

CENG364 Biomolecular Engineering

Course Objectives:

Introduce engineering students to how fundamental chemical engineering principles (such as biochemical reaction kinetics, thermodynamics of biological systems, and bioreaction networks) are relevant to a large range of biological processes of practical relevance, at cellular and industrial scale.



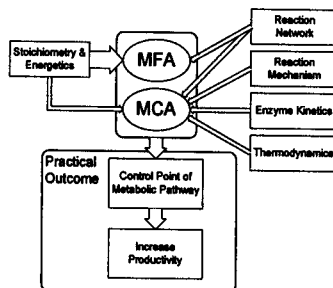
CENG364 Biomolecular Engineering

Course Objectives:

As part of an on-going CELT program, chemical engineering students will be exposed to Excel VBA programming and this will be applied to practical engineering problems within the course.



Metabolic Engineering



Course Integration

Stoichiometry	= MFA Engineering Calculations
Enzymes, Thermodynamics	= MCA Metabolic Engineering Project on Excel VBA
Metabolic Engineering	= MFA (Fed-Batch) MCA (Simple Pathways Applications)
Reaction Networks	= Systems GEPASI



Aim of lecture:

- ① Demonstrate MFA Analysis on metabolic process of moderate complexity
- ② Show major steps
- ③ Use a practical example of inhibitory production

Examples include:

(a) Recombinant E. coli \Rightarrow Acetate

(b) Yeast \Rightarrow Ethanol

(c) Animal Cells \Rightarrow Lactate and Ammonia

④ Inhibitory Products

(a) Slow the reaction rate down

(b) Reduce product yield if they are not the desirable end product.

⑤ Illustrate how practically product inhibition may be overcome by:

(a) REACTOR BASED APPROACH

(fed batch, continuous cultivation at low growth rates)

(b) ENZYME BASE APPROACH

(enzyme inhibition / deletion)

** (b) is one example of natural selection / molecular

Increased Productivity of Desired Products

Productivity increases of desired products may be achieved by:

1. Reactor Based Approaches
2. Enzyme Based Approaches
3. Metabolic Engineering



Reactor Based Approaches

The reactor is used to promote desirable reactions and to limit less favorable ones.

Fed-batch is a good example, where the production of inhibitory products (e.g. ethanol in yeast, acetate in recombinant E.Coli and Lactate in animal cells) is limited and desired products (either biomass or an end product) is favoured.



Enzyme Based Approaches

1. Enzyme Insertion or Deletion
2. Enzyme Concentration Increase
3. Enzyme Velocity Increase
4. Replacement of One Enzyme with Another
5. Modification of an Enzyme and its kinetic properties
6. Use of Enzyme Inhibitors / Activators



Metabolic Engineering Approaches

Metabolic Flux Analysis (STEADY-STATE assuming that all cellular intermediates are low in concentration and constant)

Metabolic Control Analysis (DYNAMIC but analysis undertaken under one set of operating conditions with one set of enzyme concentrations and one set of intermediate concentrations)



Metabolic Engineering Approaches

METABOLIC FLUX ANALYSIS

- Detailed Knowledge of Stoichiometry and Energetics Required
- Steady-State Process
- Can be used to indicate how overall defined metabolic improvements may be made by increasing the flux of some reactions and decreasing the flux of others
- Mechanisms Used:
 - Enzyme Insertion or Deletion
 - Enzyme Concentration Increase/Decrease
 - Enzyme Activity Increase/Decrease
 - Replacement of One Enzyme With Another
 - Modification of an Enzyme and its Kinetic Properties
 - Use of Enzyme Activators and/or Inhibitors
(Most Common in BLUE)



Metabolic Engineering Approaches

METABOLIC CONTROL ANALYSIS

- Detailed Knowledge of Stoichiometry and Energetics Required
IN ADDITION, NEED ENZYME DATA (VELOCITY -v- SUBSTRATE CONCENTRATION) FOR ALL ENZYMES - PREFERABLY FOR ALL SUBSTRATES, PRODUCTS, CELL INTERMEDIATES, ATP/NAD(H), INHIBITORS AND ACTIVATORS
- Steady-State / Pseudo Steady State Process
- Provides a "snap shot" of prevailing metabolic condition of cell at the prevailing intracellular conditions of enzyme concentration and activity and intermediates concentrations
- Can be used to identify the "rate limiting enzyme (or most likely a number of rate limiting enzymes) and suggest modifications to enzyme activity which can lead to a desired increased flux
- Mechanisms Used:
 - Enzyme Insertion or Deletion
 - Enzyme Concentration Increase/Decrease
 - Enzyme Activity Increase/Decrease
 - Replacement of One Enzyme With Another
 - Modification of an Enzyme and its Kinetic Properties
 - Use of Enzyme Activators and/or Inhibitors
(Most Common in BLUE)



Importance of Programming to Enable Metabolic Flux Analysis and Metabolic Control Analysis

- Metabolic Flux Analysis (MFA) requires the use of Matrices and Gaussian elimination to solve a metabolic pathway map. This can be done manually or may be automated by programming techniques (e.g. Excel VBA)
- Metabolic Control Analysis (MCA) requires inversion of a matrix from a relationship involving a number of matrices.
- These mathematical manipulations can be undertaken in a number of platforms (eg Mathematica, Matlab, Polymath, Excel VBA programming, etc.)



Importance of Programming to Enable Metabolic Flux Analysis and Metabolic Control Analysis

- Some of these packages are not freely available and expensive (e.g. Mathematica), have limited programming and numerical methods capacity (e.g. PolyMath) or require specialist knowledge and experience to use (e.g. Matlab). In addition, automatic repetitive calculations require programming skills.



Importance of Programming to Enable Metabolic Flux Analysis and Metabolic Control Analysis

- Students will be taught Excel VBA as a commonly available, programming language suitable for a wide range of engineering applications. All of the necessary mathematical manipulations necessary to understand MCA and MFA theories and practically apply them to practical metabolic pathway analysis and improvement are possible using this package and some elementary programming
- Although simple input/output packages are available for some applications (e.g. Gepasi for MCA), it is important that students understand the underlying basis of the calculations and the necessary solution procedures necessary for their solution.



CENG364 Projects

- Three projects will be undertaken using Excel VBA:
 - Metabolic Flux Analysis (MFA) by Gaussian Elimination of Constructed Matrices (PROJECT 2)
 - Calculation of Thermodynamic Properties at Cellular Conditions (K_{eq} of various metabolic reactions) (PROJECT 3)
 - Metabolic Control Analysis (MCA) of a Metabolic Pathway and its Comparison to Analysis Packages available (e.g. GEPASI) (PROJECT 4)

These projects will replace the mid-term exam



CENG364 Biomolecular Engineering

"MACRO-LEVEL"

(Mass and Energy Balances, Metabolic Flux Analysis)



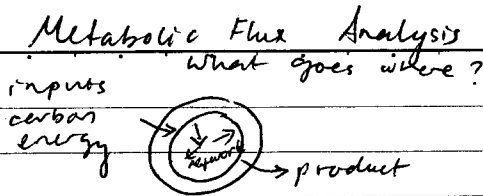
"MICRO-LEVEL"

(Enzyme Mechanisms, Mechanisms of Energetics including Transport, Cell Organelles and Cell Signaling)



SYNTHESIS OF "MACRO" and "MICRO" LEVEL
(Systems Biology, Pathways Synthesis, Metabolic Engineering)





quantitative
 amount of desired inputs +
 outputs
 CONSTRUCT INTERNAL DISTRIBUTION
 IN THE PATHWAYS.

Simple Example : E. coli
 Production of Inhibitory Product

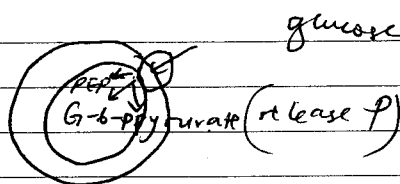
- ① MFA Analysis
 Practical way of overcoming inhibition
- ① Reactor Based (Fed-batch / Continuous)
 - ② Enzyme (Deletion / Inhibition)

Complicated system (large no. of reactions) ⇒ ① Large amount of experimental data
 ② necessary method: no longer sensible by hand ⇒ matrix solution

MFA lecture slide (case study) slide 17/47

- Information
- ① μ , yield data, product data
 - ② biosynthetic data

- (a) What compounds the cell is made of
- (b) How much of each component is needed to form 1 kg biomass (our basis)



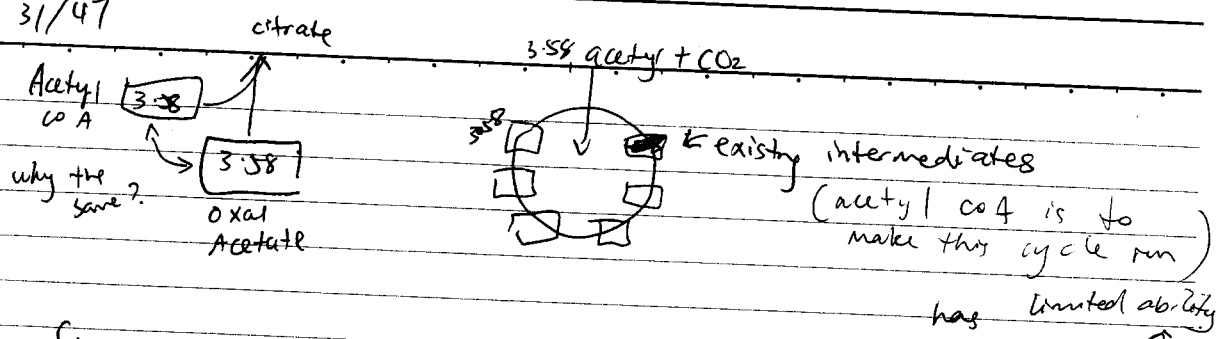
glucose comes into cell as
 glucose-6-P
 every mol of glucose taken up,
 ⇒ equivalent pyruvate
 manufactured from PEP.

★ (Phosphotransferase System (PTS))
 transfer phosphate from PEP → glucose

name: some idea of what reaction does
 replaced by a number of system based on enzyme class/
 function.
 ↳ specific to reaction.

★ Acetate is the inhibitory product

slide 31/47



Overflow metabolism = glycolysis very much faster than TCA
 ↓
 Product excretion (acetate) (inhibitory ∴ undesirable)

Case 1
 glucose net result - large glucose uptake, limited TCA cycle activity
 - large acetate excretion

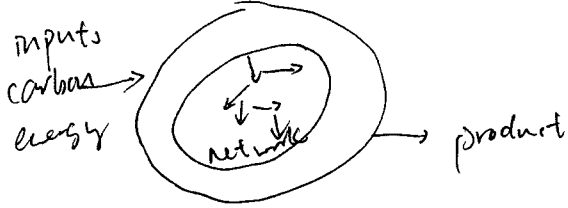
Case 2
 Pyruvate ^{worse} > even more glucose: large 3C uptake (our requirement for G-6-P production for biosynthesis)
 = limited TCA cycle capacity
 ⇒ even larger acetate production

Case 3
 fumarate enters TCA, but can process little use for GEP production. (not excessively, will cost too much energy let air go back to glucose) * Very low acetate.

Practically can't use fumarate, use glucose to adjust culture or modify enzymes.

~~Glucose~~ Glucose
 ↓
 some are slow → undesirable (stuff problem to another comp) (lactate)
 or
 another product (slow down uptake of glucose)
 desirable

what goes where



quantitative amount of defined inputs & outputs constant internal distribution in the pathway.

Complicated System \Rightarrow (large net of reaction)

- ① Amount of Experiment Data Necessary
- ② Solution method: no longer sensible "by hand"

Information so far

- ① M, Yield data, product data.
- ② Biosynthetic Data,
 - (a) what compounds the cell is made from
 - (b) how much of each compound is needed to form 1g biomass

Case 1 large glucose uptake
 net glucose result \Rightarrow limited TCA cycle activity \Rightarrow large acetate excretion.

Case 2

Pyruvate: even more large 3C uptake
~~complete~~ low requirement for G6P production for biosynthesis
 Limited TCA Cycle capacity
 Even larger acetate production.

Case 3.

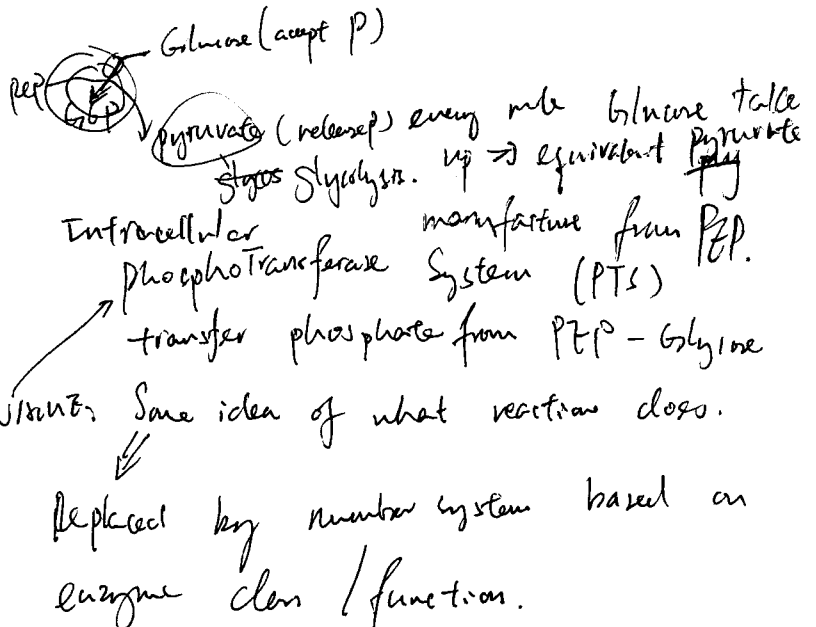
Fumarate enters TCA but only close to what the cell can process
 can't go back to glucose too much energy.

Simple Example

E. coli production of inhibitory product
 ① MFA analysis.
 practical way of necessary inhibitors.

- ① Reactor based (Fed-batch / continuous)
- ② Enzyme (deletion / inhibition)

Amount of Experiment Data Necessary
 Solution method: no longer sensible "by hand"



SPECIFIC TO REACTION



If we cut the two
→ glucose → undesirable
or ... slow down uptake
shift problem to another ~~compound~~ (an other product)

Student Name: Teh Yin Er.
 Student ID: 08520018

Y6
 11/3 (Wed.)
 (0.30 ~ 11.50 a.m.)

pg 2

PRACTICAL APPLICATION

METABOLIC FLUX ANALYSIS → 'by hand' → MATRICES
 EXCEL VBA
 (where do the material entering the cell go to??)

PRODUCT INHIBITION IS USED AS THE EXAMPLE Homework/Project
 LYSINE (a.a.)

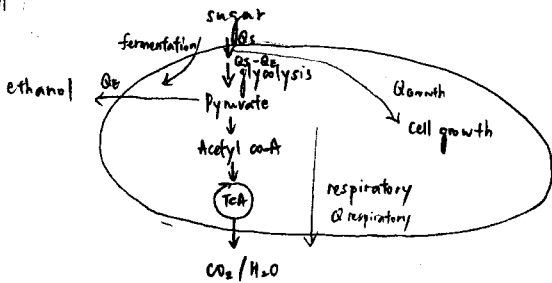
- Overcome this by
1. Doing a metabolic flux analysis to identify where the problem is
 2. Reduce inhibition effect by
 - (a) reactor based approach (Fed-Batch)
 - (b) enzyme manipulation (Enzyme deletion/Inhibition)

- Feedback by product slows down process
- Inhibitory product may not be the desired product

Examples

Recombinant bacteria (E-coli) → some desired product + ACETATE
 Yeast → ETHANOL
 Animal Cells → LACTIC ACID, NH₃ → inhibitory products

pg 9, 10, 11



Case 1: under-determined

Aim: to calculate $Q_{respiratory}$
 but only using Q_{is} and Q_{G}
 can't calculate since
 Q_{growth} is unknown.

Case 2:

only 1 solution

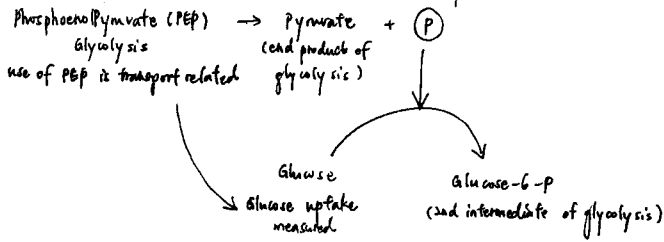
Case 3: over-determined

There is a possibility of
 multiple sets of solution

pg 22

pg 23

We assume that biosynthesis are made of these 8 precursors.



Metabolic Flux Analysis

what goes where?
We will now undertake a Metabolic Flux Analysis 'By Hand'

The purpose of this is to illustrate the calculation of carbon flow through the cell and to illustrate how knowing the fluxes can assist in identifying "limiting reactions" or bottlenecks and (potentially) offering strategies for improvement (e.g. enzyme deletion, enzyme enhancement etc)



quantitative and of defined inputs & outputs



Metabolic Flux Analysis

The development of strategies to improve metabolic performance is the basis of another technology, viz. METABOLIC ENGINEERING.

We will study the underlying theory of metabolic engineering and undertake some calculations in later lectures. Metabolic Flux Analysis should be seen as a precursor to Metabolic Engineering.



Metabolic Flux Analysis

We will also examine the concept of "Overflow Metabolism" and how practically we can manipulate the metabolism by "cell culture means". Metabolic improvement may be made by a number of available techniques, for example:

- Genetic Engineering (Enzyme deletion, enhancement, introduction)
- Cell Culture Manipulation (Batch, Fed-Batch, Continuous)
- Use of Metabolic Inhibitors



Metabolic Flux Analysis

What is a FLUX ??

The most common flux unit is mols / g biomass / h

Mols are used since the use of weights leads to some confusion as 100 mols of glucose, for example, is converted to 144 g Glucose-6-Phosphate and then in two more steps to 198 g of Glyceraldehyde-3-Phosphate and Dihydroxyacetone Phosphate.

When mols are used, 1 mol of glucose becomes 1 mol of Glucose-6-Phosphate and 1 mol each of Glyceraldehyde-3-Phosphate and Dihydroxyacetone Phosphate.



Metabolic Flux Analysis

What is Metabolic Flux Analysis?

Simply put, it is the systematic representation of an overall metabolism by a diagram linking the major catabolic and anabolic pathways together using their common biosynthetic intermediates (carbon skeletons) and having these biosynthetic intermediates form cellular components through monomers or polymers or both.

Fluxes are then assigned to the reactions in the diagram based on experimental measurements



Metabolic Flux Analysis

Flux Analysis Organises DATA, so we must define WHAT DATA WE REQUIRE

WHEN you measure everything that goes IN
WHEN you measure everything that goes OUT
AND you know the metabolic routes to connect these
THEN you can write a complete description of what is happening in the process



Metabolic Flux Analysis

Experimental measurements may be of many types:

Primary measurements:

Substrate concentration, Product Concentration, Exit Gas concentrations

Pulse-Chase Experiments with labelled substrates

NMR

Derived measurements:

Metabolic Flux Analysis

Degrees of Freedom (F)

=

Number of Substrates (S)

+

Number of Products (P)

+

Number Metabolic Intermediates Included (M)

-

Number of Constraints (C)

If $F >$ Number of Independent Experimental Measurements Available, then the system is under-determined

If $F <$ Number of Independent Experimental Measurements Available, then the system is over-determined

If $F =$ Number of Independent Experimental Measurements Available,

Metabolic Flux Analysis

Example:

How many experimental measurements are needed to determine the flux through the fermentative pathway (ethanol) and respiratory pathway of a yeast when these are the only catabolic pathways? Assume no oxygen is used for anabolism.

Case 1:

Q_S and $Q_{ETHANOL}$ known

$Q_{ETHANOL}$ can be used to calculate $Q_S^{FERMENTATION}$

$Q_S - Q_S^{FERMENTATION}$ can be used to calculate the flux of sugar going to respiration ($Q_S^{RESPIRATION}$) and to cell growth (Q_S^{GROWTH}).

Without some measurement the flux of sugar used for cell growth, the flux of sugar going to respiration cannot be determined.

This is an example of a under-determined system.



Metabolic Flux Analysis

Case 2:

Q_S and $Q_{ETHANOL}$ known, % Carbon in cell is known

$Q_{ETHANOL}$ can be used to calculate $Q_S^{FERMENTATION}$

% Carbon and Q_S can be used to calculate Q_S^{GROWTH}

$Q_S - Q_S^{FERMENTATION} - Q_S^{GROWTH}$ can be used to calculate the flux of sugar going to respiration ($Q_S^{RESPIRATION}$)

This is an example of a system capable of solution



Metabolic Flux Analysis

Case 3:

In addition to Q_S , $Q_{ETHANOL}$, % Carbon in cell being known, Q_{CO_2} is known

$Q_{ETHANOL}$ can be used to calculate $Q_{CO_2}^{FERMENTATION}$

$Q_{CO_2} - Q_{CO_2}^{FERMENTATION}$ can be used to describe $Q_{CO_2}^{RESPIRATION}$

$Q_{CO_2}^{FERMENTATION}$ and $Q_{CO_2}^{RESPIRATION}$ can be used to calculate

$Q_S^{FERMENTATION}$ and $Q_S^{RESPIRATION}$

$Q_S - Q_S^{FERMENTATION} - Q_S^{RESPIRATION}$ can be used to calculate Q_S^{GROWTH}

This could have been calculated using % Carbon data

This is an example of a over-determined systems where different combinations of data can be used to calculate what is wanted but the whole data set is unnecessary.

Excessive data = 1



Metabolic Flux Analysis

Case 4:

In addition to Q_S , $Q_{ETHANOL}$, % Carbon in cell being known, Q_{CO_2} and Q_{O_2} is known

Now five combinations of data can be used to calculate what we want :

Q_S and $Q_{ETHANOL}$ known, % Carbon (Case 2)

Q_S , $Q_{ETHANOL}$, Q_{CO_2} (Case 3)

Q_{O_2} , $Q_{ETHANOL}$, % Carbon in cell

Q_{CO_2} , Q_{O_2} and, % Carbon in cell

Q_S , Q_{O_2} and, % Carbon in cell

Excessive data = 2



Metabolic Flux Analysis

In a system is over-determined, then the redundancy of the measurements can be used to:

- Calculate the rates of non-measured metabolites
- Increase the accuracy of the available measurements by application of least squares calculation
- Identify the source the measurement error or inconsistency



Metabolic Flux Analysis

citric acid cycle

Metabolites

A and B are the measured concentrations and carbon content

Metabolite	A (g/100g)	B (g/100g)	Carbon Content (%)
Pyruvate	40.00	40.00	40.00
CO ₂	10.00	10.00	10.00
Lactate	10.00	10.00	10.00
Cell mass	10.00	10.00	10.00

The net yield from glucose is 0.50 g biomass/g glucose used.

(A) Calculate the % glucose used for Oxidation and the % glucose used for Anabolism.

The relative composition of the protein is based on the carbon content of each of the below amino acids:

Amino Acid	% Carbon	Carbon Content (%)
Arginine	6	70.00
Asparagine	6	47.00
Aspartic acid	7	47.00
Cysteine	20	30.00
Glutamic acid	11	50.00
Valine	6	60.00
Leucine	8	60.00
Glutamine	11	50.00
Alanine	7	50.00
Proline	6	47.00

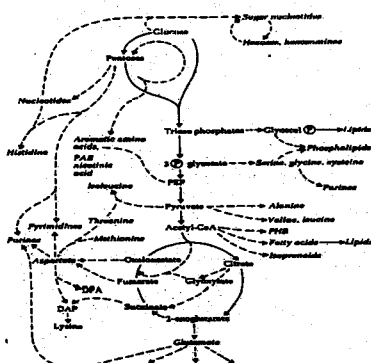
The relative number of metabolites may be assumed to average 20% from the Protein Composition Fraction and 10% from PEP in the Citric Acid Pathway.

Labels represent the mass of grams used in each unit.

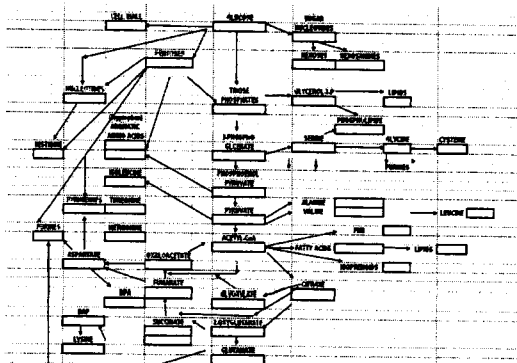
Using the correct stoichiometry, calculate the net yield, when the glucose carbon flow is based on a carbon and % carbon in cell mass.



Metabolic Flux Analysis



Metabolic Flux Analysis



Metabolic Flux Analysis

Flux Analysis of E. Coli ML308 on Glucose, Pyruvate or Fumarate As Sole Sources of Carbon

Data for the system: μ_c

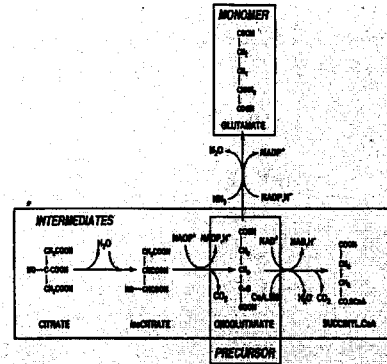
CASE 1: 11.24 mols Glucose form 1kg Biomass, 5.20 mols Acetate with CO_2 as the only other product. The specific growth rate is $0.94h^{-1}$

CASE 2: 56.12 mols Pyruvate form 1kg Biomass, 26.10 mols Acetate, 0.08 mols Lactate. The specific growth rate is $0.07h^{-1}$

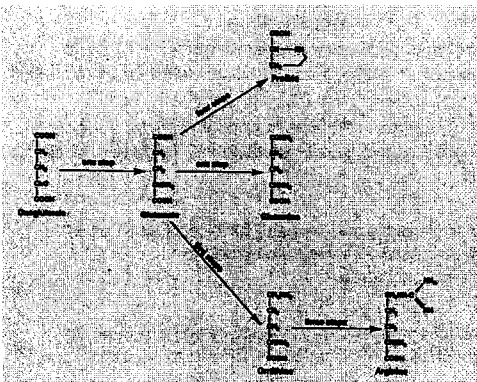
CASE 3: 26.23 mols Fumarate form 1kg Biomass, with CO_2 as the only product. The specific growth rate is $0.63h^{-1}$



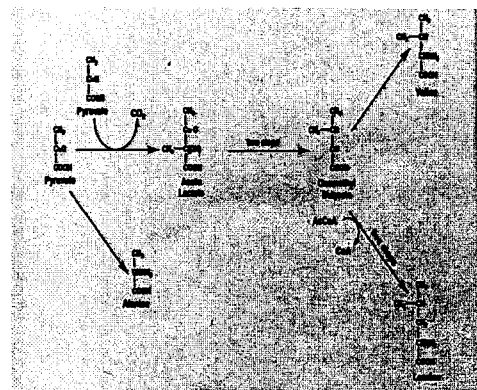
Metabolic Flux Analysis



Metabolic Flux Analysis



Metabolic Flux Analysis



Metabolic Flux Analysis

Alanine is made from 1 pyruvate

Valine is made from 2 pyruvates

Leucine is made from 1 pyruvate and one acetyl-CoA

All other cell components can be treated in the same way and can be derived from a limited number of precursors (or carbon skeletons or biosynthetic intermediates). Monomers are compounds that are directly polymerised into polymers and may or may not be equivalent to these biosynthetic intermediates.



Metabolic Flux Analysis

Metabolite number	Glucose	Pyruvate	Acetyl-CoA	Other	Total
Ala	0.000	0.000	0.000	0.000	0.000
Arg	0.000	0.000	0.000	0.000	0.000
Asp	0.000	0.000	0.000	0.000	0.000
Asn	0.000	0.000	0.000	0.000	0.000
Cys	0.000	0.000	0.000	0.000	0.000
Glu	0.000	0.000	0.000	0.000	0.000
Gly	0.000	0.000	0.000	0.000	0.000
Ile	0.000	0.000	0.000	0.000	0.000
Leu	0.000	0.000	0.000	0.000	0.000
Lys	0.000	0.000	0.000	0.000	0.000
Met	0.000	0.000	0.000	0.000	0.000
Phe	0.000	0.000	0.000	0.000	0.000
Pro	0.000	0.000	0.000	0.000	0.000
Thr	0.000	0.000	0.000	0.000	0.000
Trp	0.000	0.000	0.000	0.000	0.000
Tyr	0.000	0.000	0.000	0.000	0.000
Val	0.000	0.000	0.000	0.000	0.000
ADP	0.116	0.116	0.116	0.000	0.348
ATP	0.000	0.000	0.000	0.000	0.000
CoA	0.116	0.116	0.116	0.000	0.348
UMP	0.000	0.000	0.000	0.000	0.000
UMP	0.116	0.116	0.116	0.000	0.348
UTP	0.000	0.000	0.000	0.000	0.000
Uridic acid	0.000	0.000	0.000	0.000	0.000
UMP	0.140	0.140	0.140	0.000	0.420
Carbohydrate	1.000	1.000	1.000	0.000	3.000
Total	1.00	1.14	1.30	0.00	3.44

Information so far:

1. M₁ yield data as product data

2. Biosynthetic data

(a) what are products the cell is made from

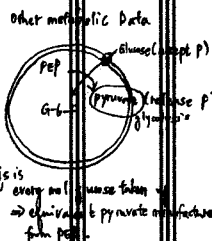
(b) how much of each average cell is needed to form 1kg biomass (on basis)



Metabolic Flux Analysis

Other relevant metabolic information:

- Glucose uptake is by the glucose phosphotransferase system (PTS). PEP is converted to Pyruvate and is used as the phosphate donor. All other pyruvate formed from PEP is undertaken by pyruvate kinase.
- 1 mol Fructose-6-P is converted to 2 mols of Triose-P - hence two boxes are included in the diagram
- Some Acetyl-CoA is used to form oxaloacetate. However, the majority of acetyl-CoA is converted to CO_2
- The majority of the precursors from the TCA Cycle come from PEP via the enzyme PEP carboxylase which converts PEP to oxaloacetate using CO_2
- The thiol ester of Acetyl-CoA is exchanged with inorganic phosphate to give acetyl phosphate which phosphorylates ADP to form acetate which is excreted via a proton

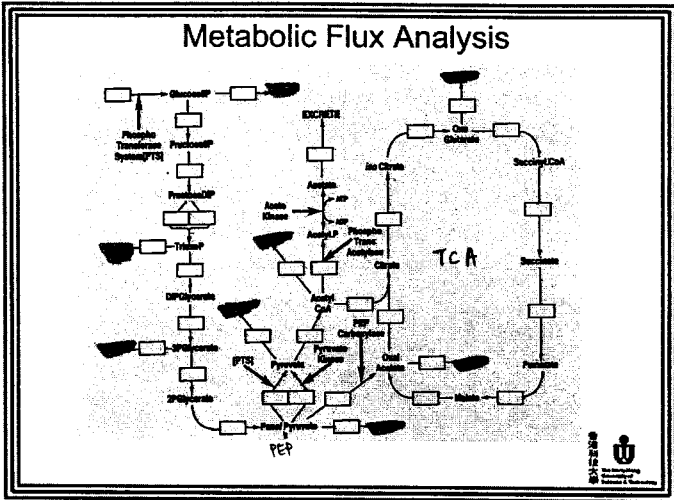


Metabolic Flux Analysis

The first step in the process is to draw a metabolic diagram which represents the known pathways and their interactions



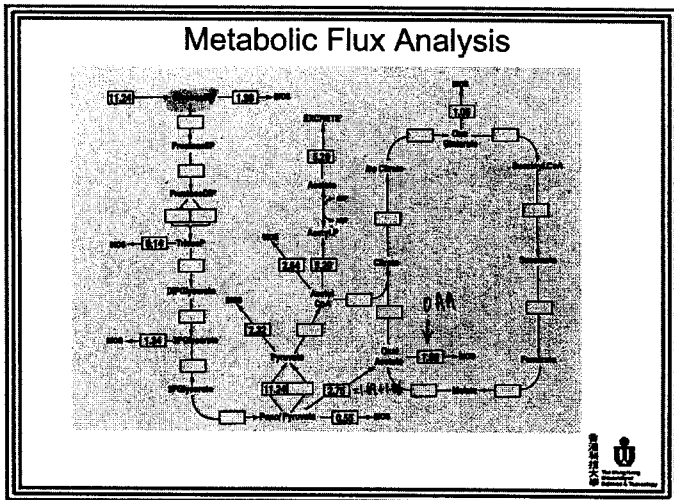
complicated system (large # of reactions) → @ out of experimental data necessary
 @ s/s method: no longer visible 'by hand' ⇒ matrix etc.



Metabolic Flux Analysis

CASE 1: 11.24 mols Glucose form 1kg Biomass, 5.20 mols Acetate with CO₂ as the only other product. The specific growth rate is 0.94h⁻¹

- When 11.24 mols of glucose is taken up, 11.24 mols of PEP must be used in its transport.
- The biosynthetic requirement from the TCA cycle is 1.69 mols from oxaloacetate and 1.06 mols from oxo-glutarate, making a total of 2.75 mols which must come from PEP to oxaloacetate conversion.
- Excretion of 5.20 mols acetate takes 5.20 mols from acetyl-CoA
- Other biosynthetic requirements are 0.14 mols from TrioseP, 1.34 mols from 3-P-glycerate, 2.32 mols from pyruvate, 0.55 mols from PEP and 2.64 mols from acetyl-CoA
- Of the 11.24 mols of glucose entering the glycolytic pathway, 1.98 mols of Glucose-6-P is taken off for biosynthesis



Metabolic Flux Analysis

The remainder is simple arithmetic:
 11.24 mols of glucose are delivered as 11.24 mols Glucose-6-P of which 1.98 mols are used for biosynthesis.

The remainder 9.26 mols is phosphorylated again and delivered as two trioseP totalling 2*9.26 mols = 18.52 mols

0.14 mols of TrioseP is used for biosynthesis and the remainder (18.38 mols) is oxidised to diphosphoglycerate.

After 1.34 mols of 3-phosphoglycerate are used for biosynthesis, 17.04 mols of PEP are available

Metabolic Flux Analysis

11.24 mols of PEP are committed to Glucose Uptake, 0.55 mols are needed for biosynthesis and 2.75 mols for anaplerotic provision of the TCA Cycle.

The balance (2.50 mols) is available for pyruvate kinase and thus converted to pyruvate, of which a total of 13.74 mols are available.

Of this 13.74 mols pyruvate, 2.32 mols are used for biosynthesis and 11.42 mols decarboxylated to Acetyl-CoA.

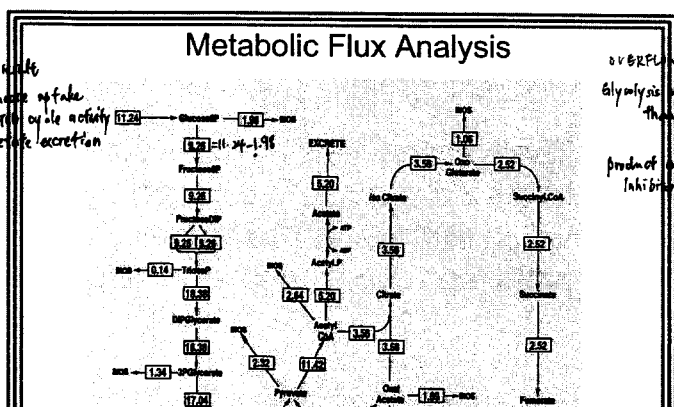
2.64 mols of Acetyl-CoA are used for biosynthesis and 5.20 mols for acetate excretion, leaving 2.52 mols malate to be oxidised and decarboxylated to oxaloacetate

Metabolic Flux Analysis

Together with the anaplerotic provision of 5.27 mols of oxaloacetate (2.75 mols from PEP and 2.52 mols from malate), 1.69 mols are used for biosynthesis and 3.58 are recycled through the TCA Cycle.

To obtain a FLUX, it is simply a matter of multiplying the current units (mols / kg dry weight) by the specific growth rate to obtain mols / g dry weight /h.

case 1:
 worse net results
 large glucose uptake
 limited TCA cycle activity
 large acetate excretion

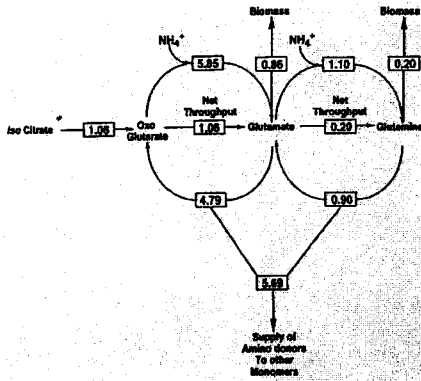


OVERFLUX METABOLISM
 Glycolysis very much faster than TCA cycle
 ↓
 product of acetation inhibitory/undesirable

Metabolic Flux Analysis

If some of the intermediates are used for other purposes, the additional load must be balanced by cyclic regeneration. The utilisation of glutamate and glutamine is a good example of this. They are amino group donors for many reactions. The throughput of oxo glutarate is 1.06 mols (0.86 to Glutamate, Proline and Arginine and 0.20 mols to Glutamine), but considerably more Glutamate and Glutamine are used as amino donors regenerating oxoglutarate and glutamate which are reaminated using ammonia and energy from the oxidative metabolism.

Metabolic Flux Analysis



Metabolic Flux Analysis

CASE 2: 56.12 mols Pyruvate form 1kg Biomass, 26.10 mols Acetate, 0.08 mols Lactate. The specific growth rate is 0.07h^{-1} .

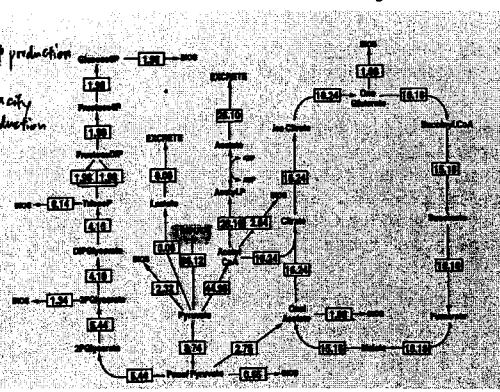
When pyruvate is used as the carbon source, PEP is made from pyruvate using pyruvate synthase.

Pyruvate is the substrate for gluconeogenesis, the process by which glucose is made from 2C and 3C compounds at an energy cost. 1.98 mols of Glucose-6P must be made from biosynthesis (as previously).

There is a large excretion of acetate (25.10 mols) and some lactate (0.08 mols).

Case 1:
pyruvate - can waste
large 3C uptake
requirement for PEP production
for biomass
limited TCA cycle capacity
very large acetate production

Metabolic Flux Analysis



Metabolic Flux Analysis

CASE 3: 26.23 mols Fumarate form 1kg Biomass, with CO_2 as the only product. The specific growth rate is 0.63h^{-1} .

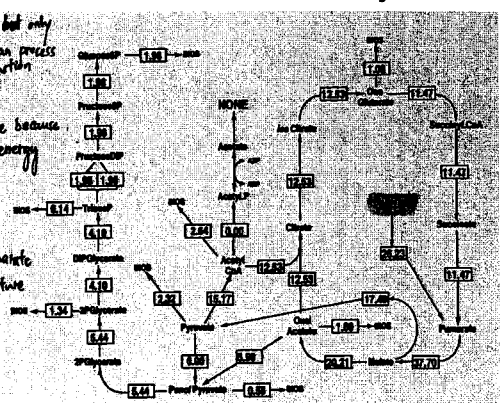
Fumarate enters directly into the TCA Cycle

All biosynthetic precursors are provided either by converting malate to pyruvate (using malate dehydrogenase) or by oxaloacetate to PEP (using PEP carboxy kinase)

There is no acetate excretion

Case 3:
fumarate enters TCA but only
use to what the cell can process
the use for G6P production
very low acetate
not go back to glucose because
it requires too much energy

Metabolic Flux Analysis



Initially, can use fumarate
to glucose to adjust culture
medium or modify

Metabolic Flux Analysis

This metabolism shows us one of the potential problems of flux analysis.

We know that 23.48 mols of C_4 must enter the glycolytic pathway from the TCA cycle (17.49 mols from malate and 5.99 mols from oxaloacetate) - however, we cannot be sure that this is the correct partitioning of the C_4 compounds.

All of the throughput could have been from malate in which case 5.99 mols PEP would need to be made from pyruvate using pyruvate synthase. Alternatively, oxaloacetate decarboxylase could operate exclusively, in which case 17.49 mols would need to be made from PEP by pyruvate kinase.

Metabolic Flux Analysis

Protocol 1. Technique of flux analysis

Data required

- Uptake of feedstocks (mole/kg dry wt biomass/h)
- Monomeric composition of biomass (mole/kg dry wt biomass)
- Biomass growth rate, μ (per hour)
- Other outputs (mole/kg dry wt biomass)

Method

- Calculate the outputs of precursors from CMPs to outputs and monomers in biomass.
- Construct a diagram of the CMPs appropriate to the uptake of inputs and provision of precursors to biomass monomers and other outputs.
- Enter the uptake of feedstocks and outputs of precursors into the basic diagram.
- Calculate and enter the consequences of uptake and anaplerosis.
- Complete the throughput diagram by calculating throughputs (mole/kg dry wt biomass) through each enzyme of the CMPs from input(s) to outputs of precursors.
- Convert the throughput diagram to a flux (mole/kg dry wt biomass/h) diagram by multiplying each throughput by the growth rate constant (μ).
- Use the flux diagram to devise strategies for intervention.

Metabolic Flux Analysis

Interpretation of Flux Analysis

Acetate excretion may be considered to be caused by limited ability of the TCA cycle to provide biosynthetic intermediates or by excessive activity of the glycolytic pathway or both. The fact that this problem does not occur when fumarate is the carbon source indicates that, in this case, the biosynthetic intermediates provision and supply are better balanced.

Metabolic Flux Analysis

Acetate excretion may be reduced by restricting the uptake of the feed (glucose). This is most easily achieved in continuous culture at low dilution rates (0.3h^{-1}).

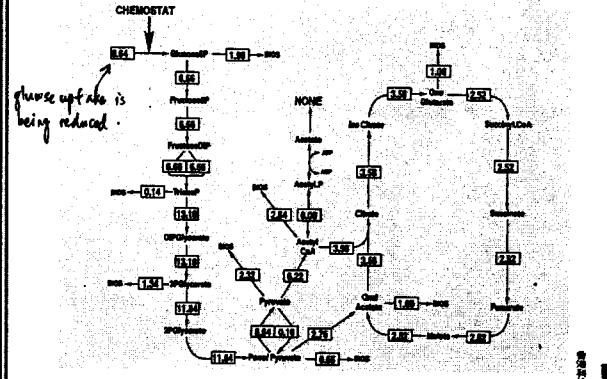
Analysis of this steady state shows that 8.64 mols of glucose are used to make 1 kg Biomass.

Comparing the glucose in batch to the glucose in continuous culture:

1. The outputs to precursors are identical
2. The throughputs in the TCA Cycle are identical
3. The throughputs in glycolysis are lowered
4. The conversion of PEP to Pyruvate is exclusively dedicated to the phosphotransferase system for glucose uptake with none involving Pyruvate Kinase



Metabolic Flux Analysis (FAVORABLE)



Metabolic Flux Analysis (INHIBITION)

Acetate excretion may also be achieved by inhibiting the enzyme (pyruvate dehydrogenase) which forms acetyl-CoA from Pyruvate and leads to Acetate Excretion.

This enzyme is inhibited by bromopyruvate.

At a concentration of $50\mu\text{mol}$, acetate excretion is totally prevented. Interestingly, the growth is also inhibited to a specific growth rate of 0.3h^{-1} , which is similar to the dilution rate in continuous culture where acetate excretion is prevented.

One method achieves the desired outcome by process manipulation



shift pathway to another comp (lactate)
where does it go?
names! PEP to lactate -> somewhere else (another product)
DESIRABLE: slow down uptake of glucose.

Metabolic Flux Analysis (DELETION)

Acetate excretion may also be prevented by a ~~deletion of the acetate uptake/excretion mechanism~~

Fluoroacetate is converted to fluorocitrate by the enzymes responsible for acetate uptake and excretion. Fluorocitrate is toxic to cells and usually they will not grow well in its presence. Selection for strains capable of growing well in the presence of Fluoroacetate may indicate the loss of the acetate uptake/excretion capacity.

Such strains do not excrete acetate.



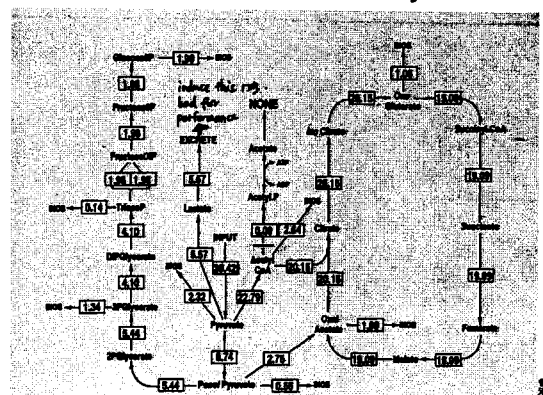
Metabolic Flux Analysis

Such strains have the following characteristics:

1. The outputs of precursors are the same
2. The uptake of pyruvate falls by 30% (acetate excretion stimulates pyruvate uptake)
3. No acetate excretion compared to 47% of the input pyruvate excreted as acetate in the wild type E Coli
4. Large stimulation of lactate excretion (14% of pyruvate uptake)
5. There is an increase in the amount of Acetyl-CoA oxidised in the TCA Cycle (more NAD(H) production) - this presumably compensates for the reducing power required to reduce pyruvate to lactate, the loss of ADP phosphorylation by acetate phosphate and the loss of the proton motive force for acetate excretion.



Metabolic Flux Analysis (UNFAVORABLE)



Metabolic Flux Analysis

Manipulation of enzymes is now very common and there needs to be some theoretical basis for determining which enzymes should be manipulated to achieve a desired outcome.

One such theoretical basis is Metabolic Control Analysis which is a central theme in Metabolic Engineering. We will study this in due course.