CM4125 Bioprocess Engineering Lab: 
Week 4: Introduction to Metabolic 
Engineering and Metabolic Flux Analysis

Instructors: David R. Shonnard¹, Susan T. Bagley²

Laboratory Teaching Assistant: Abraham Martin-Garcia

1 Chemical Engineering, 2 Biological Sciences
Michigan Technological University, Houghton, MI 49931

January 31, 2006
102 Chemical Sciences and Engineering Building
Presentation Overview

- Introduction to Metabolic Engineering
- An Example of Metabolic Engineering: Ethanol Production from Lignocellulosic Biomass Using a Genetically-Engineered *E. coli*.
- Metabolic Flux Analysis (MFA)
- Defining the Reaction Stoichiometry in MFA
- Defining the Reaction Rate Equations in MFA
- Summary
What is Metabolic Engineering?

“the directed improvement of product formation or cellular properties through the modification of specific biochemical reactions or the introduction of new ones with the use of recombinant DNA technology”


The targeting of specific biochemical reactions within the cell for modification is the essential feature of metabolic engineering. To achieve this targeting, we use Metabolic Flux Analysis (MFA).
Old Practice of Metabolic Engineering

The concept of manipulating cellular metabolism for the purpose of increasing production of a desired product is an old one. Microorganisms have been developed with enhanced metabolic features.

- DNA mutations using chemical treatment
- Selection techniques to identify strains
- Notable successes

(C. glutamicum for L-lysine production, other strains for production of amino acids, antibiotics, solvents, and vitamins.)
Modern Practice of Metabolic Engineering

"The combination of analytical methods to quantify fluxes and their control with molecular biological techniques to implement suggested genetic modifications is the essence of metabolic engineering."

- Fundamental biochemical pathway studies
- Engineering analysis of biochemical reaction pathways
- Monitoring of excreted metabolic products (HPLC)
- Monitoring of intracellular metabolites and biomass constituents (using carbon 13 labeled substrates)
- Modern molecular biology techniques

An Example of Metabolic Engineering: 
Ethanol from Lignocellulosic Biomass

Sustainable Forestry

Biomass Processing
Photo: Glacial Lakes Energy

Electricity Export and Carbon Credit

CO₂

Logistics

Vehicular Performance

Picture: 2004 H2 Alpha
A Shift to Bio-Based Products

Earth’s production of plant biomass is 8 times the current consumption of energy. The DOE estimates that US forests can provide 0.10 - 0.40 billion dry tons biomass/yr.

Harvest energy from the earth’s surface, and close the cycle for CO₂

Improved forest management, genetically improved crops, optimized bioprocessing, advanced engine technologies, and hybrid powertrains can be used in an integrated effort to displace 10 - 55% of U.S. gasoline demand (2004).
Bio-fuels Benefits

- Lower emission of climate active CO₂/mile
- Decreased demand for imported petroleum
- Job creation and economic diversification

For a 50 million gallon biomass ethanol / year facility
- ~ $100 million / year from sale of ethanol
- Stimulate $200 million / year in the local economy
- ~190 new long-term jobs + 450 spin-off jobs
- 500 construction jobs for 2 years
Process to Convert Cellulosic Biomass to Ethanol

1–3 mm

0.8% H₂SO₄
160°C
10 min

Trichoderma reesei cellulases

Genetically-engineered E. coli


or process heat and power
Composition of Dry Cellulosic Biomass

Dry Cellulosic Biomass

- Cellulose (35-50%)
  - Glucose
  - 6-C sugars
  - Hydrolysis

- Hemicellulose (20-35%)
  - Xylose
  - Arabanose
  - Mannose
  - Galactose
  - 5-C sugars
  - Hydrolysis

- Lignin (12-20%)
  - No hydrolysis
The Challenge of Fermenting all Sugars in Lignocellulosic Biomass

<table>
<thead>
<tr>
<th>Saccharomyces cervisiae</th>
<th>Escherichia coli</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Zymomonas mobilis</strong></td>
<td></td>
</tr>
<tr>
<td>Ferment glucose to ethanol</td>
<td>Can not ferment glucose to ethanol</td>
</tr>
<tr>
<td>Utilize 6C sugars only</td>
<td>Can utilize 6C and 5C sugars</td>
</tr>
<tr>
<td>Tolerant to ethanol</td>
<td>Is it easier to genetically engineer <em>E. coli</em> to ferment ethanol?</td>
</tr>
<tr>
<td>Can these microorganisms be genetically engineered to utilize 5C sugars?</td>
<td></td>
</tr>
</tbody>
</table>

*MichiganTech*
There is a competition for pyruvate among different pathways. The pathway containing the highest concentration of enzymes and where these enzymes have the highest activity (Vmax) and affinity (Km) will have the highest flux of metabolites and divert the majority of the substrate to endproduct.
Genetically Engineer This Pathway into *E. coli*

Two genes are needed. One for pyruvate decarboxylase and another for alcohol dehydrogenase. These enzymes working together in the cell will divert Pyruvate away from other fermentation products to ethanol. This would convert *E. coli* into an ethanol-producing microorganism, where before it was not!

"Principles of Biochemistry", Lehninger, Worth

![Reaction diagram](image)

**Figure 15-17**
Terminal steps in alcoholic fermentation.
Genetic Engineering of Ethanol Production in *E. coli*

A plasmid for Pyruvate decarboxylase (pdc)

A plasmid for Alcohol dehydrogenase (adh)

Ethanol Production in Sealed Cultures of *E. coli* TC4

High Performance Liquid Chromatography Profiles

G = glucose  
S = succinate  
L = lactic acid  
A = acetic acid  
U = unknown  
E = ethanol

Using a different recombinant strain of E. coli, the Ingram group obtained these results under both aerobic and anaerobic conditions. Wild type E. coli metabolizes pyruvate through PDH and PFL yielding primarily cell growth, acetate and CO2. In the recombinant strain under aerobic conditions, significant ethanol is produced because the Km for heterologous PDC from Z. mobilis is comparable to endogenous PDH and much lower than for endogenous LDH. Furthermore, Km for heterologous ADH II from Z. mobilis is about a factor of 4 lower than Km for endogenous NADH oxidase, thus effectively diverting pyruvate to ethanol. Under anaerobic conditions, again Km for PDC and ADH II are significant lower than for LDH and PFL, and the apparent Km of the native enzymes involved in NAD+ regeneration are higher than for Z. mobilis ADH II.
“Overall, overexpressed ethanologenic Z. mobilis enzymes in E. coli are quite competitive with respect to the native enzymes in channeling carbon (pyruvate) and reducing power (NADH) to ethanol.”
Metabolic Flux Analysis (MFA)

The purpose of MFA is to describe with the minimum number of equations the flow of material through a cellular metabolic network. This requires that the set of stoichiometric and/or reaction rate equations be solved for the concentrations of intracellular metabolic intermediates and/or pathway fluxes using measured values of extracellular metabolic products.

We start with a general stoichiometry for cellular reactions.
Stoichiometry of Cellular Reactions

Consider a network with \(N\) substrates that are converted to \(M\) metabolic products (extracellular and measurable) and \(Q\) biomass constituents (DNA, protein, lipids – also measurable). The network consists of \(J\) reactions involving \(K\) intracellular metabolites (not measurable, but predicted). A mole balance on the \(j\)th cellular reaction yields

\[
\sum_{i=1}^{N} \alpha_{ij} s_i + \sum_{j=1}^{M} \beta_{ij} p_j + \sum_{j=1}^{Q} \gamma_{ij} x_{macro,j} + \sum_{j=1}^{K} g_{ij} x_{met,j} = 0
\]

where subscript \(i\) refers to each substrate, product or metabolite, \(S\) is substrate, \(P\) is extracellular product, \(X_{macro}\) is a biomass constituent, and \(X_{met}\) is a pathway intermediate.
Stoichiometry of Cellular Reactions (cont.)

In the metabolic model there will be an equation like the one on the previous slide for each of the \( J \) cellular reactions. It is convenient to write the stoichiometry for all \( J \) reactions in a compact form using matrix notation.

\[
\begin{align*}
AS + BP + \Gamma X_{macro} + GX_{met} &= 0
\end{align*}
\]

where \( A, B, F, \) and \( G \) are matrices containing stoichiometric coefficients in the \( J \) reactions for the substrates, products, biomass constituents, and pathway intermediates, respectively.
Some Rules in Defining Metabolic Reactions

1. Only intracellular metabolites at **branch points** in the metabolic pathway are included in the set of reactions.
2. Reactions on **linear pathways** between branch points are not included since all of these reactions proceed at the same rate at steady state.
3. All metabolites along a linear pathway may be **lumped** into an overall reaction for that linear pathway.
4. Include all substrates and products in the set of reactions.
5. Reactants are assigned negative stoichiometric coefficients.
6. Reaction products are assigned positive coefficients.
In mixed fermentation, seven products are produced from the uptake of glucose
In mixed fermentation, eight metabolic reactions define the reaction network. A total of five intracellular metabolites are also included. Notice that no biomass constituents are included because no anabolic reactions are involved and only fueling reactions are included. With a total of 13 chemicals and only 8 reactions, the degrees of freedom for this problem is 5, thus \textbf{atleast} 5 concentrations must be measured in order to determine the other intracellular metabolites and extracellular products.
The fourth column of the G matrix contains the stoichiometric coefficients for ATP metabolism under anaerobic conditions. The third and seventh reactions in the network are responsible for ATP production. Intracellular ATP production can be measured directly by monitoring acetate production and the difference between glucose uptake and succinate production. Thus, intracellular metabolite flux can be measured by monitoring extracellular products; a key foundation of MFA. Knowing intracellular ATP provides an estimate of ATP consumption rate for cell growth and maintenance.
The redox balance (NADH balance) has to close for the conversion of sugar to different fermentation products, the matrix stoichiometric equations indicate that the uptake of glucose must be related to the formation of succinate, lactate, and ethanol.
NADH Balance in Anaerobic Fermentation
Mixed Acid Fermentation by *E. coli*


<table>
<thead>
<tr>
<th>Metabolic product</th>
<th>Moles formed per 100 mol of glucose fermented</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formate</td>
<td>2.0</td>
</tr>
<tr>
<td>Acetate</td>
<td>36.0</td>
</tr>
<tr>
<td>Lactate</td>
<td>79.5</td>
</tr>
<tr>
<td>Succinate</td>
<td>10.7</td>
</tr>
<tr>
<td>Ethanol</td>
<td>49.8</td>
</tr>
<tr>
<td>CO₂</td>
<td>88.0</td>
</tr>
<tr>
<td>H₂</td>
<td>75.0</td>
</tr>
</tbody>
</table>

NADH Consumed Determined from Stoichiometry

\[ 2 \times 10.7 + 79.5 + 2 \times 49.8 = 200.5 \]

which matches 200 moles NADH produced per 100 moles glucose consumed!

The redox balance (NADH balance) has to close for the conversion of sugar to different fermentation products. The matrix stoichiometric equations indicate that the uptake of glucose must be related to the formation of succinate (rxn 2), lactate (rxn. 4), and ethanol (rxn. 8).
Rates of Cellular Reactions

In MFA, the rates of enzyme catalyzed reactions in metabolic pathways is of even greater importance than stoichiometry. The purpose of metabolic engineering is to alter the flux of material through metabolic pathways, and therefore the ability to predict intracellular fluxes, and more importantly, to identify enzymatic reactions to modify is a primary objective.

The net specific uptake rate for the $i$th substrate is the sum of its consumption rate in all $J$ reactions.

$$r_i = \sum_{j=1}^{J} a_{ij} v_j$$

where subscript $j$ refers to each reaction in the network and $v_j$ is the rate of the $j$th reaction in the network.
Rates of Cellular Reactions (cont.)

Similarly, the net specific rate of formation of the $i$th metabolic product is

$$r_{p,i} = \sum_{j=1}^{i} \beta_{i,j} v_j$$

For the biomass constituents and intracellular metabolites we can write

$$r_{\text{macro},i} = \sum_{j=1}^{i} \gamma_{i,j} v_j$$

$$r_{\text{met},i} = \sum_{j=1}^{i} g_{i,j} v_j$$

We can write these summation equation compactly in matrix notation.

The matrix equations are a compact way to show that a vector of net component fluxes ($r$) is equal to a matrix multiplied by a vector of reaction rates ($v$), where the coefficients in these matrices are the transpose of the elements in the stoichiometric matrices (each row of the stoichiometric matrix is situated in the equivalent column of the transposed matrix). We wish in fact to determine the vector of reaction rates in order to solve for the metabolic fluxes in the reaction network.
Rates of Cellular Reactions:
Mixed Acid Fermentation by *E. coli*

The specific glucose uptake rate is given by the matrix equation

\[
\begin{pmatrix}
\nu_1 \\
\nu_2 \\
\nu_3 \\
\vdots \\
\nu_n
\end{pmatrix} = -\left( \begin{array}{cccc}
-\frac{1}{2} & 0 & 0 & 0 \\
0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 \\
\vdots & \vdots & \vdots & \vdots \\
0 & 0 & 0 & 0
\end{array} \right)
\begin{pmatrix}
\nu_1 \\
\nu_2 \\
\nu_3 \\
\vdots \\
\nu_n
\end{pmatrix}
\]

Thus the flux from glucose to PEP, which is given by \( \nu_1 \), is 2 times the specific glucose uptake rate.

Rates of Cellular Reactions: (cont.)

Mixed Acid Fermentation by *E. coli*

For the five intracellular metabolites we state these five rate equations as,

\[
\begin{align*}
T_{PEP} & = 1 -1 -1 0 0 0 0 0 \\
T_{PYR} & = 0 0 1 -1 -1 0 0 0 \\
T_{AcCoA} & = 0 0 0 0 1 0 -1 -1 \\
T_{ATP} & = 0 0 1 0 0 0 1 0 \\
T_{NADH} & = 1 -2 0 -1 0 0 0 -2 \\
\end{align*}
\]

At steady state, many of the rates on the left hand side are zero. The measurements of extracellular products and biomass components will allow for the prediction of these fluxes.

Summary of ME and MFA Concepts

- Metabolic Engineering is a new and powerful approach to understand cellular metabolism and then improve production of desired products from microorganisms.

- New microorganisms have been created with enhanced metabolic features that allows for efficient production of desired products from microbial fermentations (Ethanol from lignocellulosic biomass example)

- Metabolic Flux Analysis (MFA) allows for prediction of intracellular reaction fluxes using a set of metabolic reactions and measurements of extracellular products.