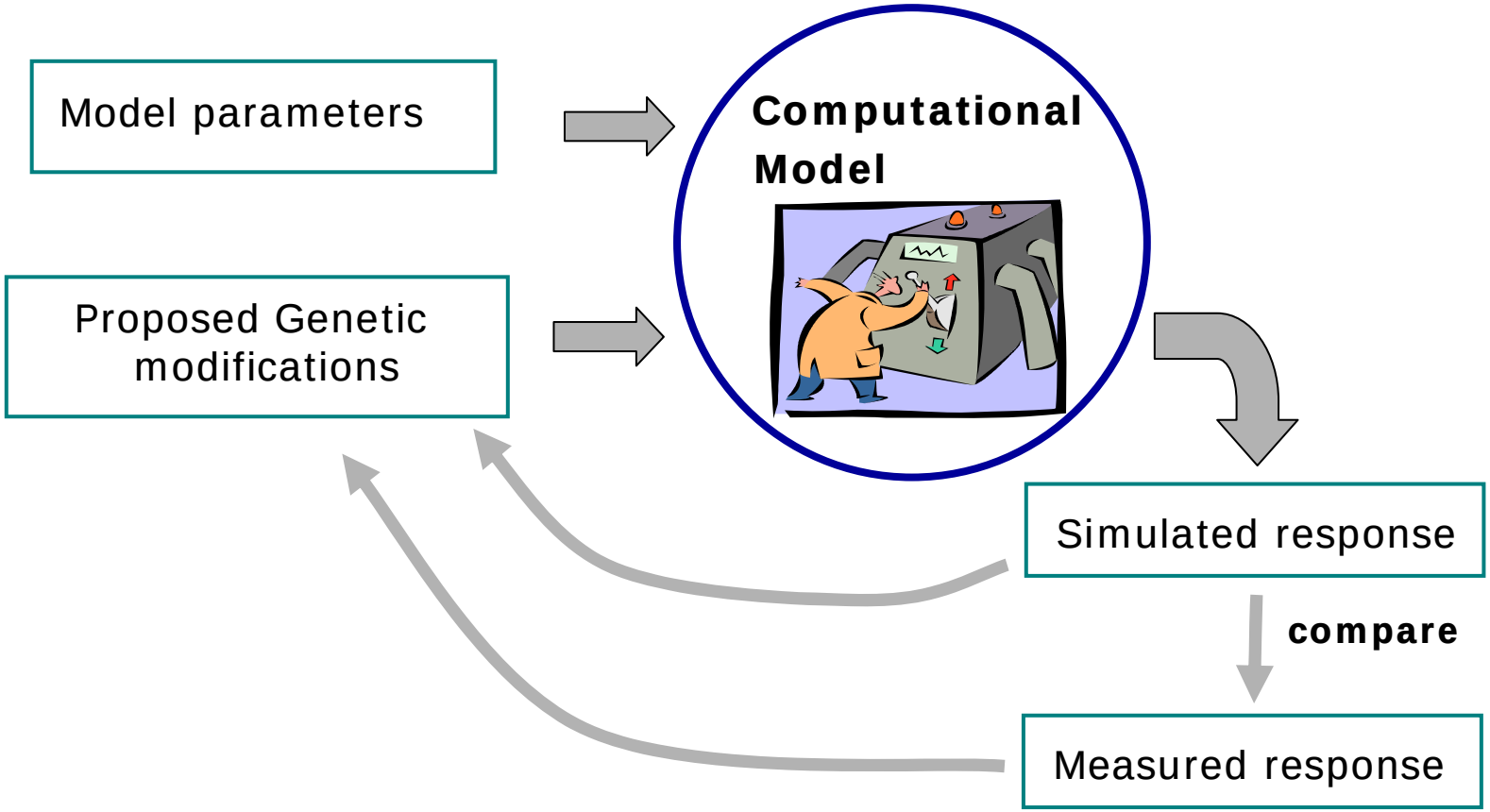
Model based approaches to fix NADH problem

- Modify balance between glutamate dehydrogenase isoenzymes
  - Delete $NADP^+$ dependent GDH1 – glutamate dehydrogenase
  - Overexpress NAD^+ dependent GDH2
  - Improves ethanol production

- Genome-scale model found 56 out of 3,500 reactions that improved ethanol yield
  - e.g. introduction of a new glyceraldehyde 3-phosphate bypassing enzyme

- Evolutionary adaptation under continuous anaerobic conditions

Iterative Metabolic Engineering



Drawbacks of ethanol as transportation fuel

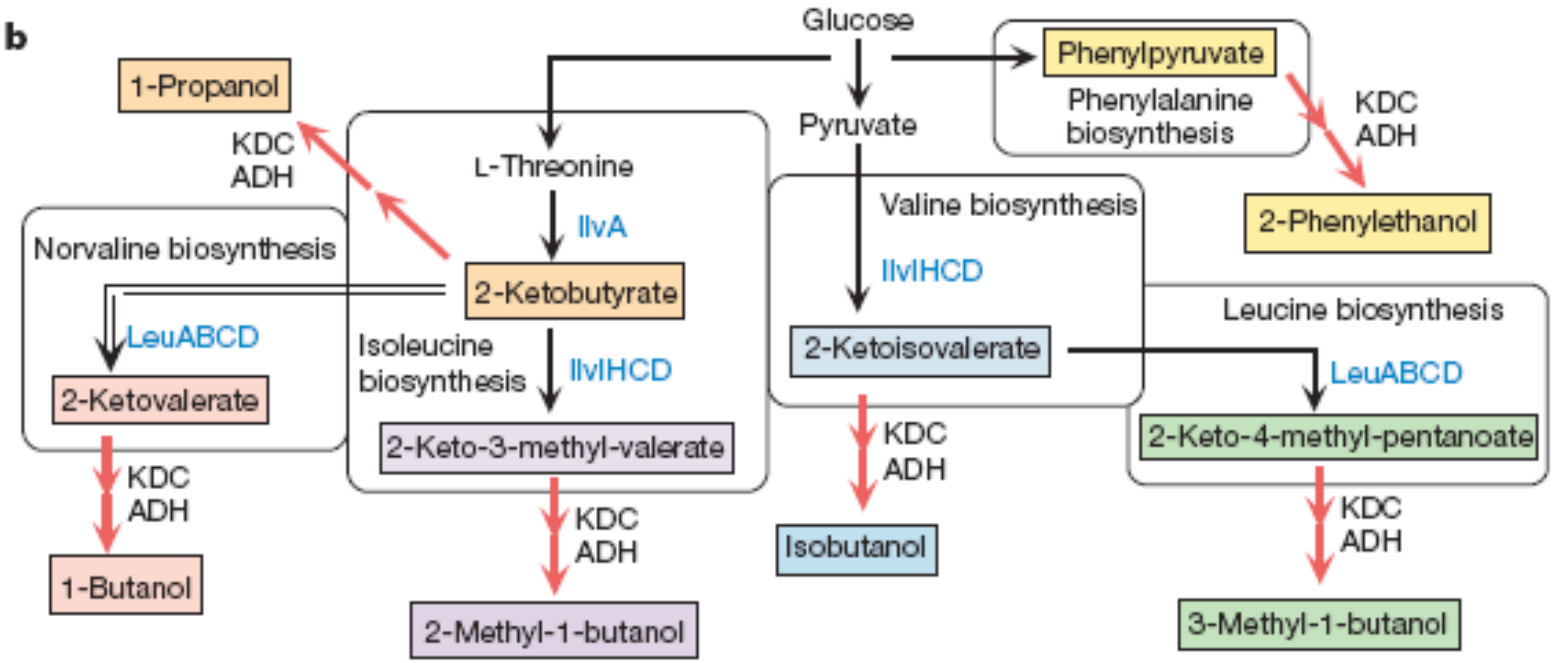
- Few vehicles can run unmodified on >10% EtOH fuel blends
- Lower energy content than gasoline
- Corrosive to metals in engines and pipelines
- Readily absorbs water
- Expensive to purify from fermentation broths

What are the alternatives?...

Butanol as a transportation fuel

- Butanol (4-carbons) is more like petrol (4-12 carbons)
- Higher energy density than ethanol (88% vs. 66%)
- Less corrosive and less water soluble than ethanol
- 85% Butanol/gasoline blends used in unmodified engines
- Can transport in the same pipelines as petrol
- Easier to integrate into the existing transportation infrastructure

Engineering production of butanol in E. Coli



Improving Butanol Yields

Genetic modification	**Isobutanol production (mM)**
Introduce 2-keto acid decarboxylase (KDC) from *lactococcus lactis*	-
Introduce alcohol dehydrogenase (ADH) from *saccharomyces cerevisiae*	4
Overexpression of *ilvHCD* genes to enhance 2-ketoisovalerate biosynthesis	23
Deletion of *adhE, ldhA, frdAB, fnr, pta* genes that contribute to by-product formation	30
Replace *ilvHCD* with *alsS* gene from Bacillis subtilis which has higher affinity for pyruvate	50
Deletion of *pflB* to decrease competition for pyruvate	300

300 mM = 22 g per litre butanol
Yield = 0.35 g butanol per g glucose = **86% of theoretical maximum**

Other directions for research

- Genetically engineered plants with less ‘biomass recalcitrance’
- Engineered multi-enzyme systems - In vitro metabolic pathways
- ‘Global transcription machinery engineering’: gTME
  - Introduce mutations into yeast transcription factors
  - Select for improved ethanol & glucose tolerance
- Longer chain alcohols & alkanes from fatty acid synthesis routes

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