Bioprocesses and Downstream Processing

ChE-437

Part 1

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EPFL

Hes·so VALAIS WALLIS
Haute Ecole Spécialisée de Suisse occidentale
Fachhochschule Westschweiz
University of Applied Sciences Western Switzerland

Course information

ChE-437 / **4 crédits**
Enseignant(s): Crelier Simon, Eyer Kurt, Zinn Manfred
Langue: Anglais

Summary

This course aims at a more advanced coverage of the basic aspects discussed in module ChE-311. It is however of a stand-alone nature, and even students who have little knowledge on bioprocess development shall benefit as well from this module.

Exam

When there are less than 10 students, the exam will be oral (30 min).
For more students, the exam will be written and last 3 hours.

By the end of the course, you should be able to:

- ✓ Integrate concepts and knowledge from various domains (biology, process engineering, (bio)chemistry)
- ✓ Discuss the merits, disadvantages and characteristics of the different types of bioreactors as well as their mode of operation
- ✓ Dimension unit operations
- ✓ Interpret data or observations from case studies
- ✓ Choose an appropriate fermentation or purification strategy
- ✓ Predict the outcome or the performance of a unit operation or specific equipment
- ✓ Justify your choices and assumptions
- ✓ Solve calculation problems

Your lecturers:

Manfred Zinn

- Upstream processing: introduction and basics in cultivation
- Bioprocess design for batch, fed-batch, and chemostat cultures
- Special bioprocesses and applications
- Paper studies including short presentations by students

Kurt Eyer

- Bioreactors & Fermenters: Basics
- Characterization of biological reactor systems
- Scale-up procedure: science or art?
- Applied examples and economic aspects of industrial bioprocesses

Simon Crelier

- Downstream processing: introduction and liquid-solid separation
- Cell lysis and precipitation
- Liquid-liquid extraction
- Adsorption and chromatography
- Membrane-based separations
- Polishing steps and latest trends

Module ChE-437 Bioprocesses & Downstream Processing
Tuesday, 8:15 pm to 12:00 pm, Room CHB331

Date	Subject	Lecturer
18.02.25	Upstream processing: introduction and basics in cultivation	M. Zinn
25.02.25	Bioprocess design for batch, fed-batch and chemostat cultures	M. Zinn
04.03.25	Special bioprocesses and applications	M. Zinn
11.03.25	School trip	M. Zinn & S. Crelier
18.03.25	**Case studies, student presentations**	M. Zinn
25.03.25	Introduction and basics of bioreactors / Fermentors; Biopharmaceuticals	K. Eyer
01.04.25	Characterization and selection of biological reactor systems	K. Eyer
08.04.25	Scale-up of biological processes	K. Eyer
15.04.25	Applied examples and economic aspects of industrial bioprocesses	K. Eyer
29.04.25	Overview of DSP in biotechnology: past, present and future trends	S. Crelier
06.05.25	Physical separations: beyond the basics / Revamping Protein Precipitation	S. Crelier
13.05.25	Liquid/liquid extraction / Case studies in adsorption and chromatography	S. Crelier
20.05.25	Membrane-based separations / PAT in downstream processing	S. Crelier
27.05.25	Polishing steps: refolding, crystallization, drying / DSP from lab to production scale	S. Crelier

23 students enrolled (17.2.2025)

Paper presentations by students on 18.3.2025

- There will be 8 papers to be discussed (to chose from 8 publications on Moodle). All papers are studied beforehand.
- 3 Students form a group and present one paper (ppt. max. 15 min).
- The goal is to critically read, understand the techniques used (M&M) and reflect on the significance of the publication.

More readings (free & online)

History of Biotechnology: https://www.whatisbiotechnology.org/index.php/timeline/index/2620

Glossary in Biotechnology:
https://www.fao.org/3/x3910e/x3910e.pdf

Biotechnology Science News:
https://www.sciencedaily.com/news/top/

Biotech Business:
https://www.labiotech.eu/
https://www.swissbiotech.org/

Networks:
https://biotechnet.ch/
https://bioalps.org/
https://www.efbiotechnology.org/

Agenda

1. Drivers in biotechnology
2. The cell
3. Cell preservation
4. Batch cultivation
5. Medium design

1. Drivers of Biotechnology

https://disruptiveinvesting.io/wp-content/uploads/2021/07/14.0-p.jpg

Early biotechnology: Food biotechnology

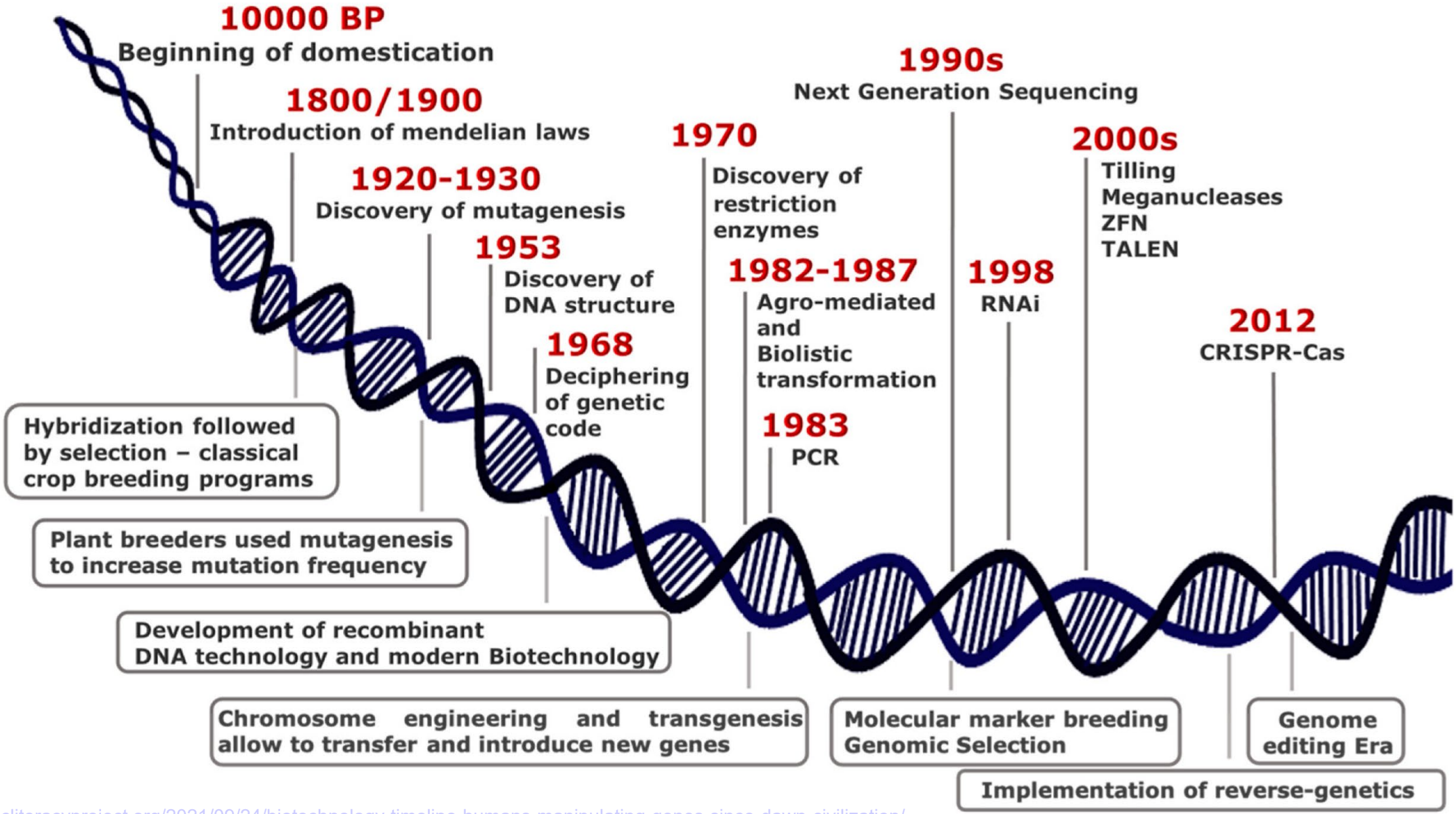
The temples in Mesopotamia issued workers with daily rations of barley beer, the staple drink of Mesopotamia. The tablet was impressed with five different types of numerical symbol. From Mesopotamia, Iraq. Late Uruk Period, 3100-3000 BCE. (The British Museum, London)

https://www.worldhistory.org/image/4849/mesopotamian-beer-rations-tablet/

A funerary model of a bakery and brewery, dating the 11th dynasty, circa 2009-1998 B.C. Painted and gessoed wood, originally from Thebes.

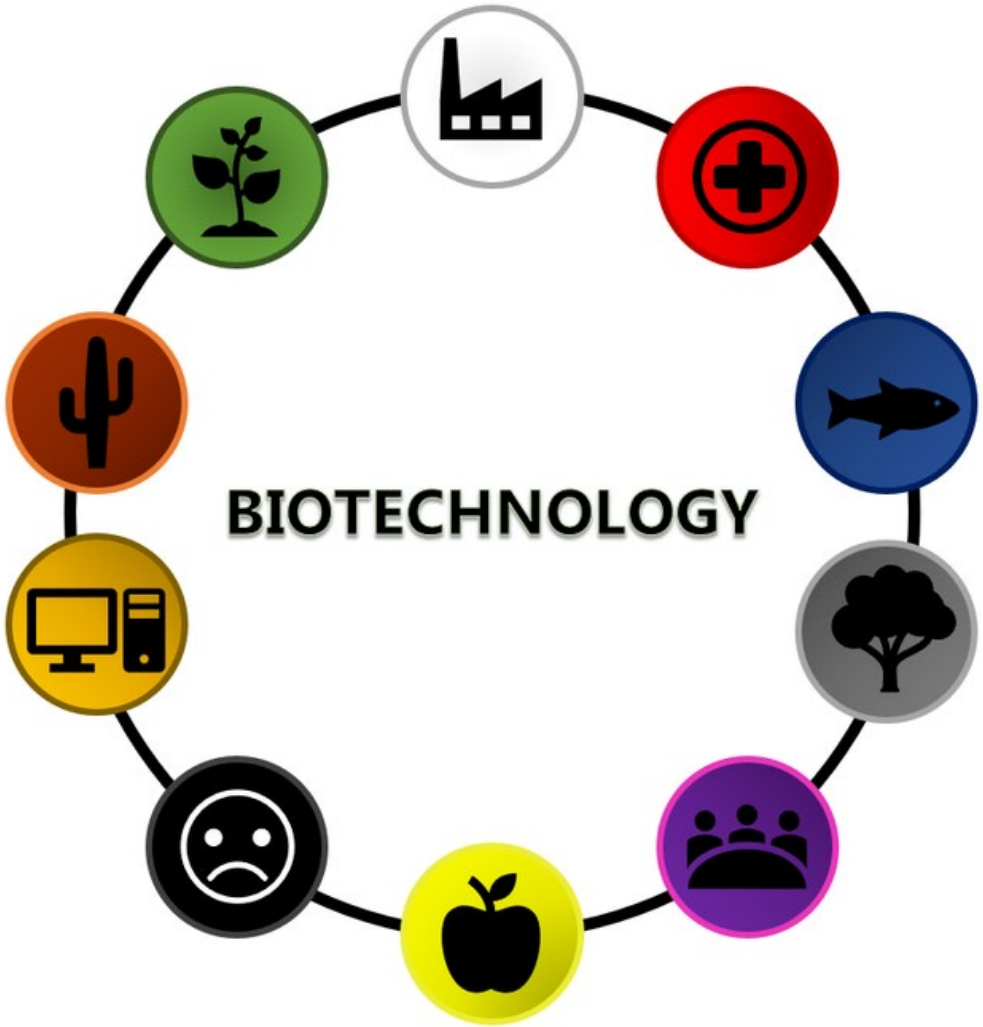
https://www.worldhistory.org/image/573/ancient-egyptian-brewery-and-bakery/

Important milestones in molecular biology



https://geneticliteracyproject.org/2021/09/24/biotechnology-timeline-humans-manipulating-genes-since-dawn-civilization/

The different colors in biotechnology

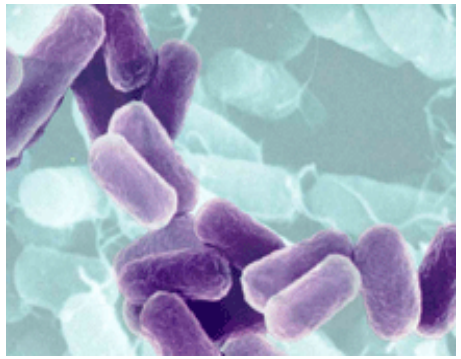


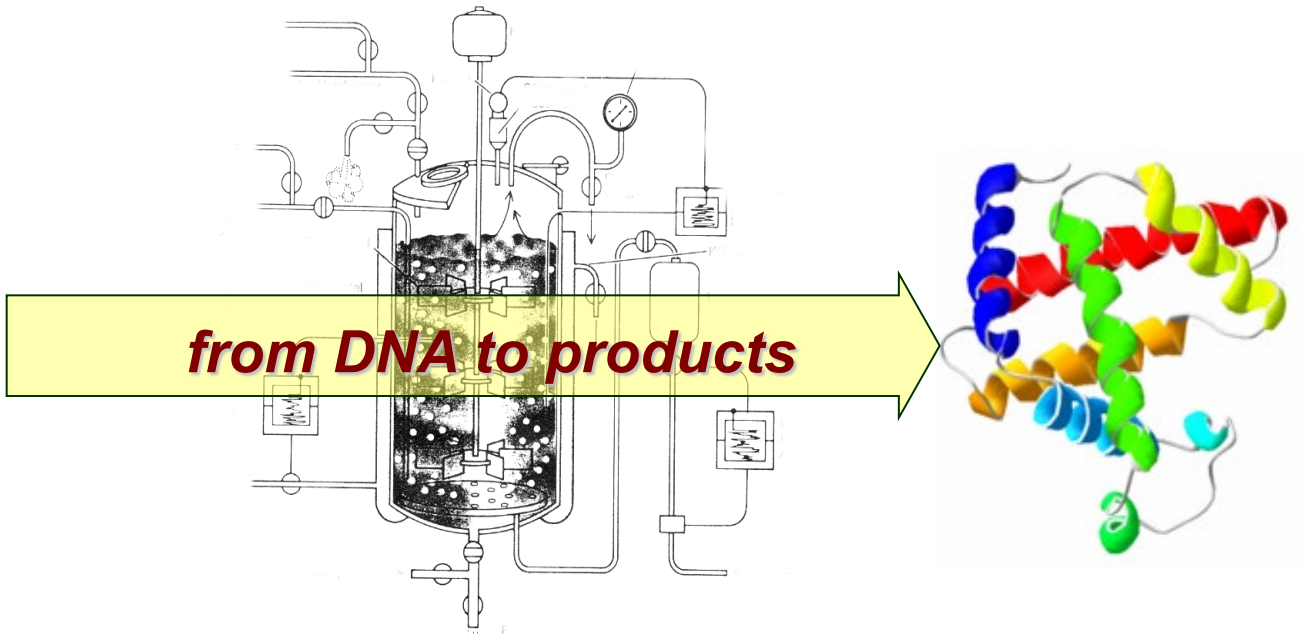
Red	Medicine and human health
White	Industrial processes involving microorganisms
Green	Processes improving agriculture
Blue	Marine biotechnology
Yellow	Food and nutrition
Grey	Envirnomental biotechnology
Gold	Bioinformatics, computer science
Brown	Biotechnology of dessert and dry regions
Violet	Law, ethics, philosophy
Dark	Bioterrorism, biological warfare

https://steemit.com/steemstem/@jepper/all-the-colors-of-biotechnology

DaSilva, E.. The Colours of Biotechnology: Science, Development and Humankind. *Electronic Journal of Biotechnology*, North America, 724 07 **2012**

The basic tenet in industrial bioprocess engineering





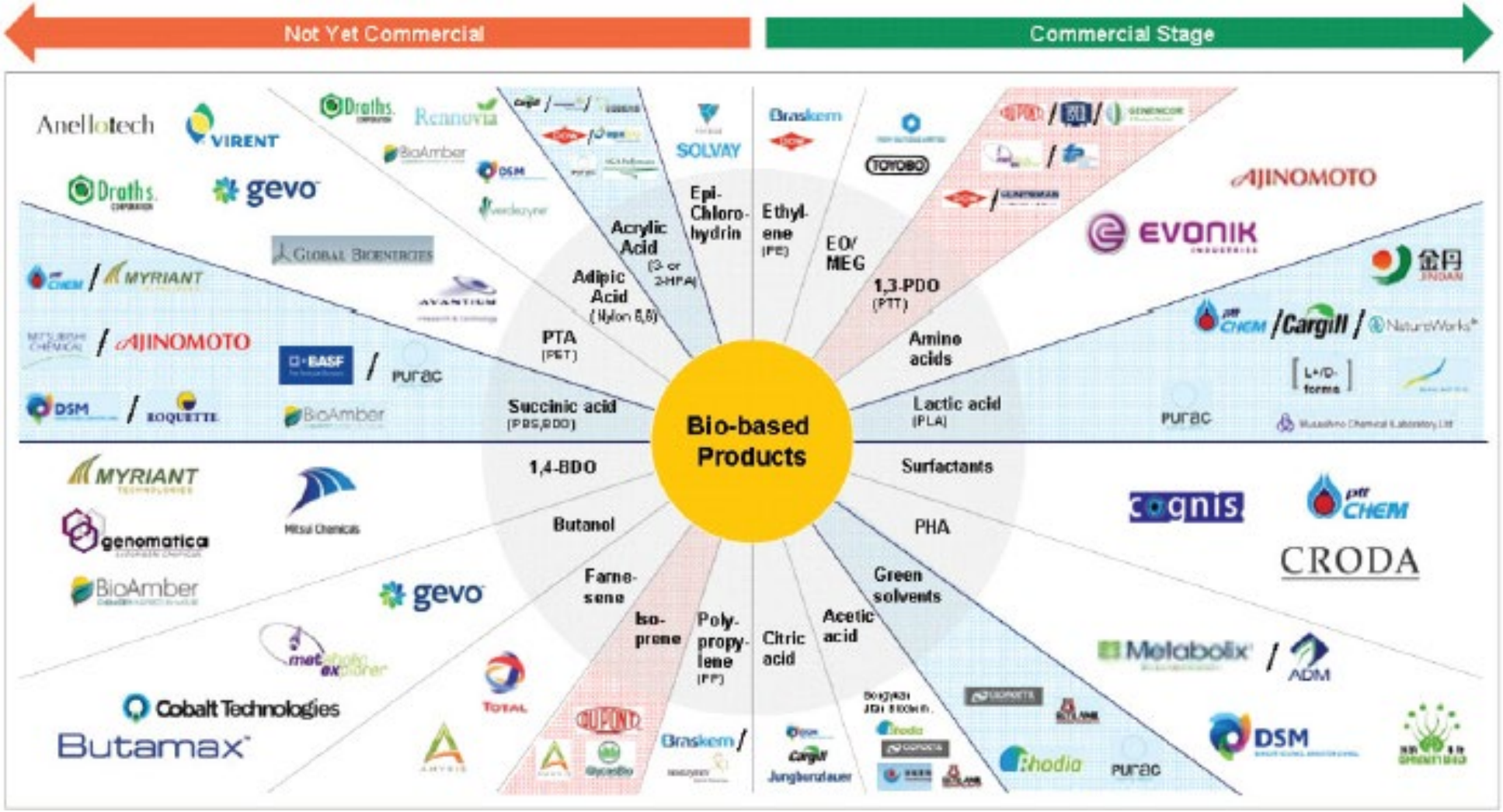
- Genetic engineering
- Activity screening

- Bioprocessing
- Enzymatic catalysis

- Downstream processing

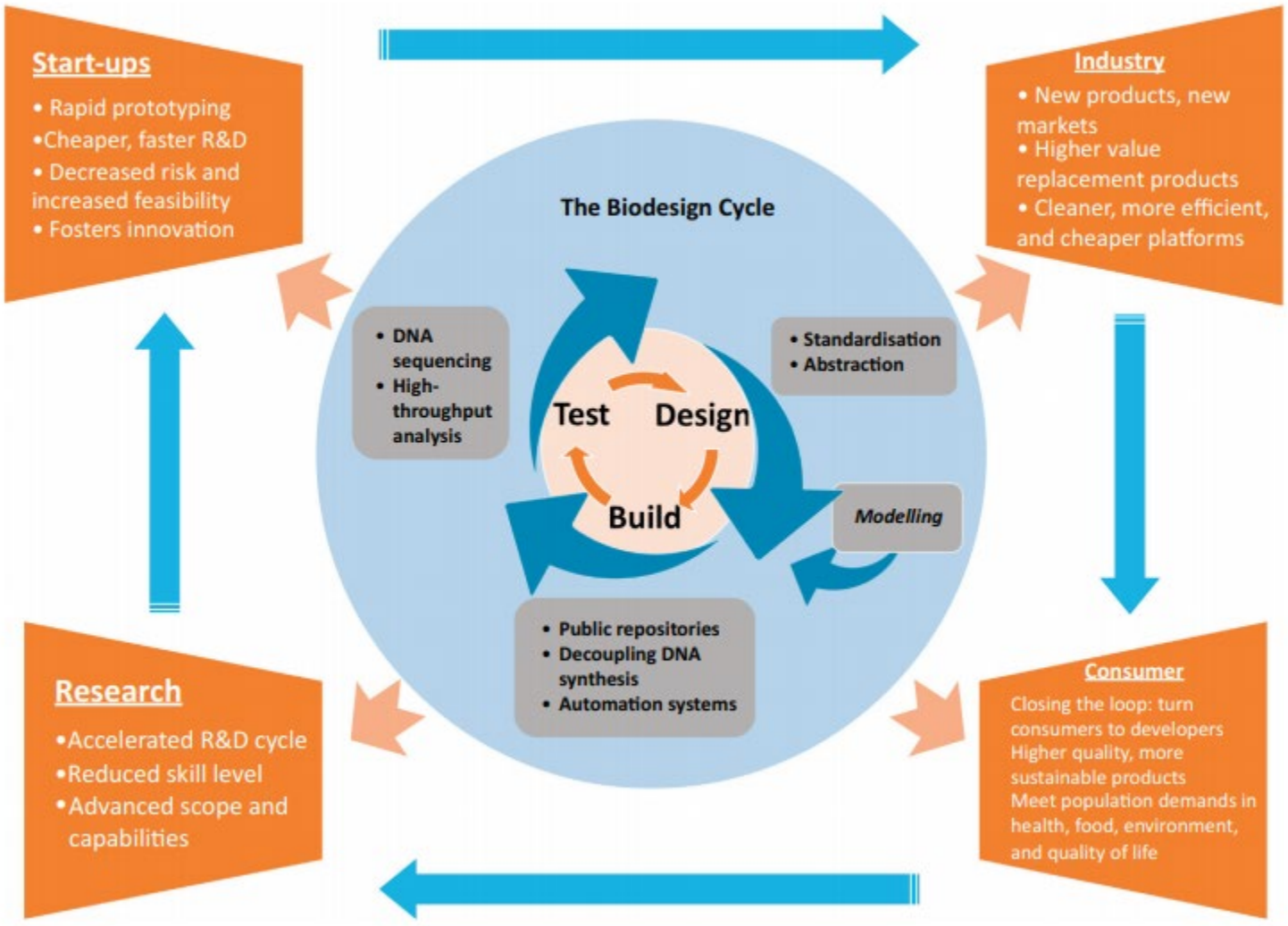
Chemical & Bioanalytics

Typical products of white biotechnology



Industrial Biotechnology: Past and Present." National Research Council. 2015. *Industrialization of Biology: A Roadmap to Accelerate the Advanced Manufacturing of Chemicals*. Washington, DC: The National Academies Press. doi: 10.17226/19001.

Synthetic Biology: A new economic engine



Flores Bueso Y, Tangney M. 2017. Synthetic Biology in the Driving Seat of the Bioeconomy. Trends in Biotechnology. 2017/05/01/;35:373-378.

Swiss biotechnology is innovative

Jan Lucht

scienceindustries | Head Biotechnology

The life sciences sector (pharmaceuticals, vitamins and diagnostics) represents the largest export industry of Switzerland. In 2022, it accounted for 39% of total Swiss exports and added a record CHF 109.6 billion (+0.6% over 2021) to the foreign export trade.

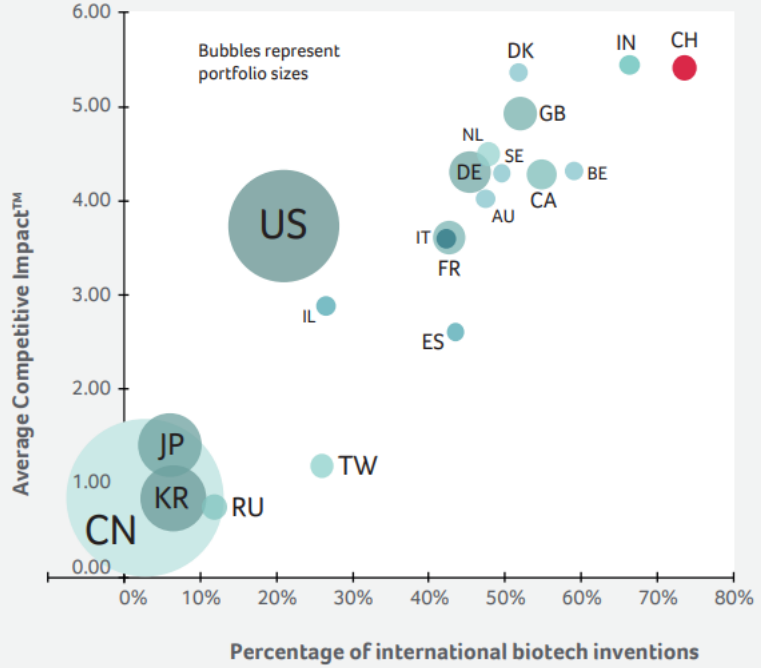
Christian Moser

Swiss Federal Institute of Intellectual Property | Patent Expert

David Rees

Swiss Federal Institute of Intellectual Property | Patent Expert

Inventors who are resident in Switzerland contribute disproportionately to the global biotech patent portfolio, in terms of both quantity and quality. The claim that Swiss biotech inventions provide global solutions is justified by their high impact, combining technological relevance with a broad coverage of global markets. Three out of four biotech patent applications listing Swiss inventors are international inventions. This exceptionally high rate, which applies equally to private companies and to public research institutions, illustrates the strong international collaboration network of Swiss-based inventors.

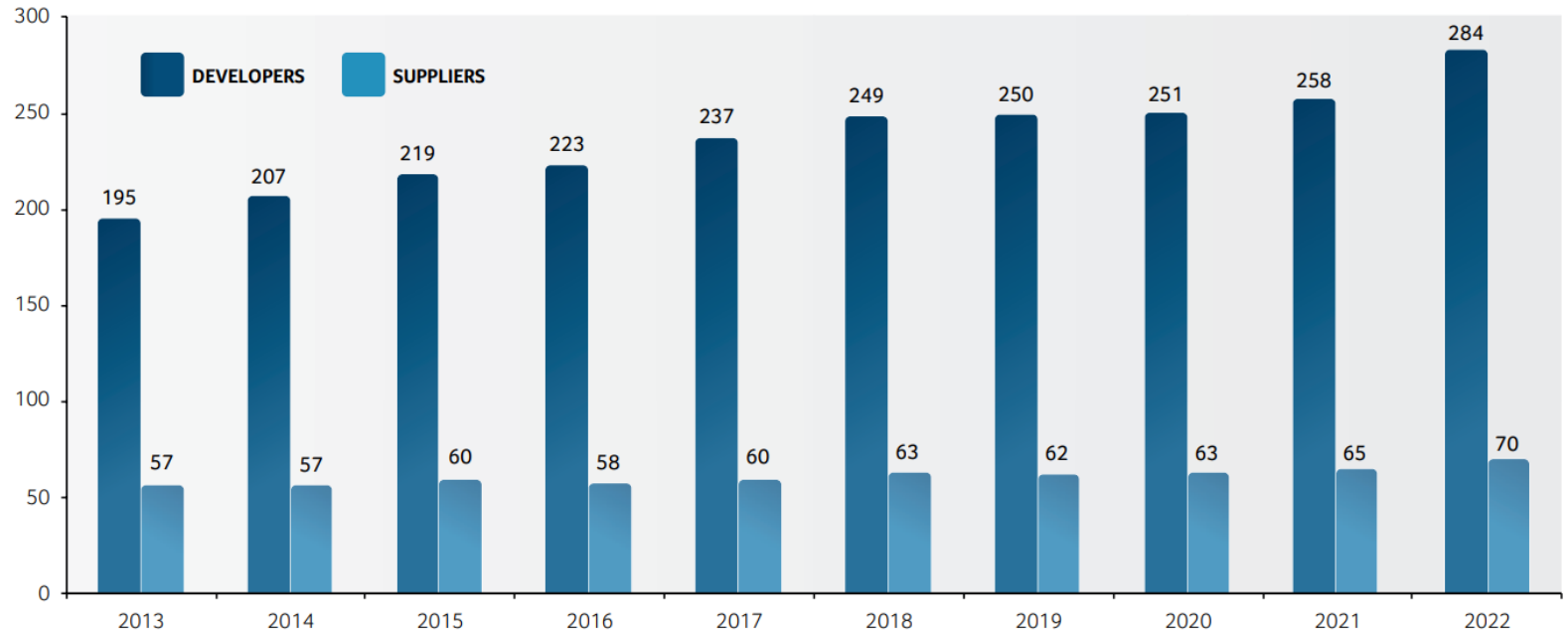


Invented in		Biotech patents 2022
CN	China	192'802
USA	USA	96'502
KR	South Korea	33'922
JP	Japan	32'100
DE	Germany	13'721
GB	United Kingdom	8'836
FR	France	8'599
CA	Canada	6'735
RU	Russian Federation	5'109
CH	Switzerland	4'767
TW	Taiwan	4'540
NL	Netherlands	4'444
IT	Italy	3'135
IN	India	3'106
IL	Israel	2'921
AU	Australia	2'756
DK	Denmark	2'752
ES	Spain	2'629
BE	Belgium	2'584
SE	Sweden	2'435

Figure 4: Percentage of international biotech inventions and Competitive Impact™ for top 20 inventor countries (data for the reporting date December 31, 2022)

Biotechnology in Switzerland

Number of biotech companies in Switzerland 2013-2022

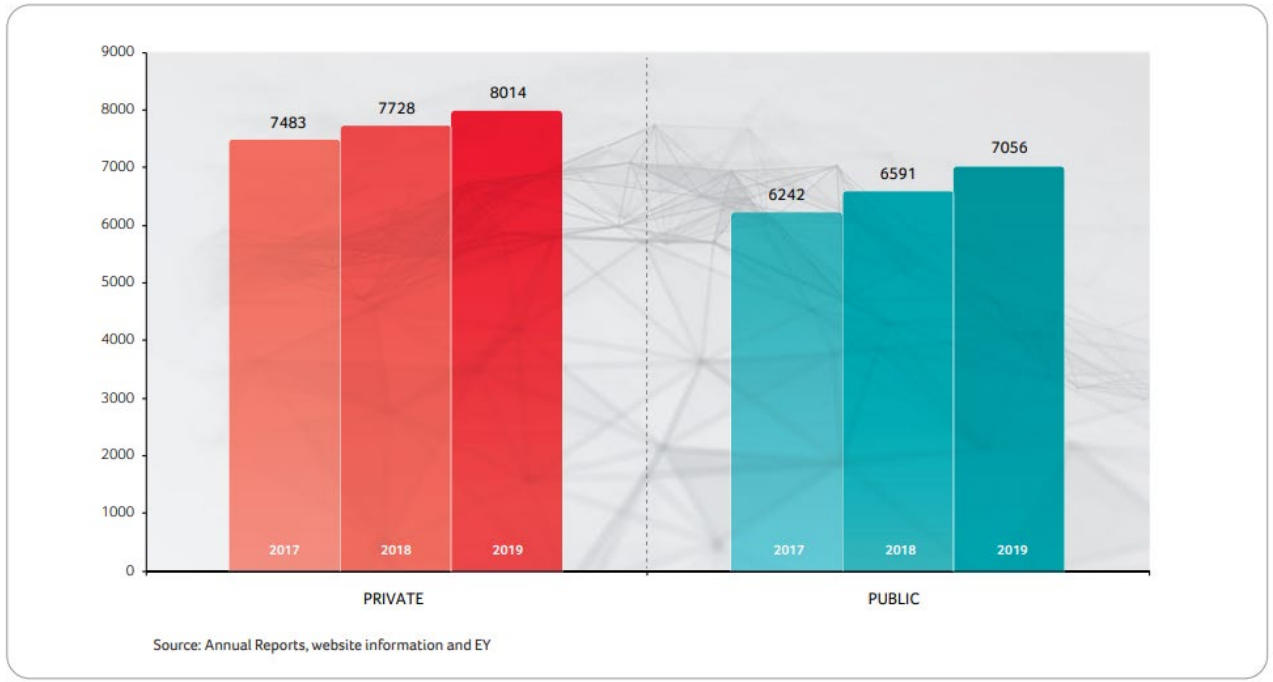


Source: Swiss Biotech Association, website information, EY

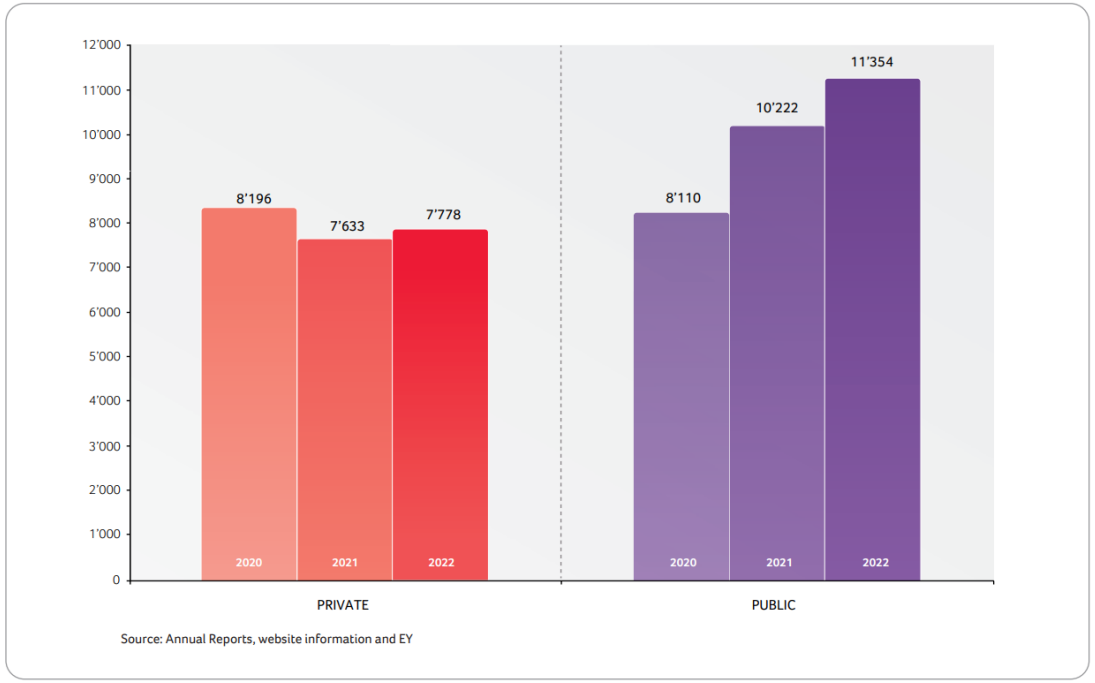
Swiss Biotech Report – Swiss Biotech

Biotechnology in Switzerland

Number of Swiss Biotech Employees 2017-2019



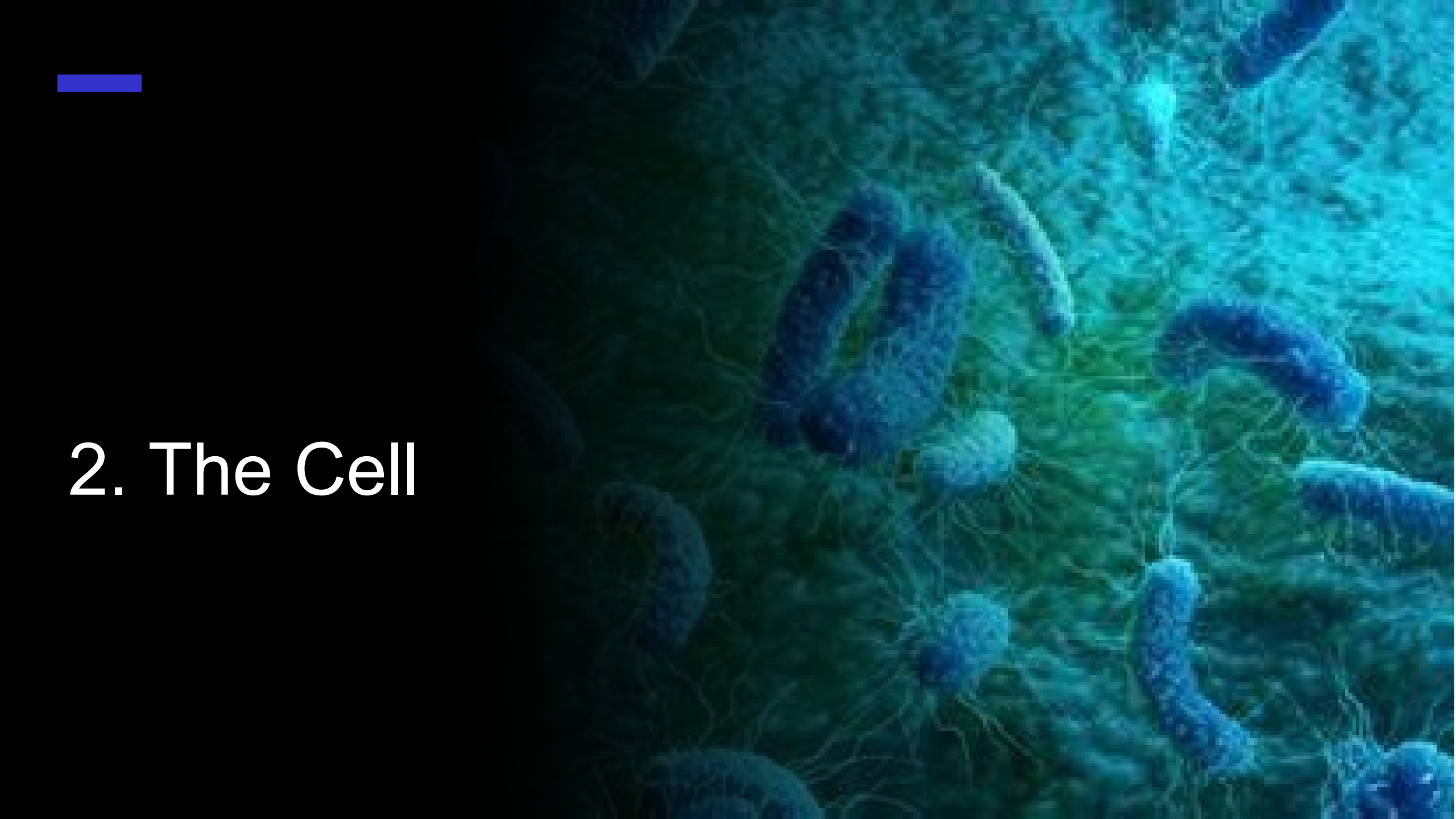
Number of employees in Swiss R&D biotech companies 2020-2022



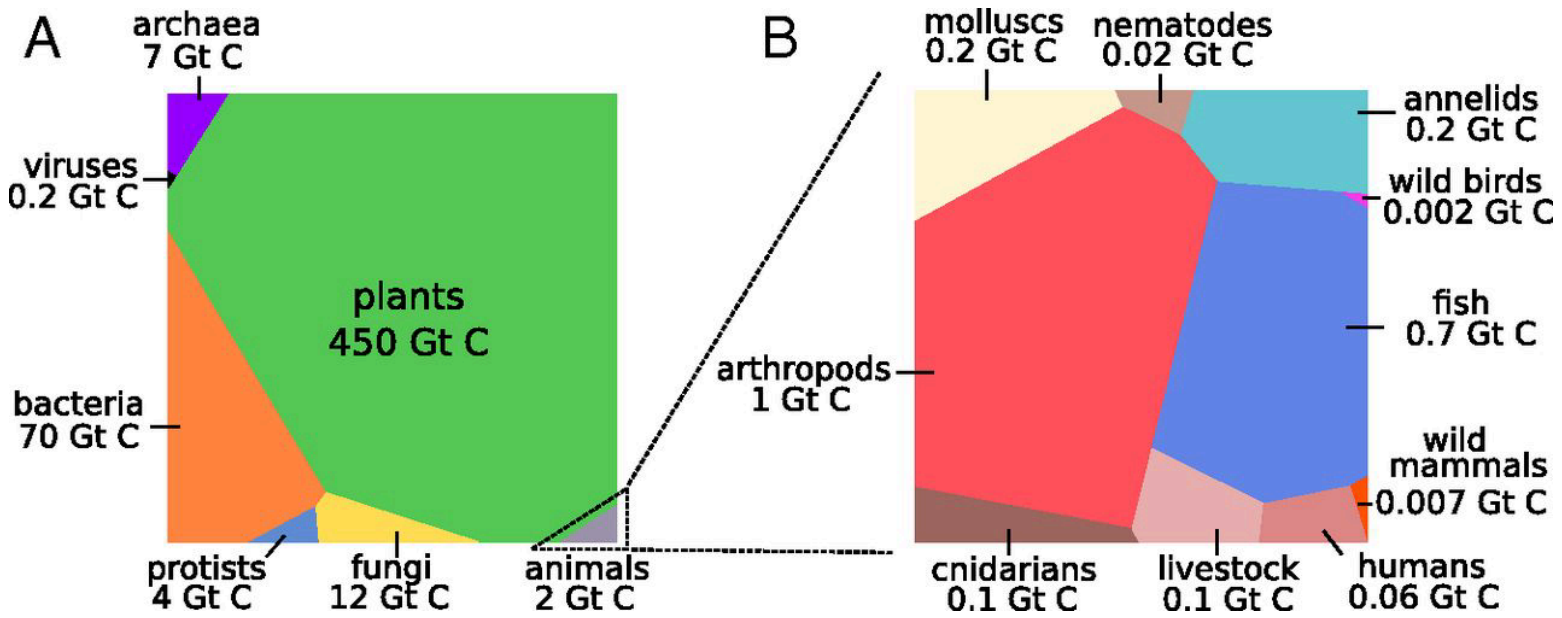
=> Good job opportunities for you!

Swiss Biotech Report – Swiss Biotech

2. The Cell



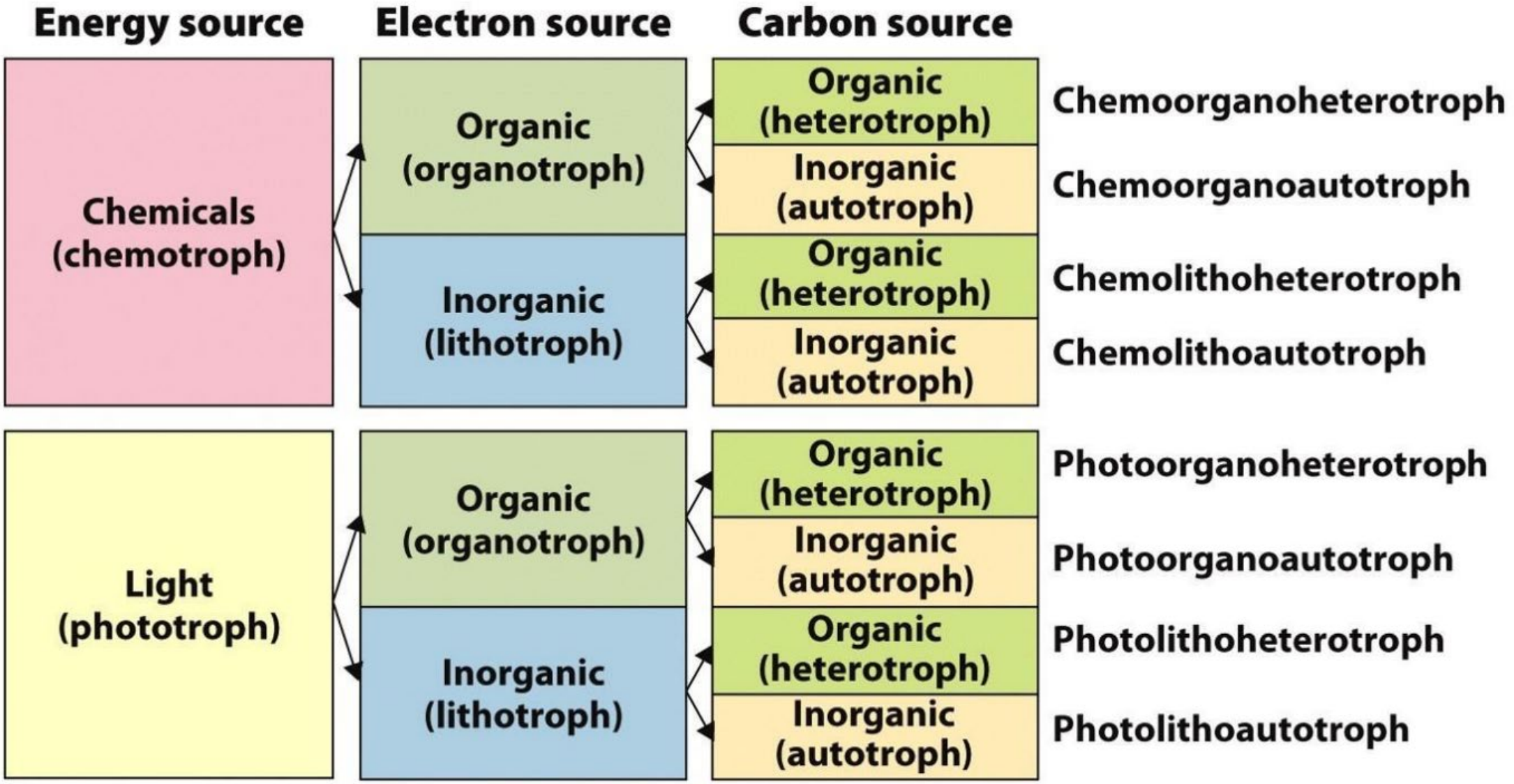
Distribution of biomass on earth



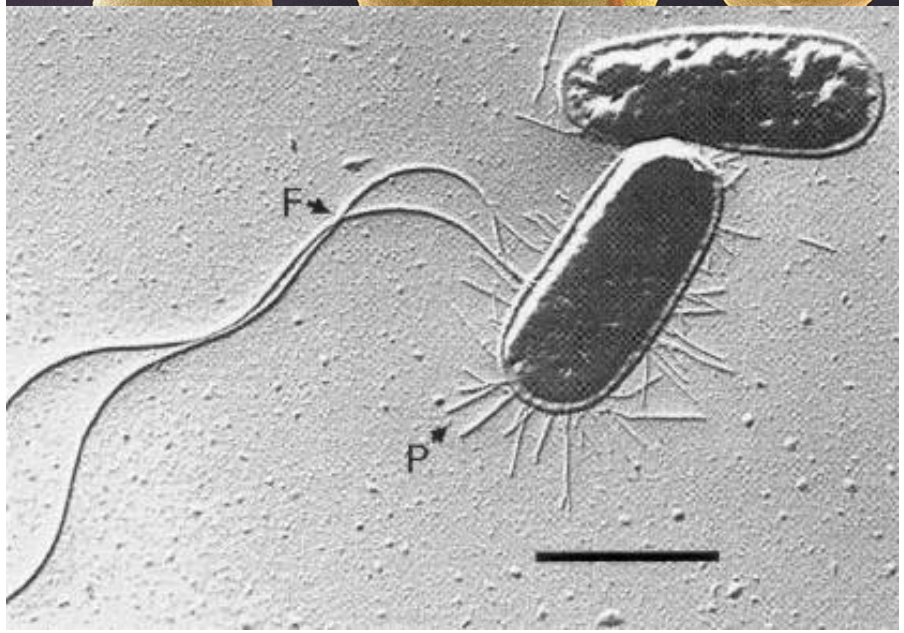
Graphical representation of the global biomass distribution by taxa. (*A*) Absolute biomasses of different taxa are represented using a Voronoi diagram, with the area of each cell being proportional to that taxa global biomass (the specific shape of each polygon carries no meaning)
(*B*) Absolute biomass of different animal taxa.

https://www.pnas.org/content/115/25/6506

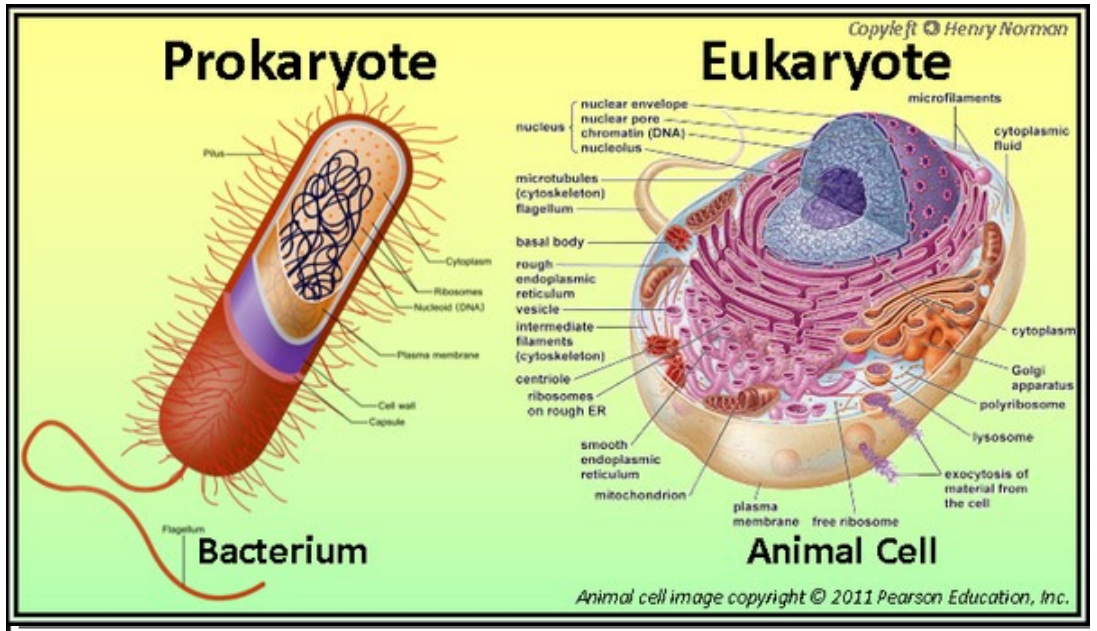
Classifications of metabolically active cells



Average composition of single cells



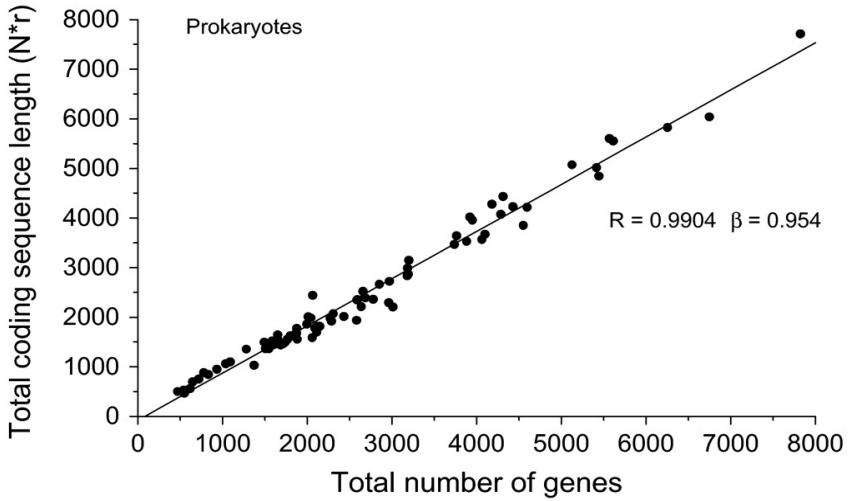
https://www.quora.com/What-is-the-difference-between-bacteria-cells-and-animal-plant-cells

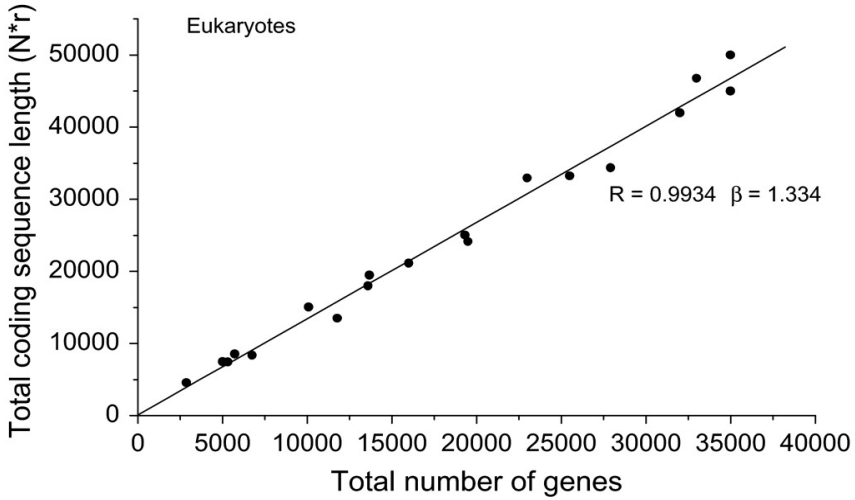


		E. coli	*S. cerevisiae*	Mammalian Cell
% total weight	**Water**	70	80	70
% dry weight	**DNA**	3	0.1–0.6	1
	RNA	20	6–12	4
	Proteins	50–55	35–60	60
	Lipids	7–9	4–10	13
	References	[15,34,35]	[36–40]	[35]

doi:10.1371/journal.pone.0067590.t002

Translation efficiency: Prokaryotes vs. eukaryotes





Analysis of regression of total coding sequence length on the number of genes in 81 prokaryotic species and 19 eukaryotic species.

Genetic engineering of prokaryotic species seems to be easier.

Molecular Biology and Evolution, Volume 23, Issue 6, June 2006, Pages 1107–1108, https://doi.org/10.1093/molbev/msk019

Escherichia coli

Overall macromolecular composition of an average *E. coli* cell in **aerobic** balanced growth **at 37°C** in glucose minimal medium, with doubling time of 40 minutes and 1 pg cell wet weight (≈0.9 μm^3 cell volume). Adapted with modifications from F. C. Neidhardt et al., "Physiology of the bacterial cell", Sinauer, 1990 (BNID 104954). Modifications included increasing cell dry weight from 284 fg to 300 fg and total cell mass from 950 to 1000 fg as well as rounding other values to decrease the number of significant digits such that values reflect expected uncertainties ranges. Under different growth rates the volume and mass per cell can change several fold. The relative composition changes with growth rate but not as significantly. For a given cell volume and growth rate, the uncertainty in most properties is expected to be on the order of 10-30% standard deviation. Original values refer to B/r strain, but to within the uncertainty expected, the values reported here are considered characteristic of most common *E. coli* strains. Data sources can be found at BNID 111490. An independent source for slower growth rates can be found at BNID 111460.

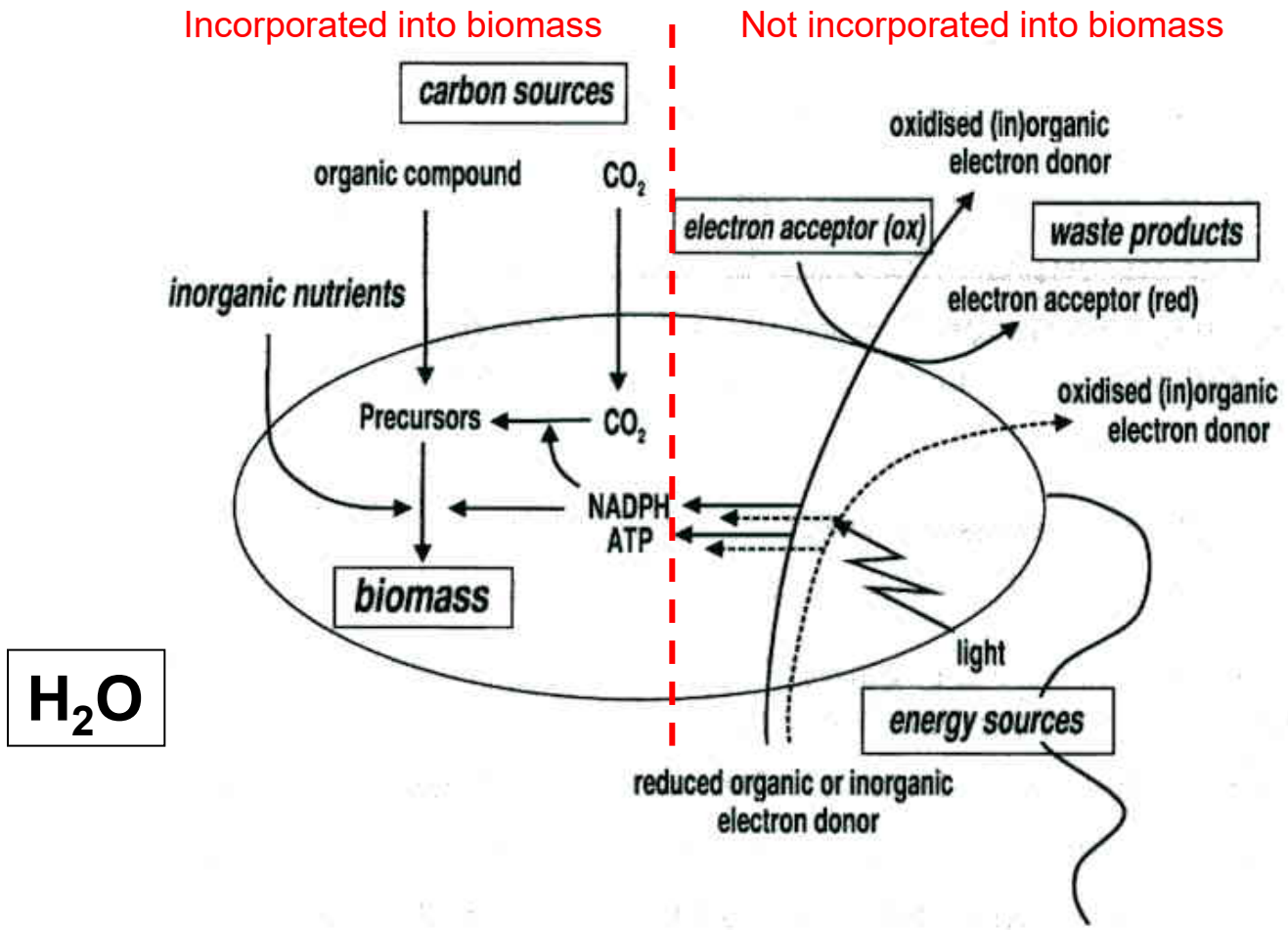
macromolecule	percentage of total dry weight	weight per cell (fg)	characteristic molecular weight (Da)	number of molecules per cell
protein	55	165	3×10^4	3,000,000
RNA	20	60		
23 S rRNA		32	1×10^6	20,000
16 S rRNA		16	5×10^5	20,000
5 S rRNA		1	4×10^4	20,000
transfer		9	2×10^4	200,000
messenger		2	1×10^6	1,400
DNA	3	9	3×10^9	2
lipid	9	27	800	20,000,000
lipopolysaccharide	3	9	8000	1,000,000
peptidoglycan	3	9	$(1000)_n$	1
glycogen	3	9	1×10^6	4,000
metabolites and cofactors pool	3	9		
inorganic ions	1	3		
total dry weight	100	300		
water (70% of cell)		700		
total cell weight		1000		

composition rules of thumb

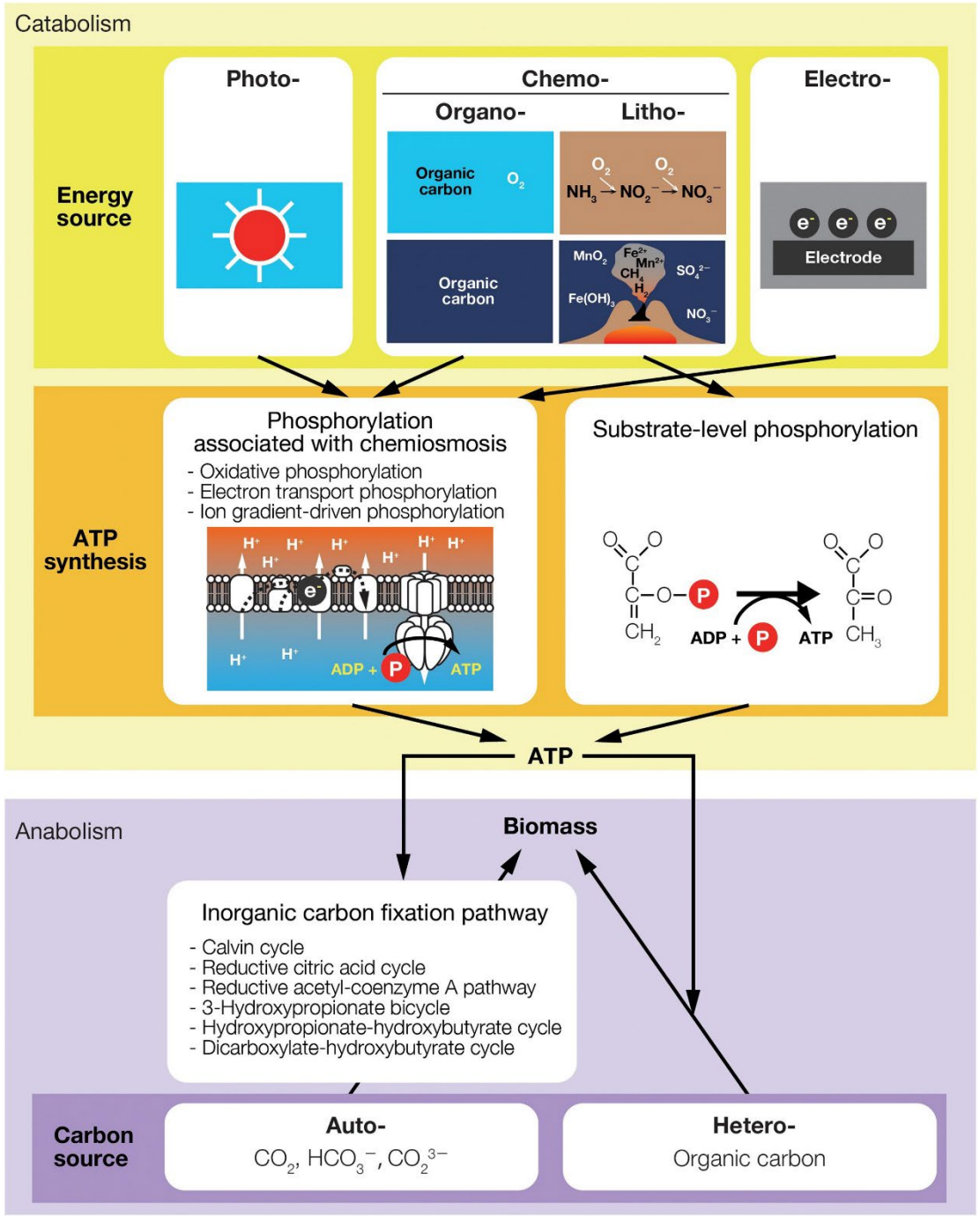
- carbon atoms ~10^{10}
- 1 molecule per cell gives ~1 nM conc.
- ATP required to build and maintain cell over a cell cycle ~10^{10}
- glucose molecules needed per cell cycle ~3×10^9 (2/3 of carbons used for biomass and 1/3 used for ATP)

http://book.bionumbers.org/what-is-the-macromolecular-composition-of-the-cell/

Physiological function of nutrients



Simplified sketch of the physiological function of nutrients for the growth of microorganisms.



The Yin and Yang of metabolism

Dissimilation: The oxidation of a reduced (in)organic compound to provide energy for biosynthesis and cell maintenance.

Assimilation: The incorporation of a compound into biomass.

Seto M, Iwasa Y. How Thermodynamics Illuminates Population Interactions in Microbial Communities. Frontiers in Ecology and Evolution. 2020;8.

A few definitions

Metabolism:	Transformation of substances in the cell to gain precursors for cell components and energy.
Catabolism:	Breakdown of nutrients into smaller fragments (ev. energy gain).
Amphibolism:	Intermediary metabolism (synthesis of organic acids and phosphate esters = building blocks); *anaplerotic sequences* when no transfer from catab. to anab. is possible.
Anabolism:	Synthetic metabolism (synthesis of polymers from building blocks).

Metabolism = (Catabolism + Amphibolism) + Anabolism

The amphibolism has 13 key compounds

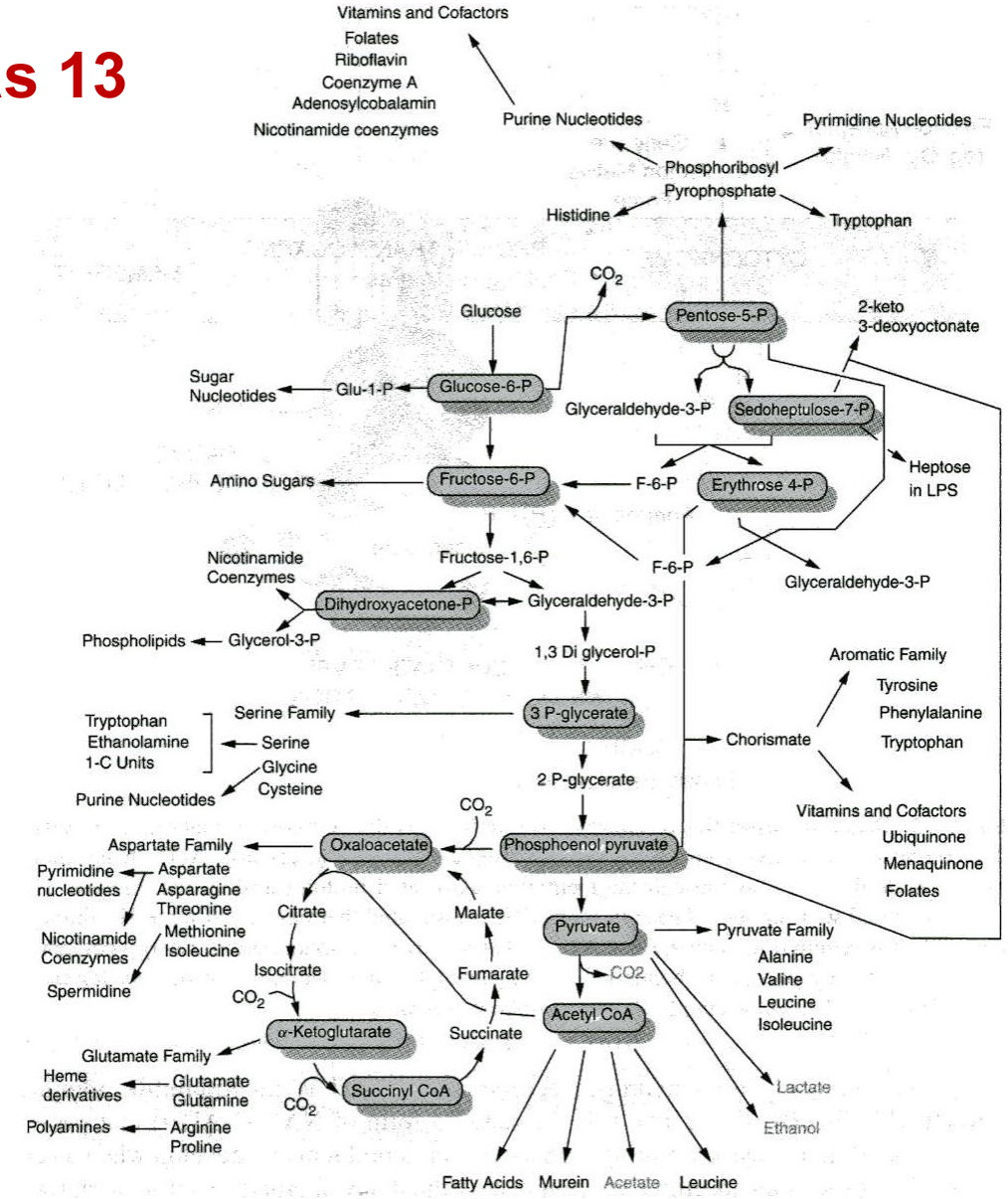
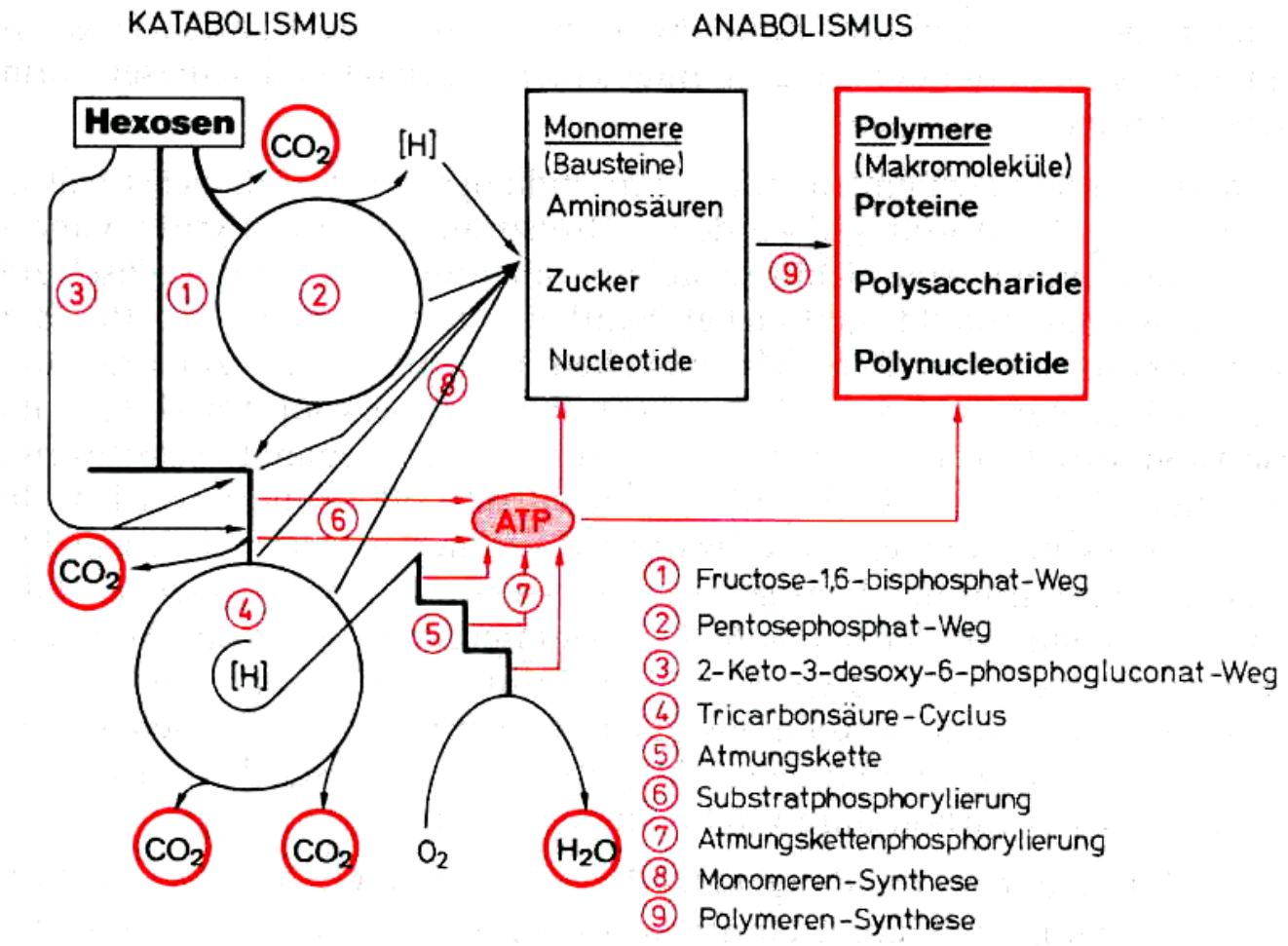


Fig. 1-11. Biosynthetic pathways leading to the amino acids and related compounds. The oblong-circled intermediates are the 13 key compounds that serve as biosynthetic precursors for a variety of essential end products.

A. G. Moat, J.W. Foster, M.P. Spector, 2002. Microbial Physiology ISBN 0-471-39483-1

Metabolism in aerobic cells

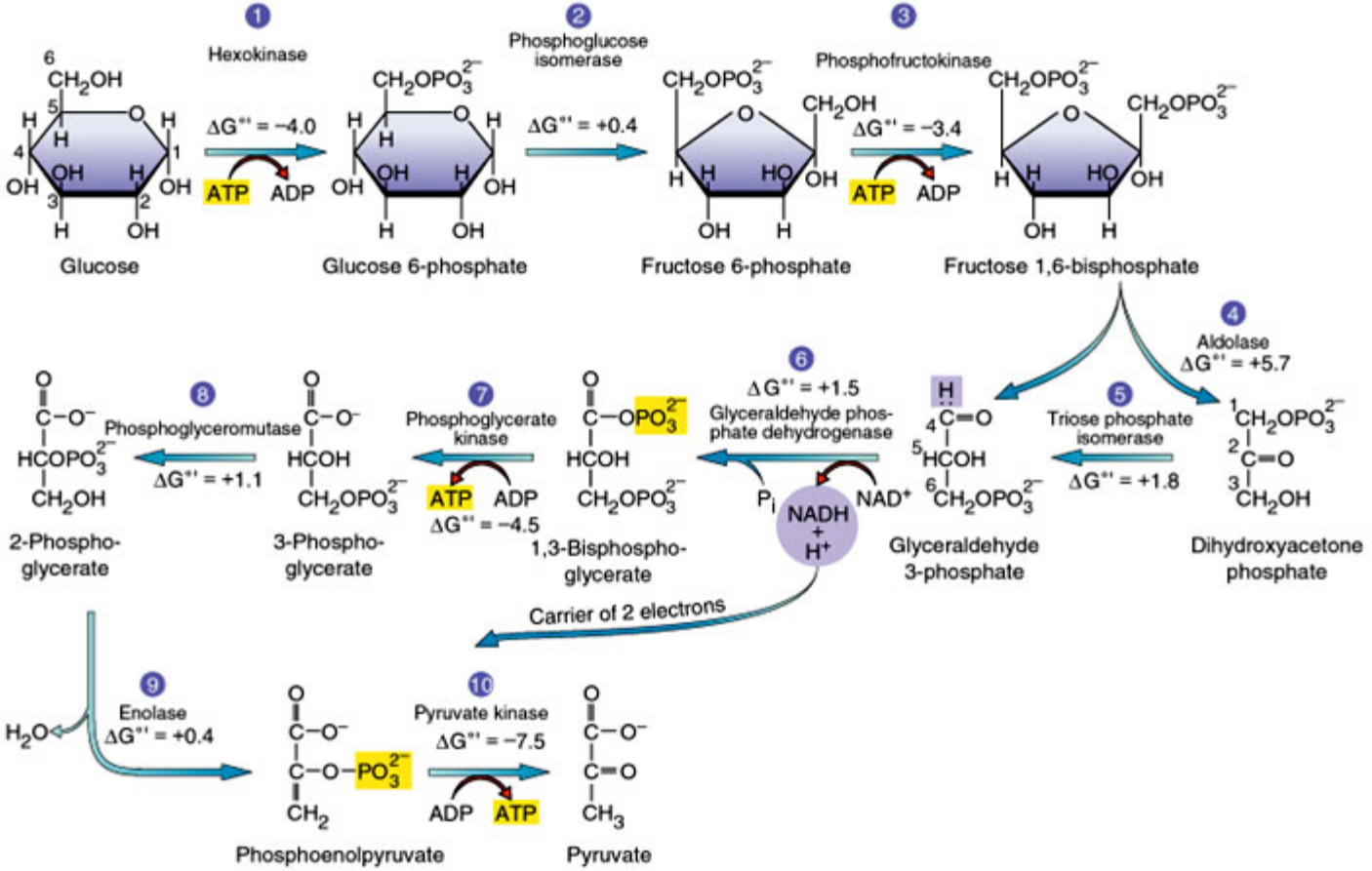


Glycolysis
PP pathway
Entner-Doudoroff
Krebs Cycle
Respiratory chain
Substrate chain phosph.
Respiratory chain phosph.

Schlegel, 7. überarbeitete Auflage

Fructose-1,6-bisphosphate pathway

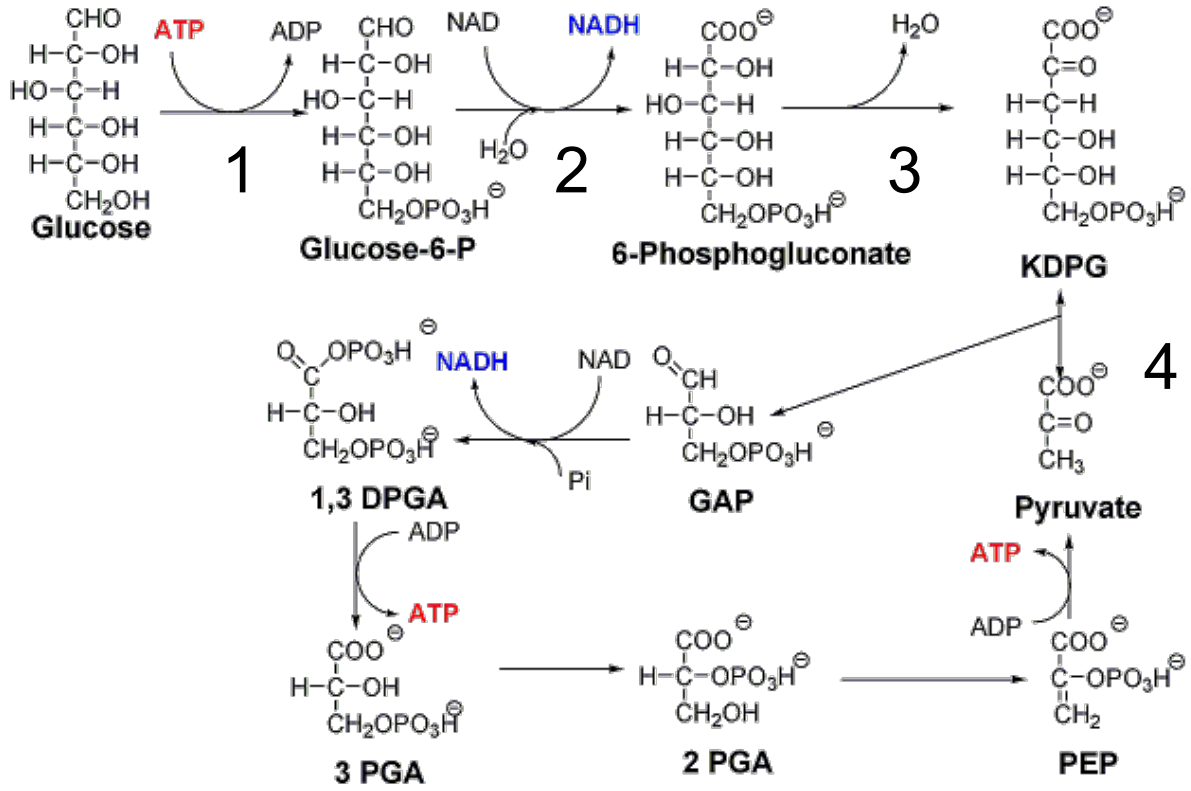
(Embden-Meyerhof-Parnas pathway, Glycolysis)



$$\text{Glucose} + 2NAD^+ + 2ADP + 2P_i \longrightarrow 2\text{Pyruvate} + 2NADH + 2ATP$$

http://4.bp.blogspot.com/_t38RPovEIS8/TRYWdZrSt7I/AAAAAAAAADA/O_h0g2Fve9Q/s1600/glycolysis_pathway.jpg

Entner-Doudoroff pathway in many Pseudomonads



(1) Hexokinase; (2) glucose-6-phosphate dehydrogenase; (3) phosphogluconate dehydrase; (4) 2-keto-3-deoxy-6-phosphogluconate aldolase.

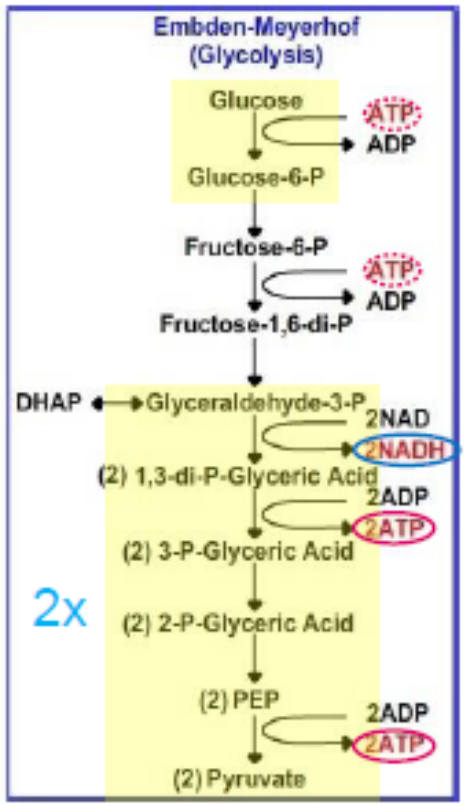
$$\text{Glucose} + 2NAD^{+} + 1ADP + 1P_i \longrightarrow 2\text{Pyruvate} + 2NADH + 1ATP$$

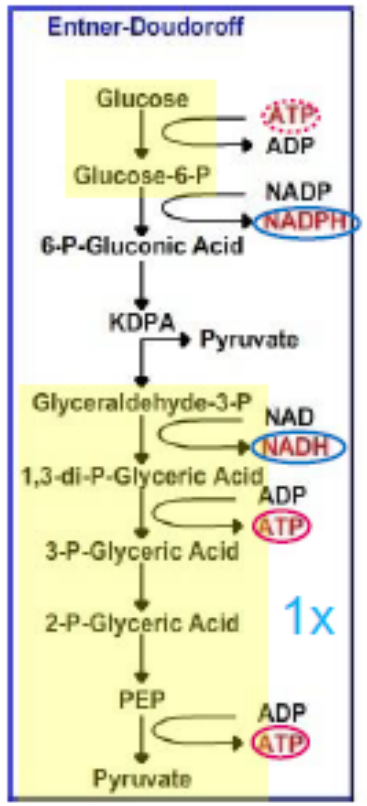
EMP (glycolysis) *vs.* ED pathways

ATP and NADH balances:

-2 ATP (consumed)
+4 ATP (produced)
= +2 ATP

+2 NADH (produced)
= +2 NADH





ATP and NADH balances:

-1 ATP (consumed)
+2 ATP (produced)
= +1 ATP

+2 «NADH» (produced)
= +2 NADH

Glucose + 2NAD$^+$ + 2ADP + 2P_i
→ 2Pyruvate + 2NADH + 2ATP

Glucose + 2NAD$^+$ + 1ADP + 1P_i
→ 2Pyruvate + 2NADH + 1ATP

Pentose-phosphate pathway

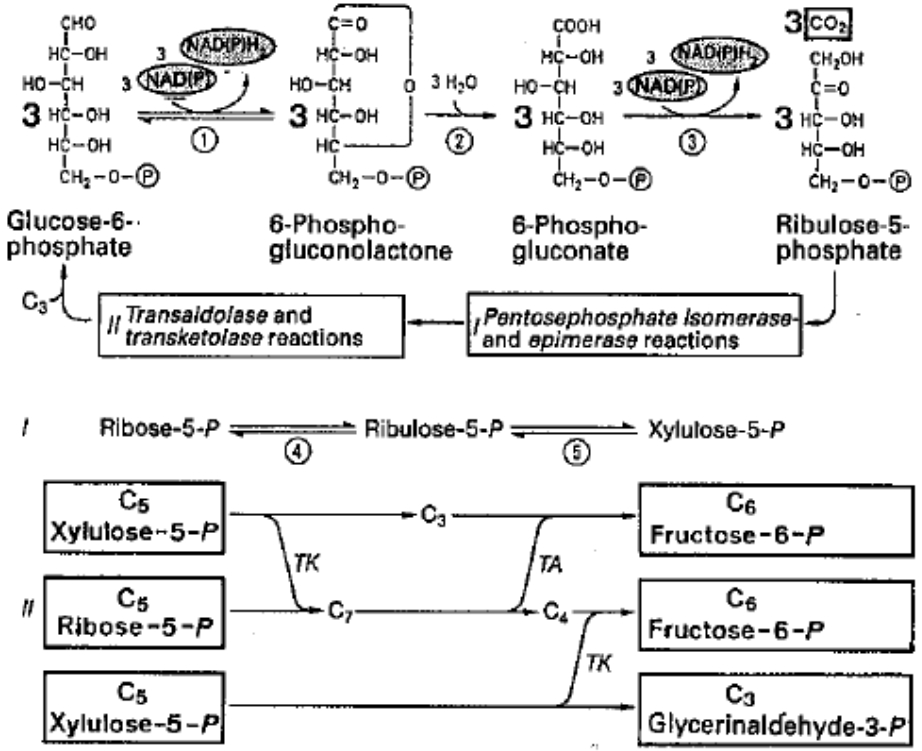
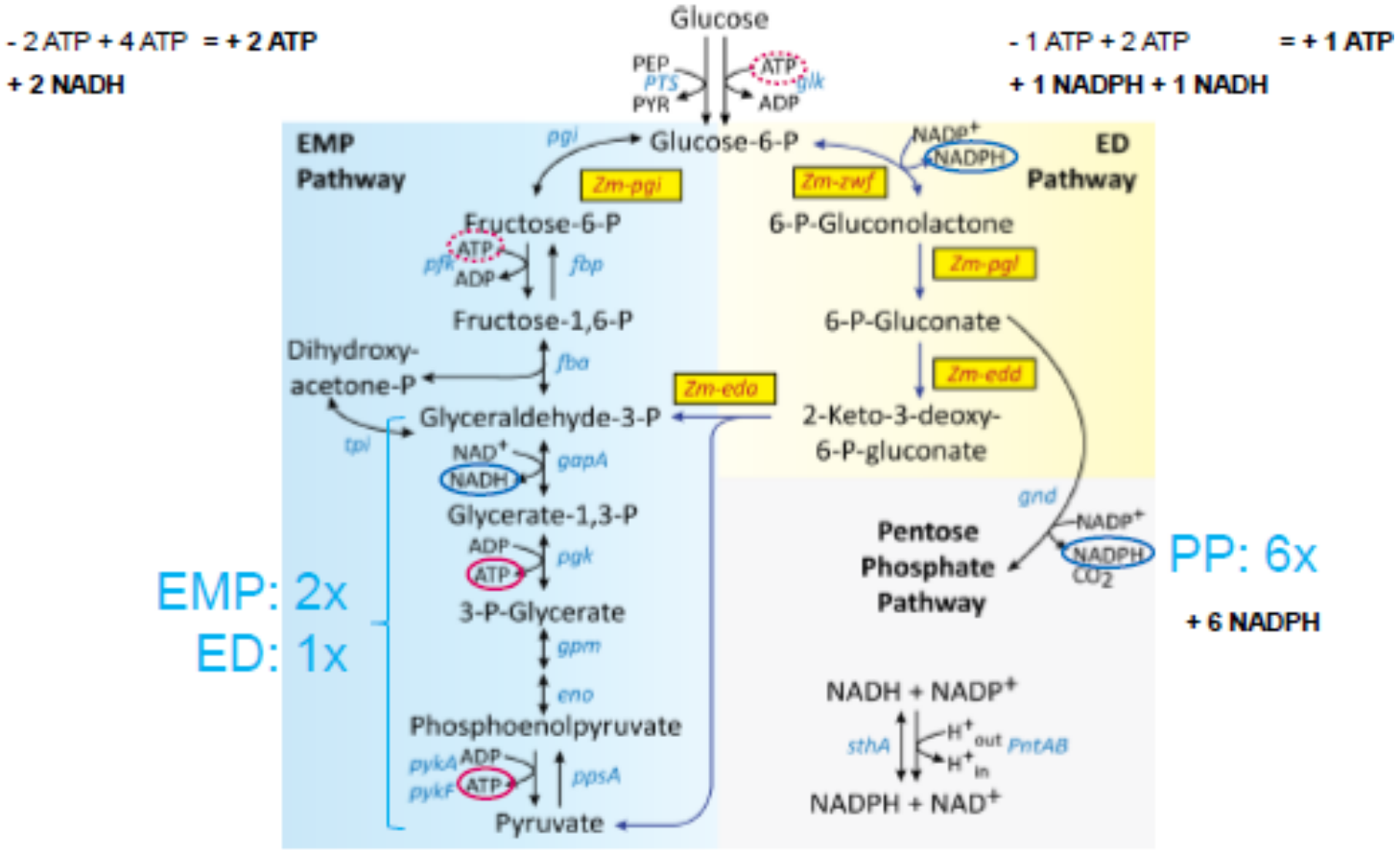


Fig. 7.4. *The pentose-phosphate pathway for the oxidative catabolism of glucose-6-phosphate.*

The oxidative steps culminate in the formation of ribulose-5-phosphate. The ribulose-5-phosphate exists in an enzyme-catalysed equilibrium with ribose-5-phosphate and xylulose-5-phosphate. The pentose-phosphates are converted to two fructose-phosphates and one glyceraldehyde-phosphate by the actions of transketolase and transaldolase. These reactions are completely reversible; in the reverse direction, they participate in the ribulose-monophosphate cycle of formaldehyde fixation and in the ribulose-bisphosphate cycle of carbon dioxide fixation, as well as in other cyclical processes. The enzymes involved are: (1) glucose-6-phosphate dehydrogenase; (2) lactonase; (3) 6-phosphogluconate dehydrogenase; (4) phosphoribose isomerase; (5) ribulose-5-phosphate-3-epimerase; TK, transketolase; TA, transaldolase.

Glucose + 1ATP + 6$NADP^+$ ⟶ Glyceraldehyde 3-P + ADP + 6NADPH + $3CO_2$

EMP *vs.* ED *vs.* PP pathways



http://dx.doi.org/10.1016/j.ymben.2015.03.001

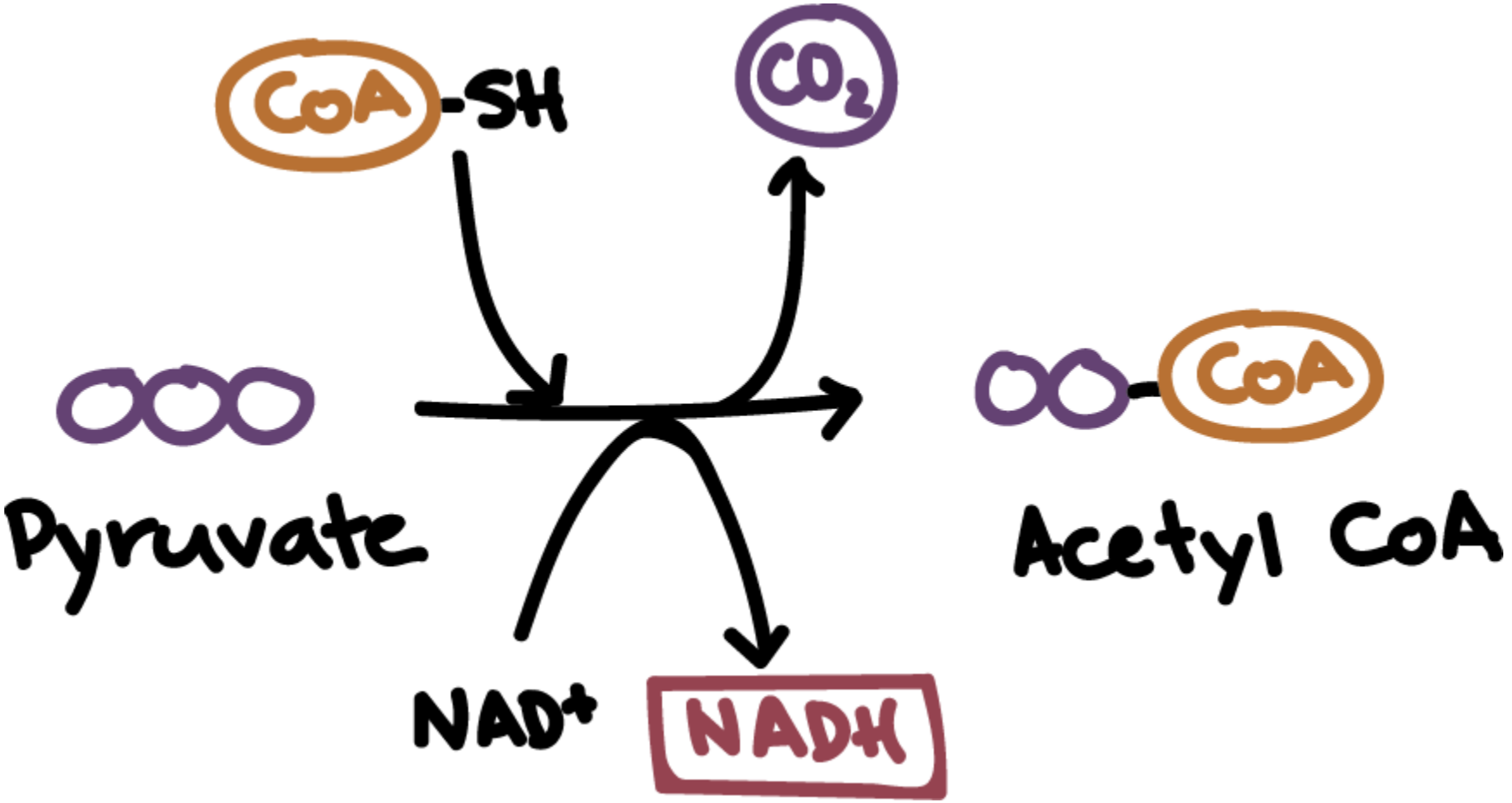
Table 7.3. *The participation of different pathways in the catabolism of hexose (%)*

EMP PP ED

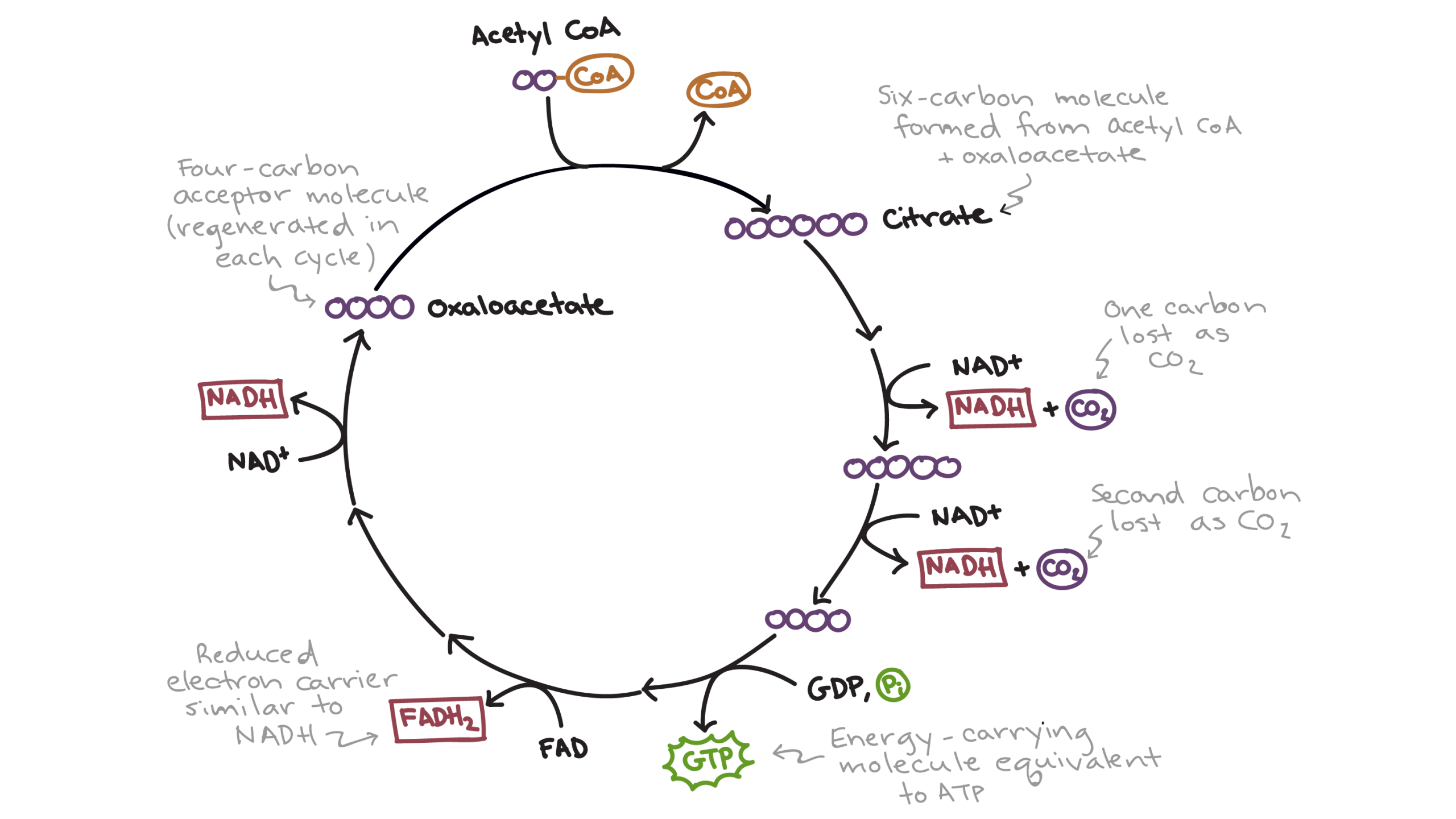
Species	Fructose-1,6-bisphosphate pathway (%)	Pentose-phosphate pathway (%)	2-keto-3-deoxy-6-phosphogluconate pathway (%)
Candida utilis	70–80	30–20	
Streptomyces griseus	97	3	
Penicillium chrysogenum	77	23	
Escherichia coli	72	28	
Bacillus subtilis	74	26	
Pseudomonas aeruginosa		29	71
Gluconobacter oxydans		100	
Pseudomonas saccharophila			100
Atcaligenes eutrophus			100

Ng CY, Farasat I, Maranas CD, Salis HM. Rational design of a synthetic Entner–Doudoroff pathway for improved and controllable NADPH regeneration. Metabolic Engineering. 2015;29:86-96.

Oxidative phosphorylation



https://www.khanacademy.org/science/biology/cellular-respiration-and-fermentation/pyruvate-oxidation-and-the-citric-acid-cycle/a/pyruvate-oxidation


Acetyl CoA
CoA
CoA
Six-carbon molecule formed from acetyl CoA + oxaloacetate
Citrate
Four-carbon acceptor molecule (regenerated in each cycle)
Oxaloacetate
One carbon lost as CO_2
NAD+
NADH + CO_2
Second carbon lost as CO_2
NAD+
NADH + CO_2
GDP, P_i
GTP
Energy-carrying molecule equivalent to ATP
FAD
$FADH_2$
Reduced electron carrier similar to NADH
NAD+
NADH

Krebs cycle

(Citric acid cycle)

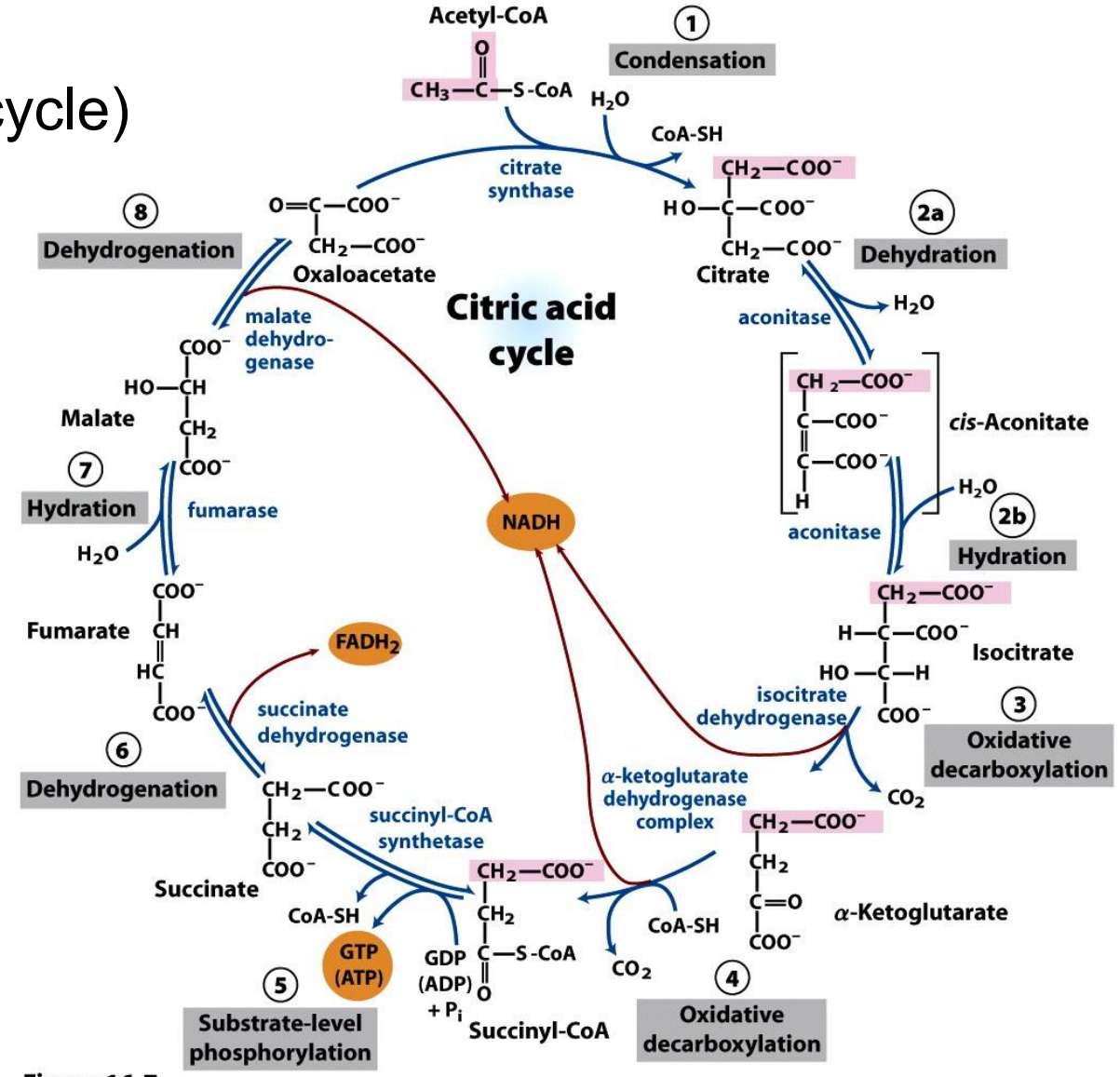


Figure 16-7
Lehninger Principles of Biochemistry, Fifth Edition

$$\text{Acetyl-CoA} + 3NAD^+ + FAD + GDP(ADP) \longrightarrow 2CO_2 + \text{coenzyme A} + 3NADH + FADH^+ + GTP(ATP)$$

Oxidative and substrate-level phosphorylation

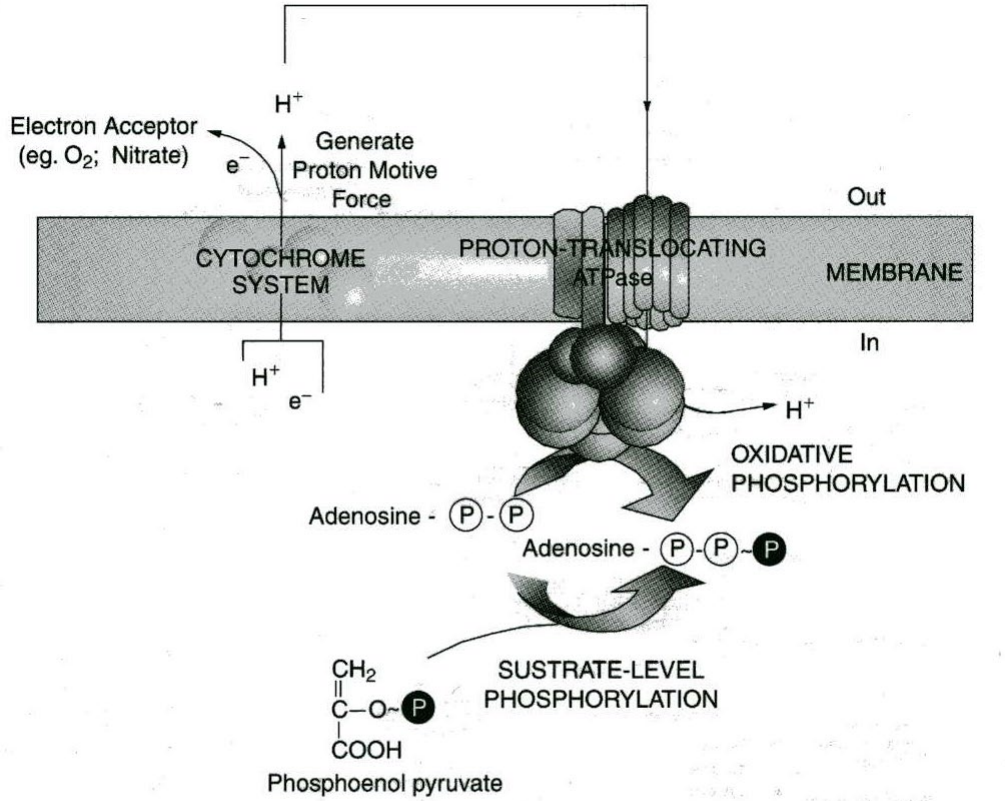


Fig. 1-12. Reactions essential to energy production. Oxidative phosphorylation. The energy that comprises the proton motive force can be harnessed and used to generate ATP when protons from outside the cell pass through the membrane-associated proton-translocating ATPase. The energy released will run the ATPase in reverse. It is estimated that passage of three H^+ through the ATPase is required to generate one ATP. Substrate-level phosphorylation. Energy contained within high-energy phosphate bonds of certain glycolytic intermediates can be transferred to ADP, forming ATP. The example shows phosphoenolpyruvate.

Environmental pH is important

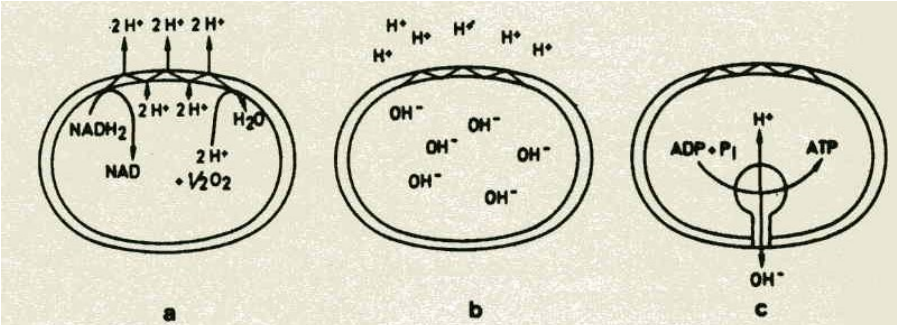


Fig. 7.8. *The respiratory chain and electron-transport phosphorylation in and at the cytoplasmic membrane of protocytes and the inner membrane of mitochondria.*

(a) $NADH_2$ oxidation and proton extrusion; (b) electrochemical gradient between inner and outer surfaces; (c) regeneration of ATP as a consequence of the backflow of protons.

$$\Delta p = \frac{\Delta \mu_{H^+}}{F} = \Delta\Psi - Z * \Delta pH$$

Both the pH gradient and the electrical membrane potential gradient exert a pull in the direction of the cell interior on the extruded protons = proton motive force Δp.

$\Delta\Psi$: Electrical membrane potential

Z=2.3 RT/F (59mV at 25°C)

The proton potential can be based entirely on the H difference or on the membrane potential or on both.

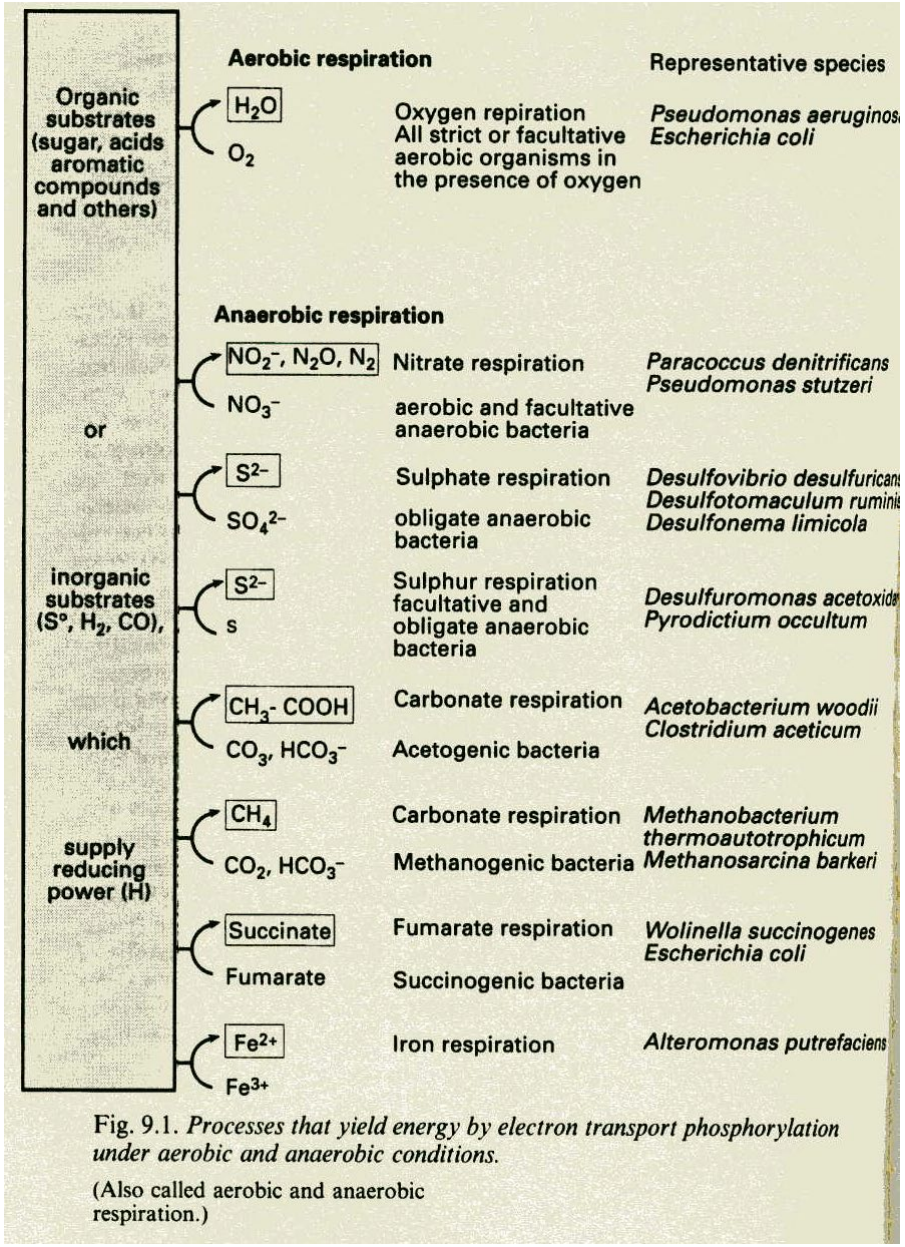


Fig. 9.1. *Processes that yield energy by electron transport phosphorylation under aerobic and anaerobic conditions.*

(Also called aerobic and anaerobic respiration.)

Combined energy gain

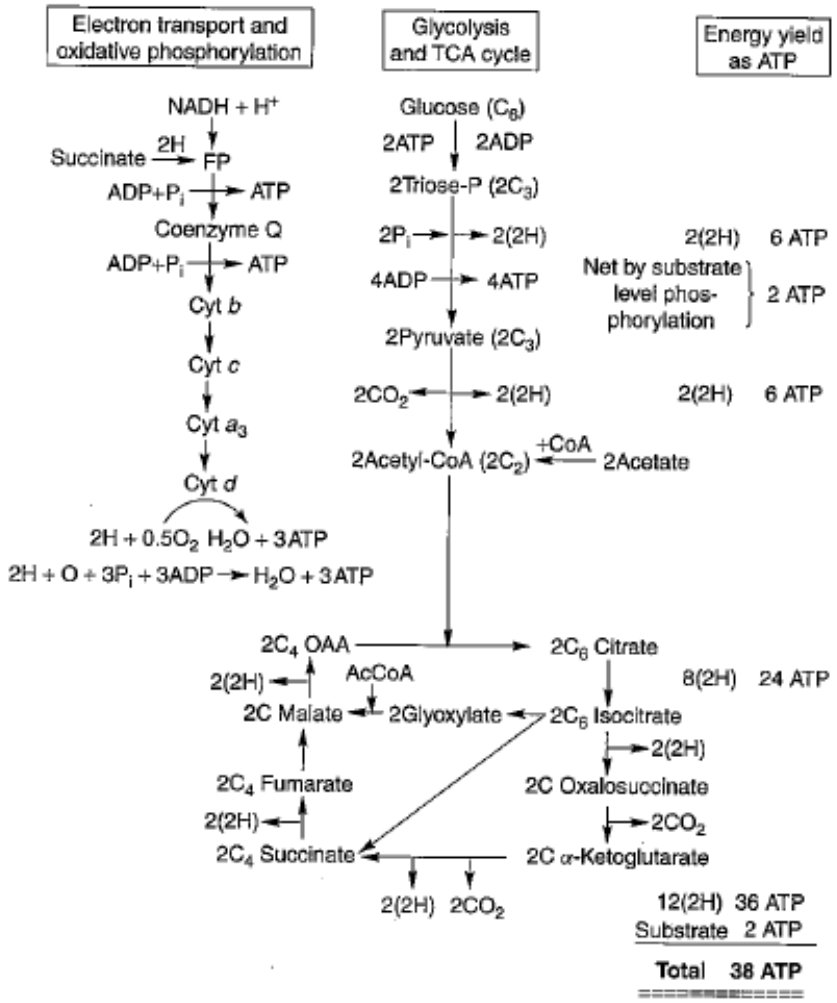
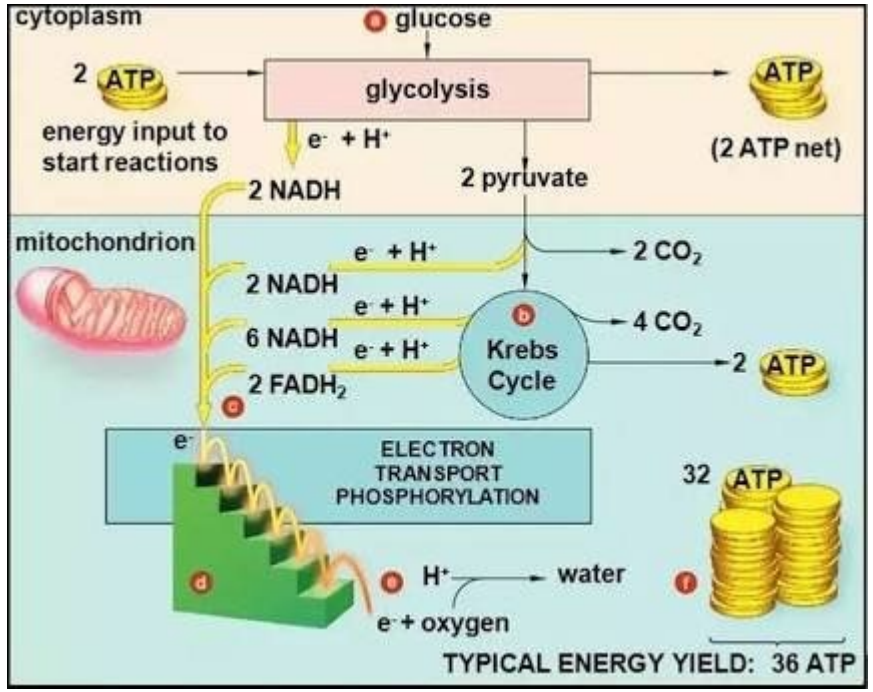


Fig. 9-9. Theoretical energy yield as ATP from glycolysis and the TCA cycle. The calculations shown here assume that each pair of hydrogen atoms (2H) released from the substrate yields 3 ATP. The reaction shown as $ADP + P_i \rightarrow ATP$ represents the action of ATP synthase. Two turns of the TCA cycle are required to completely oxidize the 2 acetyl-CoA derived from glucose. Each 2H generated by the system yields 1 molecule of water ($2H + 0.5O_2 \rightarrow H_2O$). Overall reaction: $C_6H_{12}O_6 + 38P_i + 38ADP + 6O_2 \rightarrow 6CO_2 + 6H_2O + 38ATP$. Total ATP from TCA cycle: $12(2H) + 6O_2 + 36P_i \rightarrow 6H_2O + 36ATP$.



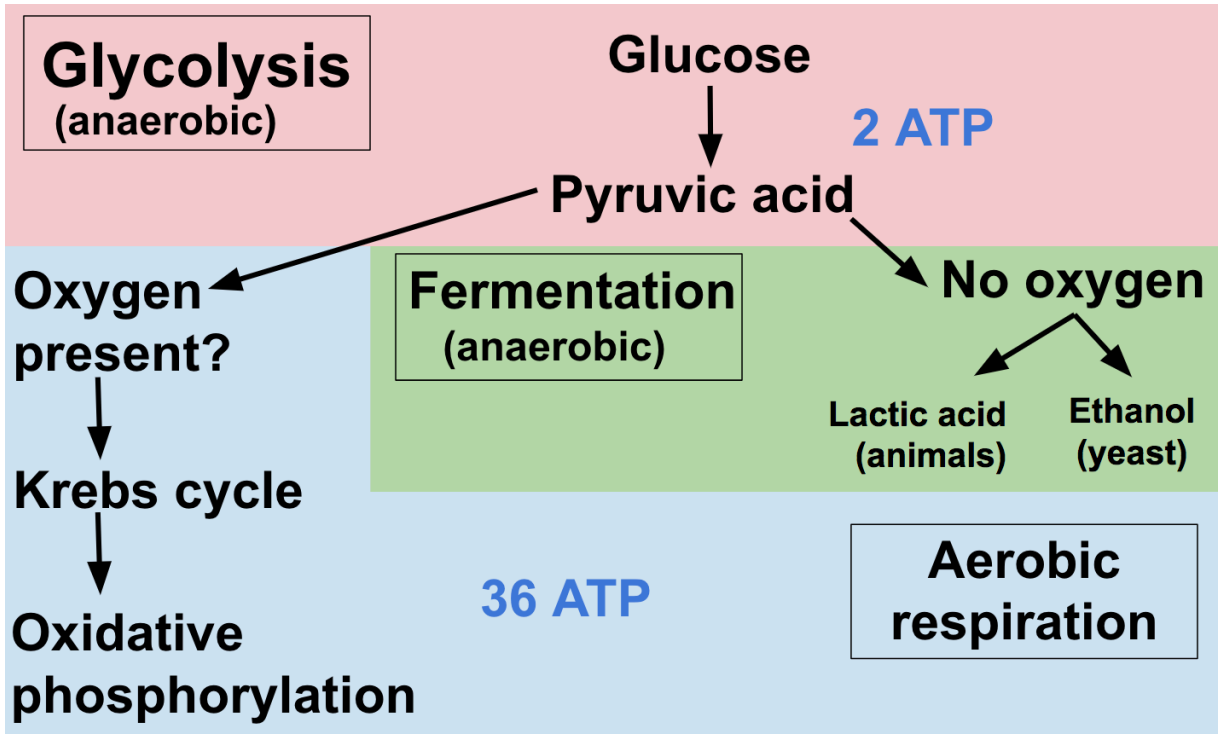
P/O ratios:

$NADPH + 3ADP + 3P_i + 0.5O_2 \rightarrow NADP^+ + 3ATP + H_2O$

$NADH + 3ADP + 3P_i + 0.5O_2 \rightarrow NAD^+ + 3ATP + H_2O$

$FADH + 2ADP + 2P_i + 0.5O_2 \rightarrow FAD^+ + 2ATP + H_2O$

Consumption of glucose under aerobic and anaerobic growth conditions



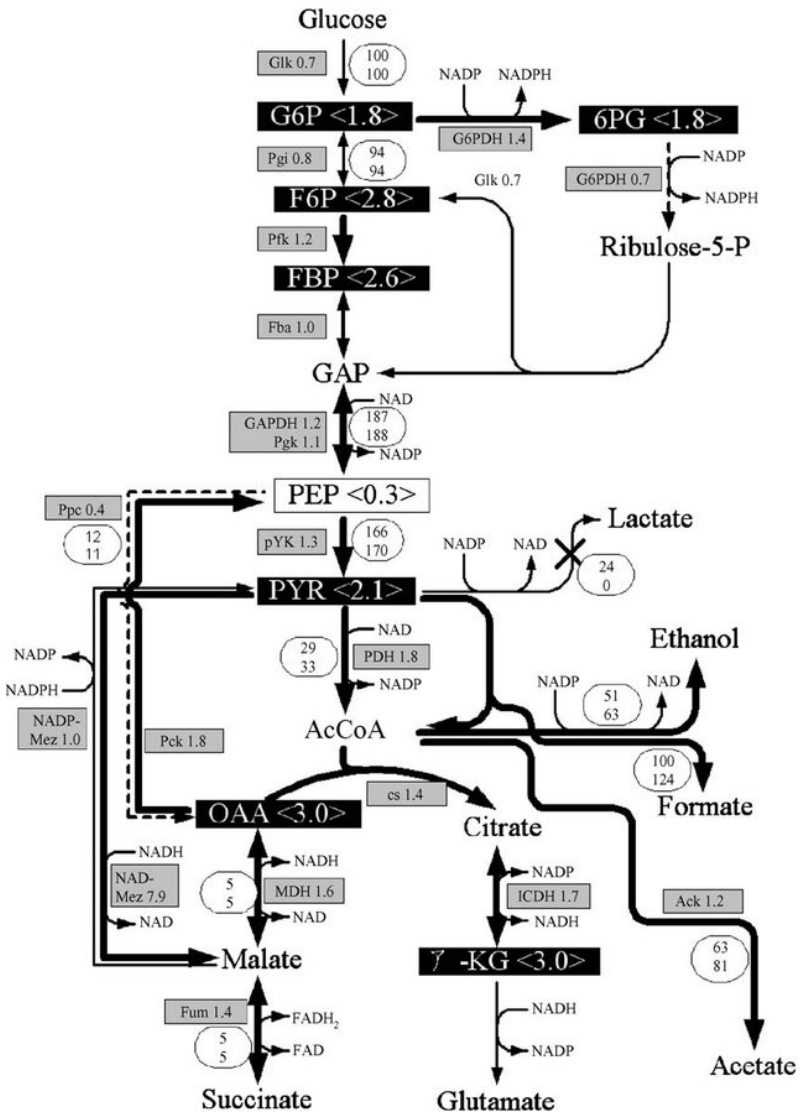
Anaerobic respiration is a normal part of cellular respiration. Glycolysis, which is the first step in all types of cellular respiration is anaerobic and does not require oxygen.

If oxygen is present, the pathway will continue on to the Krebs cycle and oxidative phosphorylation.

However, if oxygen is not present, some organisms can undergo fermentation to continually produce ATP.

https://www.khanacademy.org/science/in-in-class-11-biology-india/x9d1157914247c627:respiration-in-plants/x9d1157914247c627:fermentation-and-the-amphibolic-pathway/a/hs-cellular-respiration-review

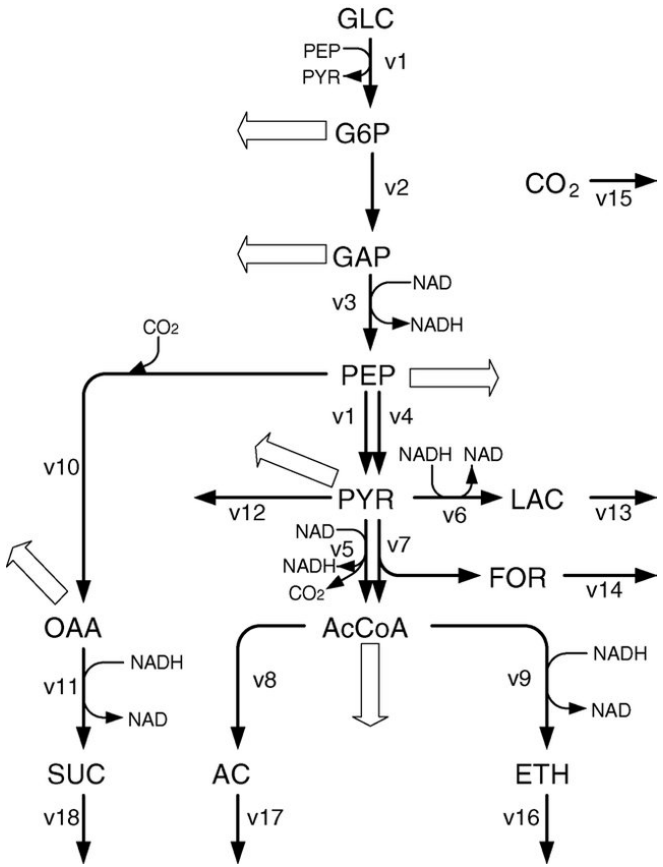
Anaerobic growth of *Escherichia coli* (flux model)



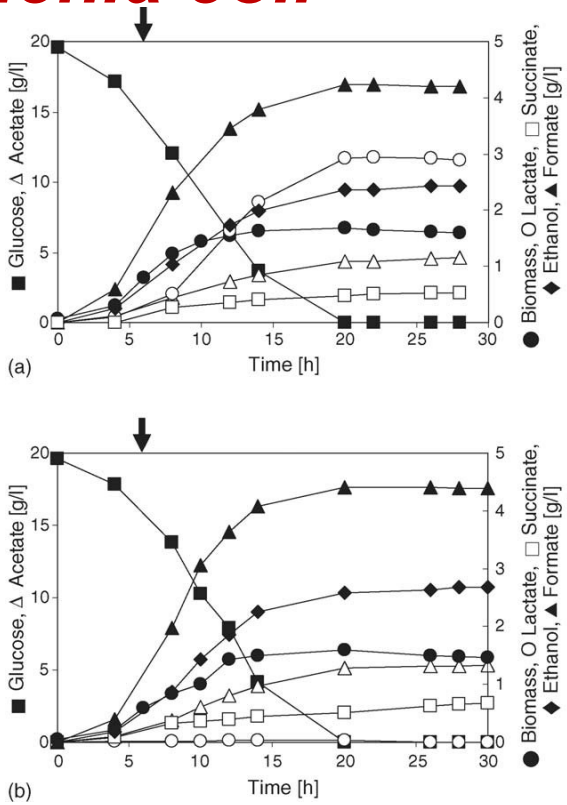
Anaerobic metabolism of glucose in *E. coli*. The value beside the intracellular metabolite and the enzyme in grey box is the relative value for *ldhA* mutant compared to the parent strain. The oval box represents the flux data: upper value, parent strain; lower value, ldhA mutant. The cross represents knockout of *ldhA* gene. Thick line, upregulation; dotted line, downregulation.

Kabir MM, Ho PY, Shimizu K. Effect of *ldhA* gene deletion on the metabolism of *Escherichia coli* based on gene expression, enzyme activities, intracellular metabolite concentrations, and metabolic flux distribution. Biochemical Engineering Journal. 2005;26(1):1-11.

Anaerobic growth of engineered *Escherichia coli*



Fermentative pathways of *Escherichia coli* grown on glucose. '⇨' indicates biomass formation flux. The fluxes through each pathway are designated v1-v18. Abbreviations: PYR, pyruvate; LAC, lactate; GLC, glucose; G6P, glucose-6-phosphate; GAP, glyceraldehydes-3-phosphate; PEP, phosphoenol pyruvate; AcCoA, acetyl-CoA; ETH, ethanol; SUC, succinate; OAA, oxaloacetate; BIO, biomass; FOR, formate; AC, acetate.



Batch cultivation results of parent *E. coli* (a) and lactate dehydrogenase (*ldhA)* mutant *E. coli* (b) grown on glucose under anaerobic condition. The values presented here are the mean values from four independent measurements (duplicate measurements from duplicate experiments). The arrow indicates the sampling time for RT-PCR analysis, measurement of enzyme activities, and intracellular metabolites.

Kabir MM, Ho PY, Shimizu K. Effect of *ldhA* gene deletion on the metabolism of *Escherichia coli* based on gene expression, enzyme activities, intracellular metabolite concentrations, and metabolic flux distribution. Biochemical Engineering Journal. 2005;26(1):1-11.

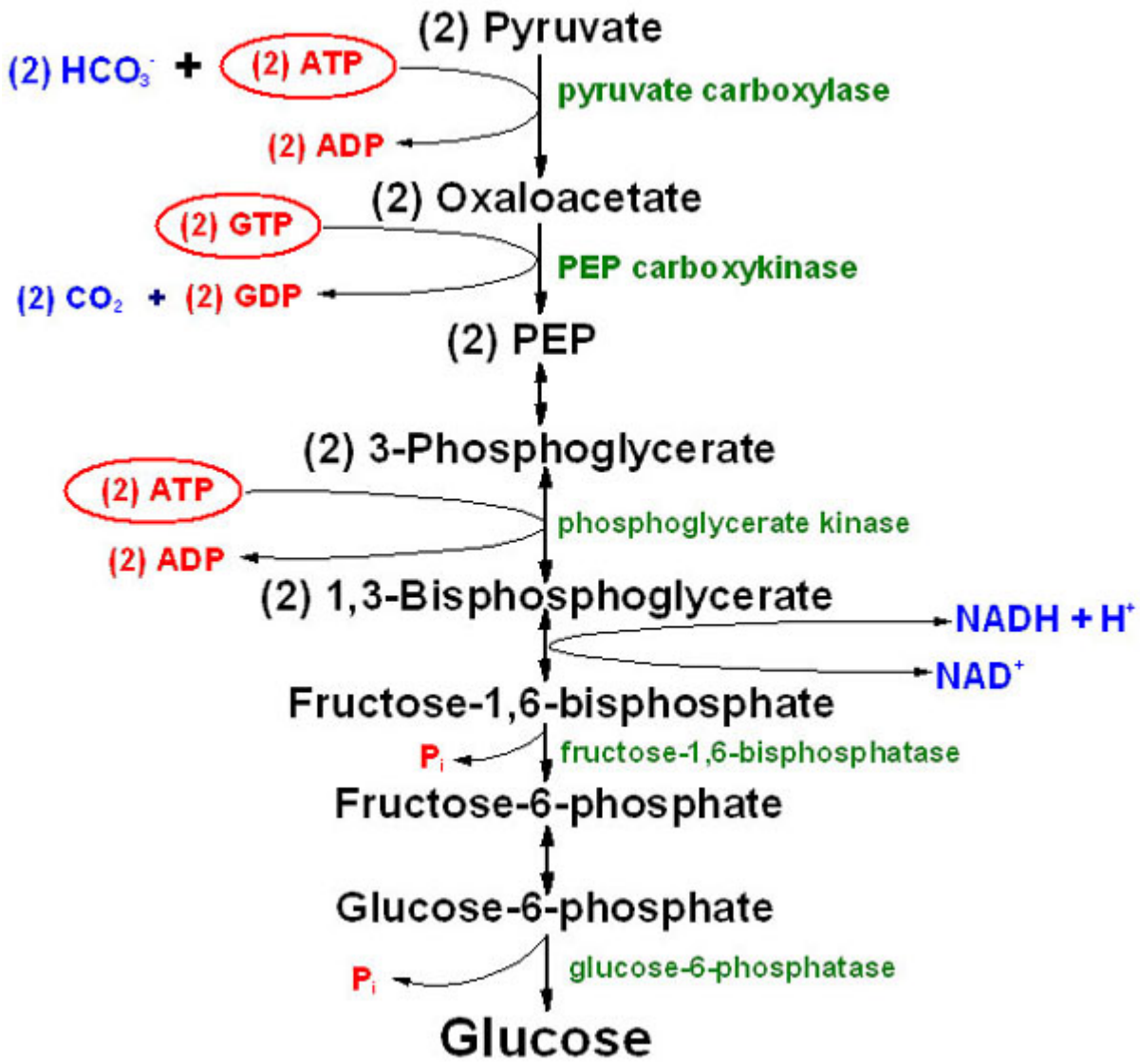
The capacity of bacteria for biosynthesis

TABLE 5.2 The Biosynthetic Capabilities of a Bacterial Cell[a]

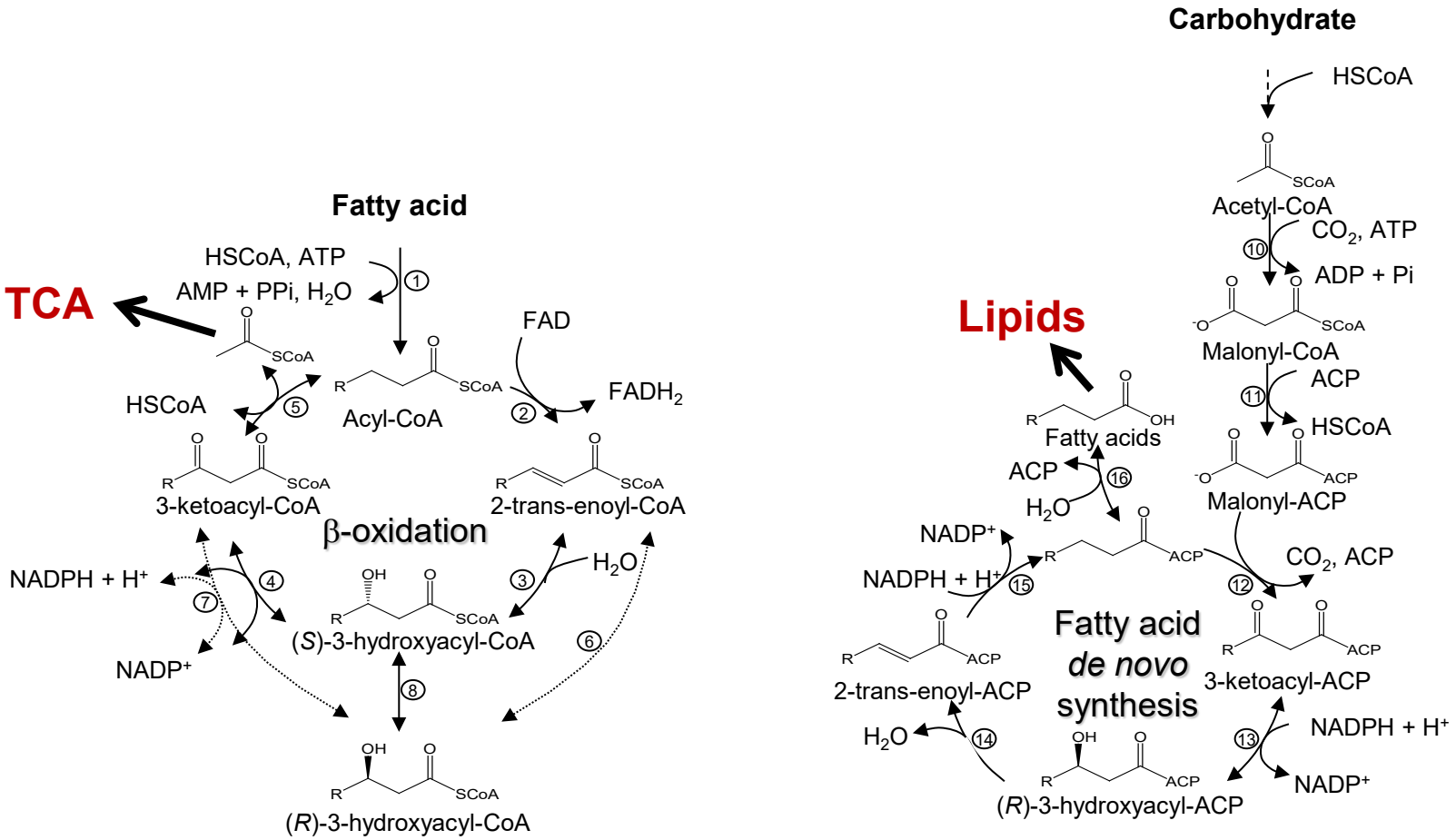
Chemical component	Percent of dry weight	Approximate molecular weight	Number of molecules per cell	Number of molecules synthesized per second	Number of molecules of ATP required to synthesize per second	Percent of total synthetic energy required
DNA	5	2,000,000,000	1	0.00083	60,000	2.5
RNA	10	1,000,000	15,000	12.5	75,000	3.1
Protein	70	60,000	1,700,000	1,400	2,120,000	88.0
Lipids	10	1,000	15,000,000	12,500	87,500	3.7
Polysaccharides	5	200,000	39,000	32.5	65,000	2.7

[a] Reprinted with permission from Lehninger (1971). *Escherichia coli* is about $1 \times 1 \times 3$ μm in size; it has a volume of 2.25 μm^3, a total weight of 10×10^{13} g, and a dry weight of 2.5×10^{-13} g. The rates of biosynthesis were averaged over a 20 minute cell division cycle.

Gluconeogenesis



Fatty acid degradation and fatty acid *de novo* synthesis



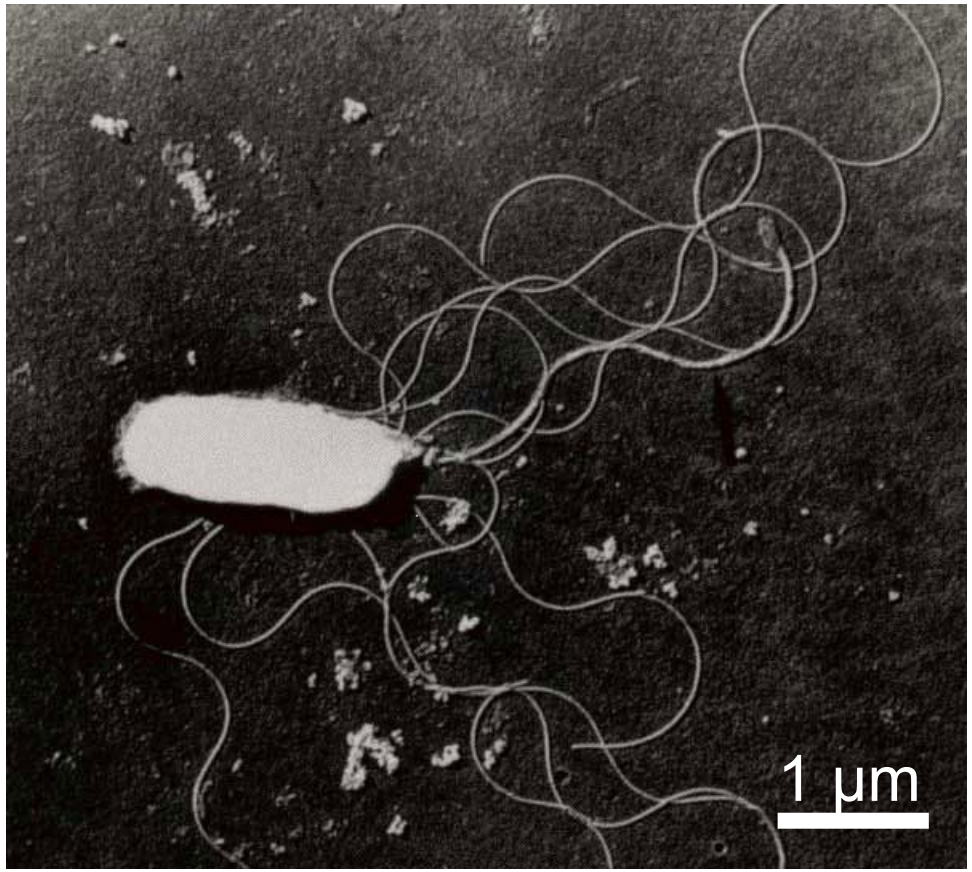
Questions

- Why do different growth pathways exist?
- Where do we obtain informations on metabolic pathways?
- What is the meaning of 5.3.1.9? (see kegg)
- What are anapleurotic sequences?
- Can the cell control the cellular flux of metabolites?

Energy (ATP) is required for anabolism but also for cell maintenance

The cell needs maintenance energy for various activities:

- Transformation of cellular materials (recycling)
- Activation of enzymatic reactions (phosphorylation)
- Maintenance of an electrochemical gradient between inside and outside of the cell
- Cell movement: World record owns *Vibrio cholerae:* 12 mm min^{-1} (ca. 50x body length/sec)



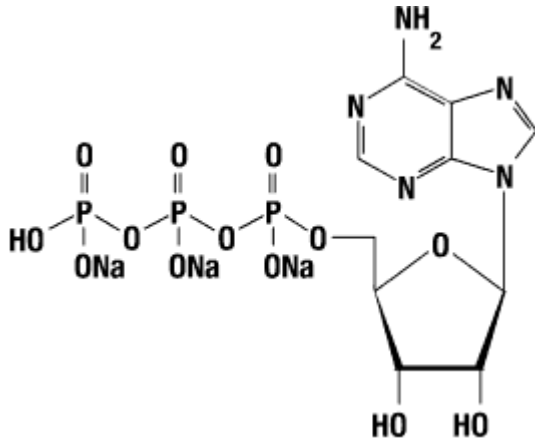
Escherichia coli

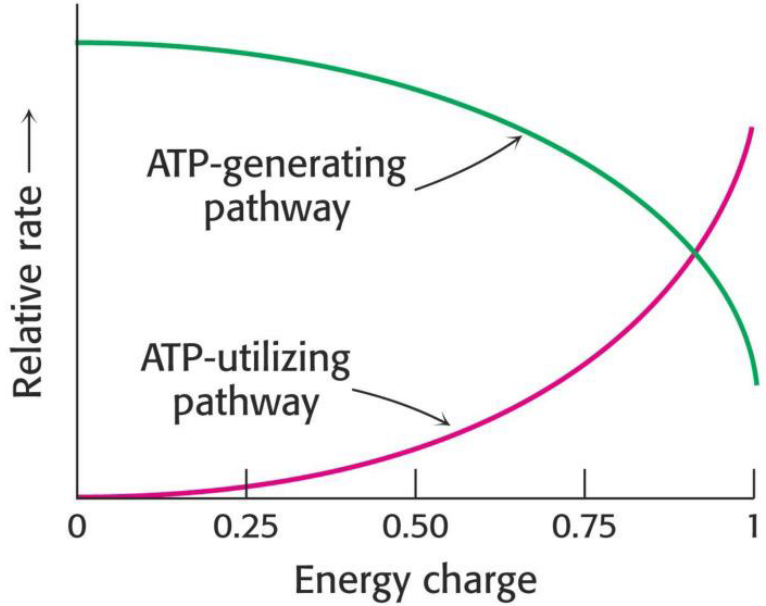
The energy charge

The energy unit in biochemistry: Adenosin triphosphate (ATP)

$$ATP \rightleftharpoons ADP + P_i$$

$$\Delta G = -30.6 \text{ kJ}$$





The energy charge:

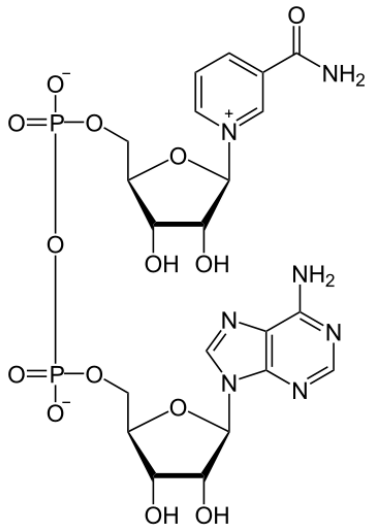
$$\frac{[ATP]+0.5[ADP]}{[ATP]+[ADP]+[AMP]}$$

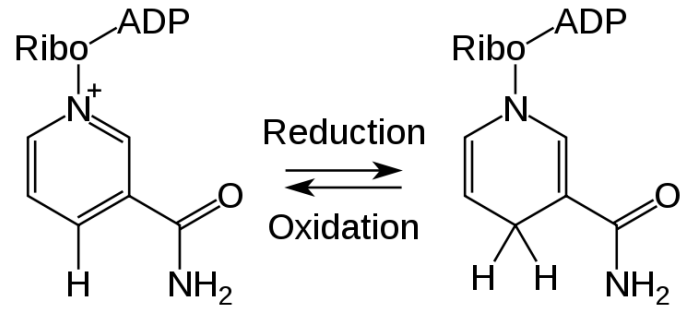
High energy charge regulates metabolism.

High conc. of ATP inhibit the relative rates of a typical ATP-generating (catabolic) pathway and stimulate the typical ATP-utilizing (anabolic) pathway (The energy charge, like the pH of a cell, is 'buffered'!).

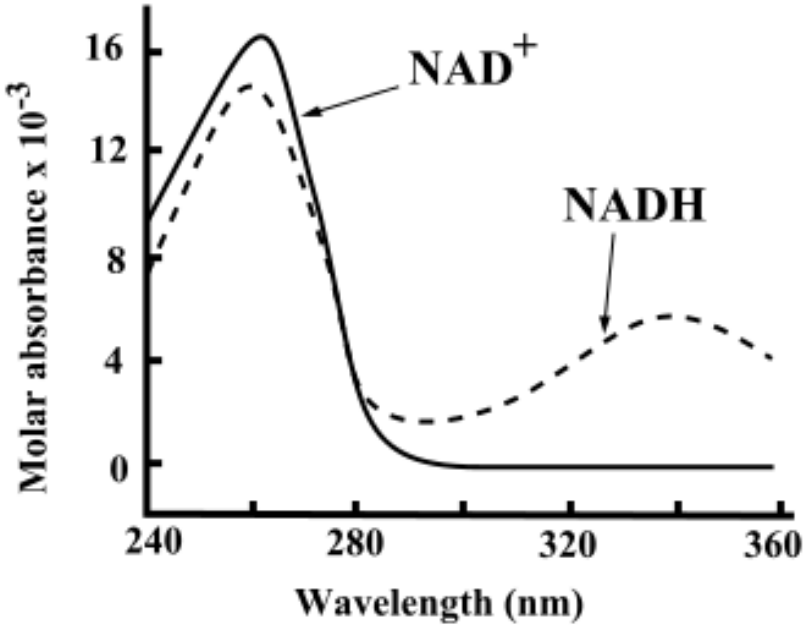
E. coli has an energy charge of 0.8 during exp. growth phase that slowly decreases to 0.5 during stationary growth phase. The cells die when the value is <0.5.

Nicotinamide adenine dinucleotide

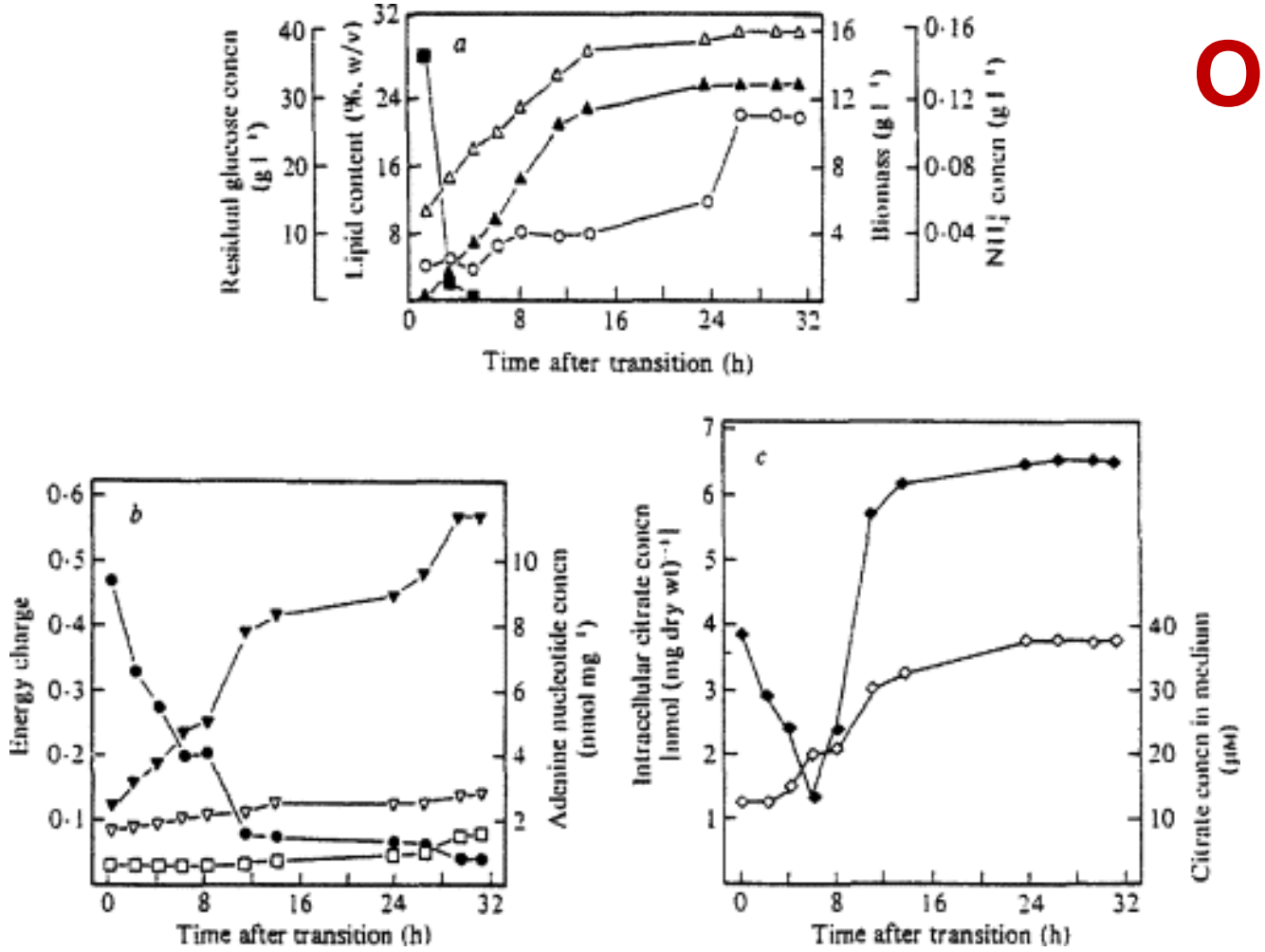


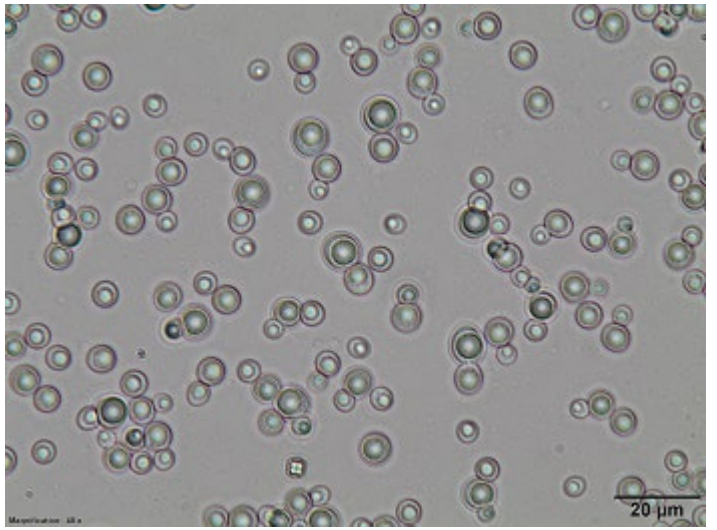


Nicotinamide adenine dinucleotide, abbreviated NAD^+, is a coenzyme found in all living cells. The compound is a dinucleotide, since it consists of two nucleotides joined through their phosphate groups. One nucleotide contains an adenine base and the other nicotinamide.



Oil produced by yeasts



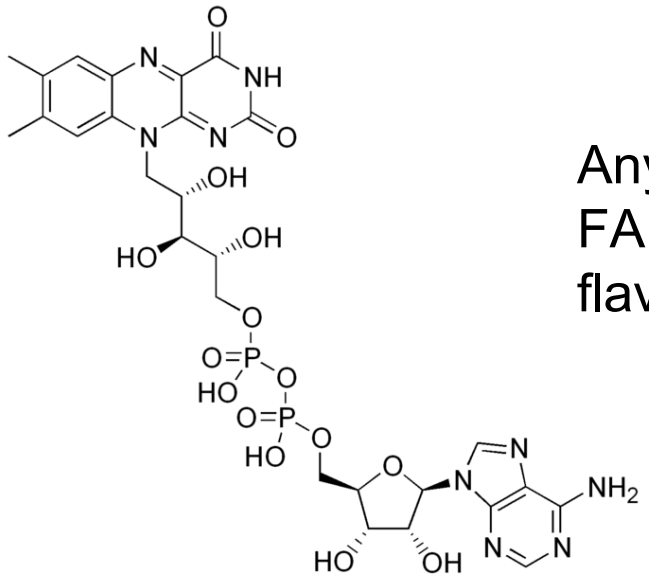


Lipomyces starkeyi

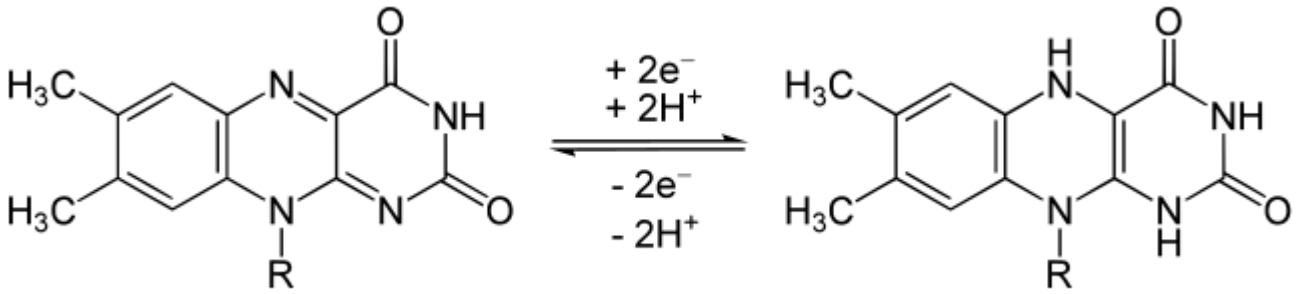
Ratledge C, Wynn JP. The Biochemistry and Molecular Biology of Lipid Accumulation in Oleaginous Microorganisms. In: Laskin AI, Bennett JW, Gadd GM, editors. Advances in Applied Microbiology. 51: Academic Press; 2002. p. 1-52.

Pattern of lipid accumulation in the oleaginous yeast, *Lipomyces starkeyi*, during transition from carbon-limited growth to nitrogen-limited growth. The yeast was in steady-state continuous culture growing at a constant rate of 0.06 h^{-1}; at zero time the medium was switched and effectively all residual NH_4^+ was consumed in about 3–4 h; the biomass began to increase immediately from time zero but lipid accumulation did not commence until after 8 h (A) during which time the AMP concentration had dropped by 80% (B) and citrate had begun to accumulate (C). (A) Biomass (Δ), lipid content of cells (○), concentration of NH_4^+ (■) and glucose (▲) in medium; (B) intracellular concentration of AMP (●); ADP (∇), ATP (□) and energy change (▼); c): intracellular (♦) and extracellular (◊) concentrations of citrate.

Flavin adenine dinucleotide (FAD)



Any oxidoreductase enzyme that uses FAD as an electron carrier is called a flavoprotein.



Questions

- What is essential for growth?
- What‘s the difference between a living and a dead cell?
- How can we determine whether a cell is still alive?
- What means VBNC?
- Can we cultivate all cells in a bioreactor?

3. Preservation of cells

https://delivery-files.atcc.org/api/public/content/164341-Norm_-Barker_Agar-Plates.jpg

Importance of maintaining and preservation of microorganisms

- There have been done significant efforts for **isolating strains** that are performing special tasks (e.g. overexpression of enzymes) and have been adapted to particular growth conditions.
- An important goal is to **preserve such strains** for scientific but also for industrial applications. The strains have to be pure and should keep their properties (reduction of mutation).
- Large **strain collections** have been established in industry and by academic and governmental organizations.

Choice of method of preservation

There are different methods for the conservation of microorganisms having advantages and disadvantages. All the characteristics have to be compared and carefully evaluated.

The key characteristics that have to be considered:

- Conservation of viability
- Modification of characteristical properties
- Genetical stability
- Purity: only one strain! (= axenic culture)
- Costs
- Number of strains to be preserved
- Value of strains to be conserved
- Distribution and transportation of cultures
- Frequency of utilization of strain

Clear statements on preservation

Take home messages:

1. There is no optimal preservation for all characteristics.

2. Each method is a compromise of costs, labour intensity and preservation stability.

3. Each microorganism behaves differently.

4. The optimal method has to be found by trial and error or on base of literature study.

Methods for conservation (1)

- It is recommended to store a valuable strains using more than 3 different methods.
- It is recommended to do test cultivations in order to assess the fitness of the strains after conservation. Suitable methods are spread plating and liquid cultures.
- There are 4 fundamental techniques used to conserve cells:

1. Subculturing/ transfer of cultures (e.g. kefir)
2. Drying
3. Lyophylization
4. Freezing

Methods of conservation (2)

1. Subculturing

Regular transfer of cells on a fresh medium. The frequency of subculturing depends on the stability of the cells. A survival rate of 10% is acceptable if cells can be grown on liquid or solid medium.

2. Drying of cells

Cultures are usually dried at ambient temperatures in the vacuum. Frequently a matrix component is added, e.g. silicon or plastic beads for quicker drying and also an emulsifier, e.g. skim milk. The conservation is usually performed in glass vials in a desiccator at low temperature (4°C). The glass vials can be heat sealed.

This method is particular suitable for fungi.

Methods of conservation (3)

3. Lyophilisation

The cells in a culture are concentrated, mixed with a protecting agent, e.g. glycerol, BSA for the avoidance of too quick drying. Drying is perforemd in a vacuum chamber (lyophiliser) with cooling coil. The samples are kept at low temperature in the beginning (-20° to -160°C).

About 95% of water can be eliminitated after 4 -6 hours. Subsequently the vials are closed and frequently stored under nitrogen atmosphere or another inert gas.

This technique is most popular and is suitable for a long-time storage of cells. Most of the cells of culture collections are conserved by such a procedure.

Methods of conservation (4)

A large number of parameters influence the survivability of cells after lyophilization :

- **Age of inoculum:** The cells are surviving better when they are treated in the late exponential/early stationnary state.
- **The medium for culturing the cells** : In general a defined medium is better than a rich medium because of oxidation stability.
- **The culture medium** can help to increase the protection of cells: glucose-serum, « lait écrémé », saccharose, etc.).
- **The period of drying and the level of drying:** It is generally accepted that about 1% of relative humidity is helping to increase the survivability.
- **The medium for rehydration** : A medium rather diluted appears to be favourable for bringing the cells back to life.
- **Intrinsic factors:** e.g. about 75% of all tested yeasts strains of *S. cerevisiae* have a survivability of only 5% . However, there are strains that have a survivability of more than 95%.

Method of conservation (5)

4. Frozen stocks

- Conservation of microorganisms at temperatures between -20° and -196°C→ simplest method.
- Before freezeing the cells, protective agents have to be added:
  - The cryoprotecting agents are in most cases glycerol and DMSO, or a mix of glycerol-DMSO. It has been described that also a solution containing proteins, skim milk, or BSA (*bovine serum albumin*) help to increase the survivability.
- For thawing the cells, it is best to thaw them quickly at the temperature of normal growth (<40°C).

Method of conservation(6)

Investment and efficiency of different methods

Method	Costs [1] (material/ apparatus)	Costs of personnel	Survivability	Genetical stability
Periodical transfer of cultures	low	high	A few days up to 1 year	low
Paraffine coverage	low	medium	0.5 - 10 years	low
Drying with silica gel	low	medium	1- 5 years	good[2]
Lyophilization	high	medium	5 - 30 years	good[2]
Conservation by freezing (frozen stocks):				
at -20°C	medium	low	0.5 - 5 years	variable
at - 80°C	high	low	5 years	good
at -196°C	high	low	unlimited	very good

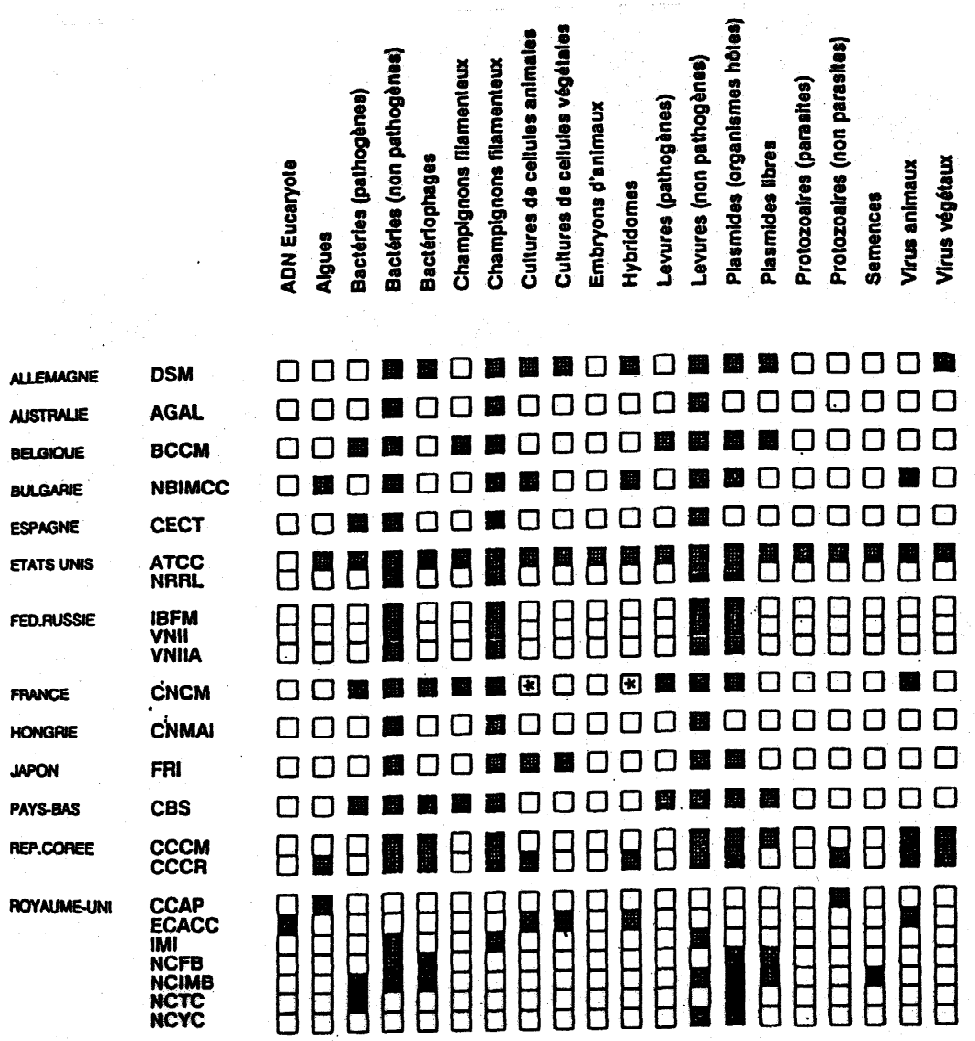
1) For maintenance of a medium sized culture collection and over a prolonged conservation period.
2) A too strong removal of water can dammage DNA.

Public strain collections

http://www.wfcc.info/ccinfo/home/

A large number of microorganisms will be conserved in public strain collections. The most important ones are:

- **ATCC** American Type Culture Collection (USA)
- **BCCM** Belgian Coordinated Collections of Microorganisms
- **CBS** Centralbureau voor Schimmelcultures (Niederland)
- **NRRL** Agriculturual Research Service Culture Collection (USA)
- **DSM** Deutsche Sammlung von Mikroorganismen und Zellkulturen (Deutschland)
- **CCoS** Culture Collection of Switzerland



Improvement of productivity

The optimization of a bioprocess can be improved at three levels:

- improvement of the fermentation (e.g. preculture, feeding strategy)
- medium optimization
- strain development

Engineering

This approach includes:

1. improvement of functionality of bioreactors for a maximal productivity. One has to assess the influence of pH, temperature, the transfer of biomass, the cell concentration, the morphology of the cells, the osmotic pressure, the rheology, etc.
2. the design of a bioreactor for a maximum productivity.
3. extraction and purification of a product (*downstream processing*)

Improvement of the productivity

Composition of the growth medium

The medium composition plays a crucial role for an optimal production. The statistical approach (*design of experiments*) helps to determine the most significant parameters for cultivation in a most efficient way.

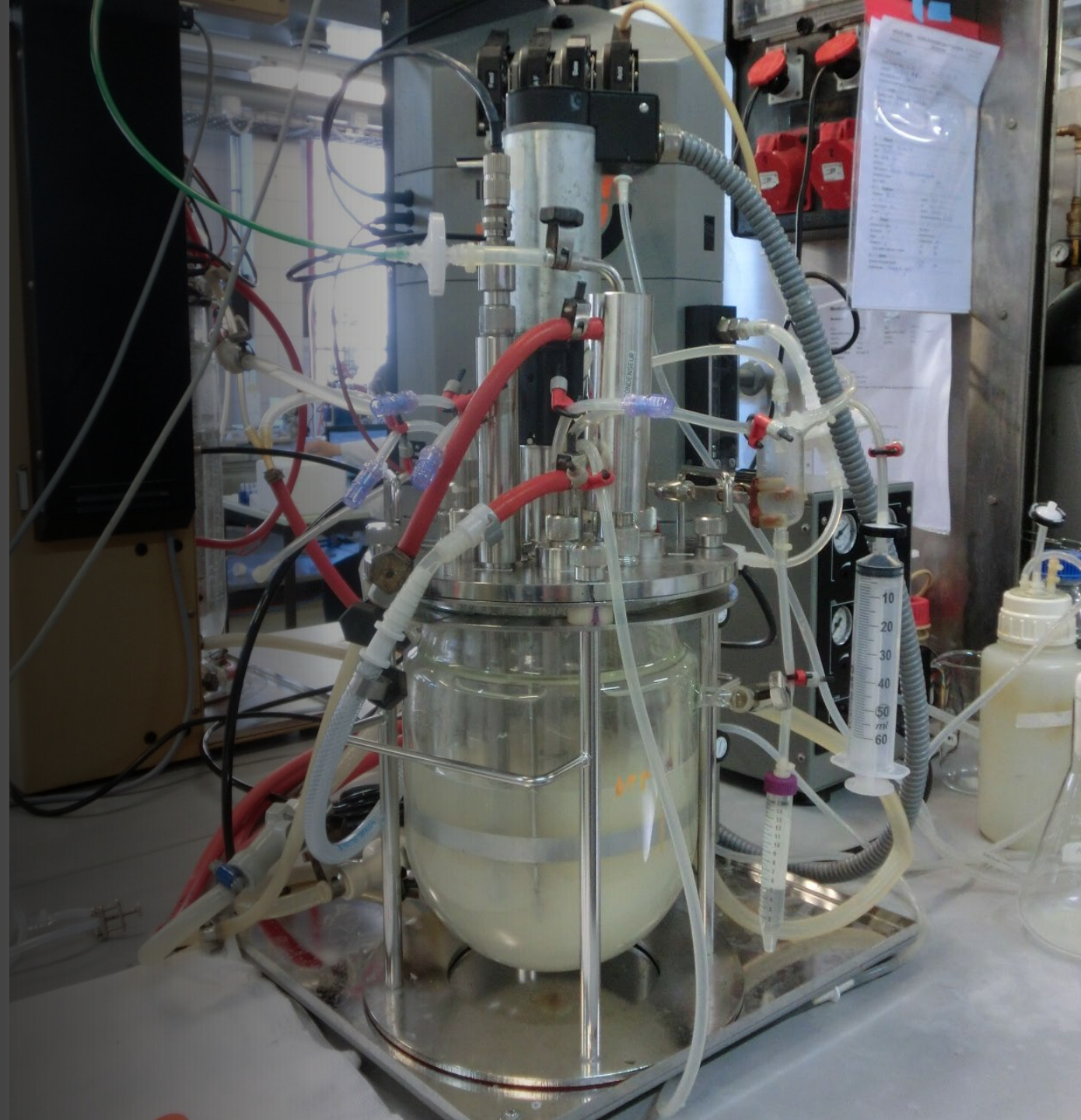
Improvement of cells

There is a possibility to increase the productivity by genetical engineering. The objectives are:

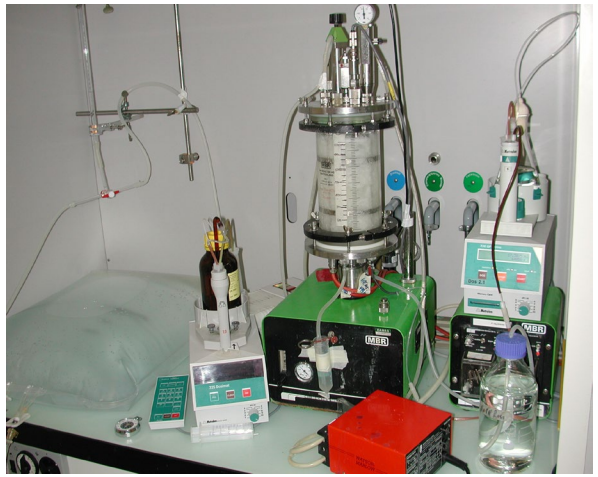
- Increase of the product yield
- The maintenance of the yield

Techniques:
- Selection
- Selection followed by a mutation: sequential mutagenesis
- Genetical engineering: directed evolution

4. Batch culture



Requirements for successful batch cultures



Checklist for a successful batch cultivation:

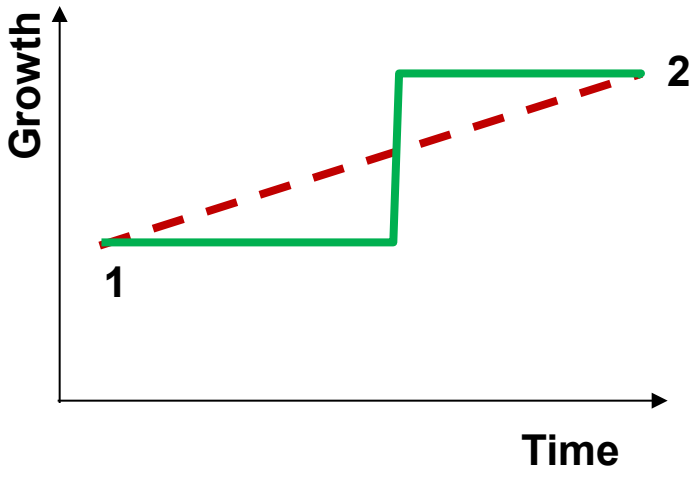
1. Viable inoculum (still growing preculture)
2. Energy source
3. Nutrients for biomass synthesis
4. Abscence of inhibitors which prevent rapid growth
5. Suitable physicochemical conditions

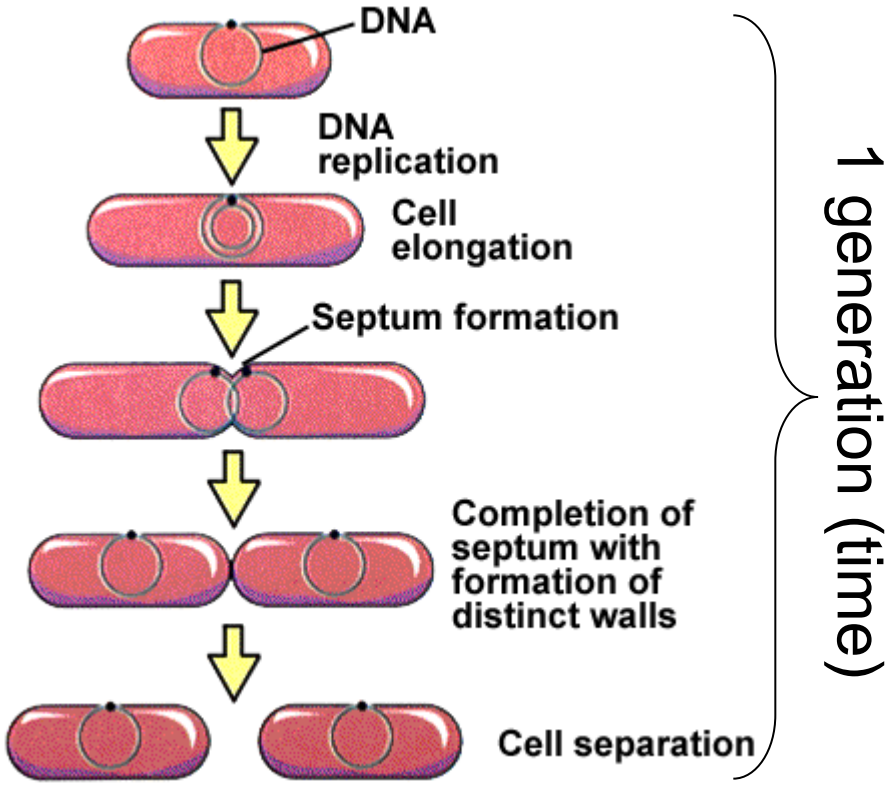
Microbial growth

Growth = increase in # of cells
(by binary fission)
generation time: 10 min – days

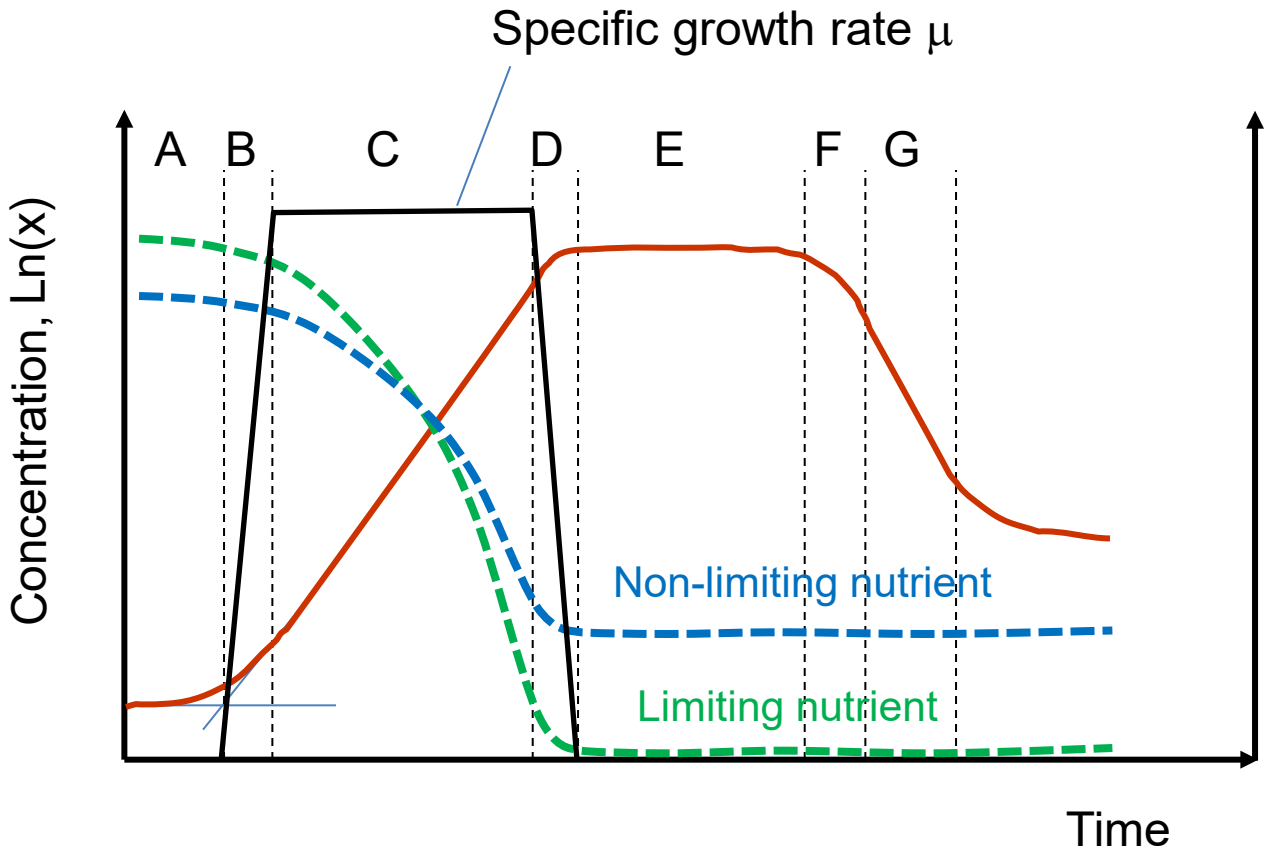
or

Growth = increase in dry biomass





Growth phases in a batch culture



A. Lag phase
B. Accelerating growth
C. Exponential growth
D. Phase of growth deceleration
E. Stationary phase
F. Phase of accelerated decay
G. Exponential death phase

Mathematical expression of Monod's modell

1st equation of Monod:

$$\frac{dx}{dt} = \mu \cdot x$$

The speed of growth is proportional to the concentration of the biomass.

2nd equation of Monod:

$$\mu = \mu_{max} \frac{s}{s + K_s}$$

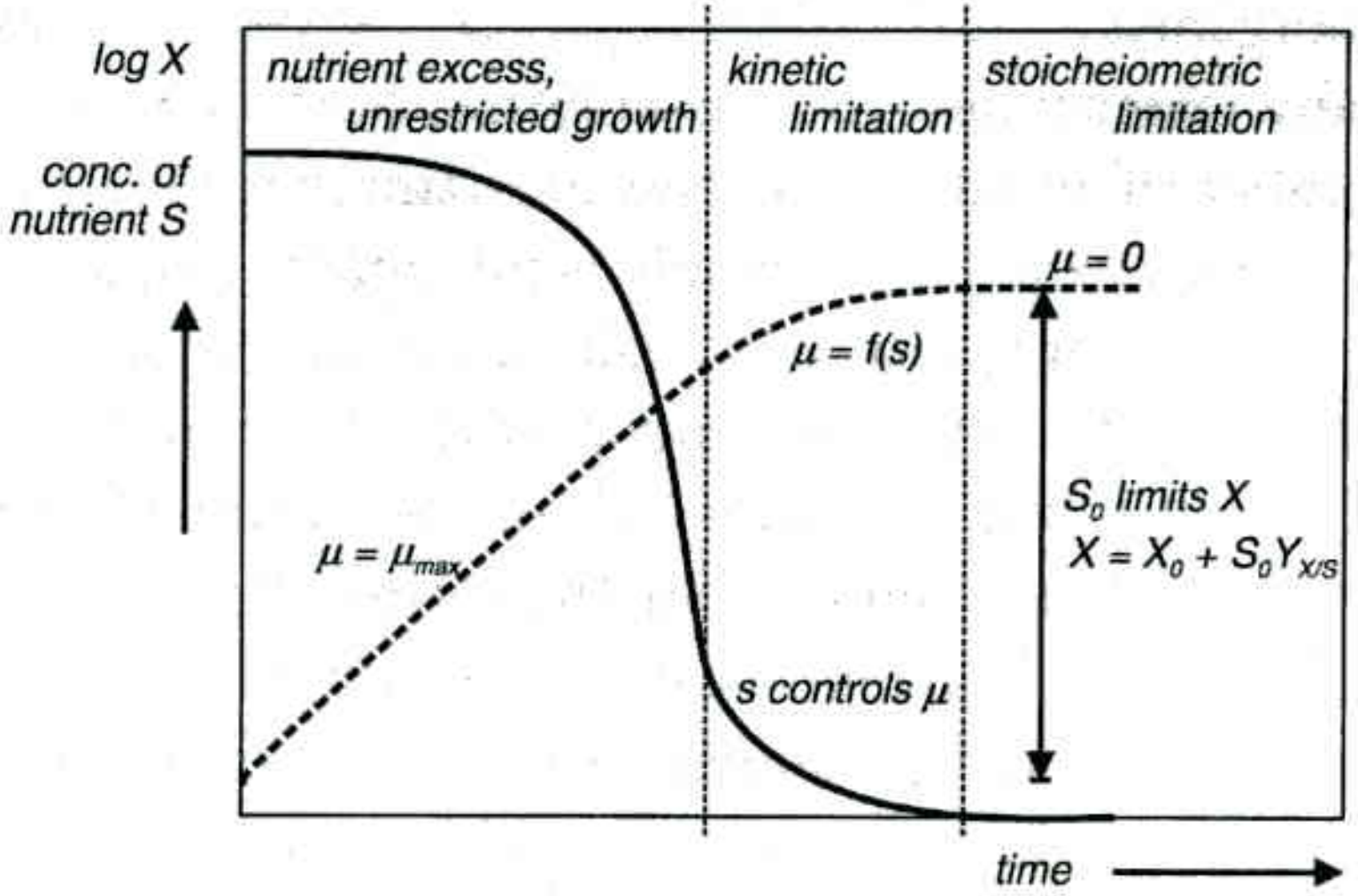
The specific growth rate depends only on the concentration of the limiting substrate according to the Michaelis-Menten kinetics.

3rd equation of Monod:

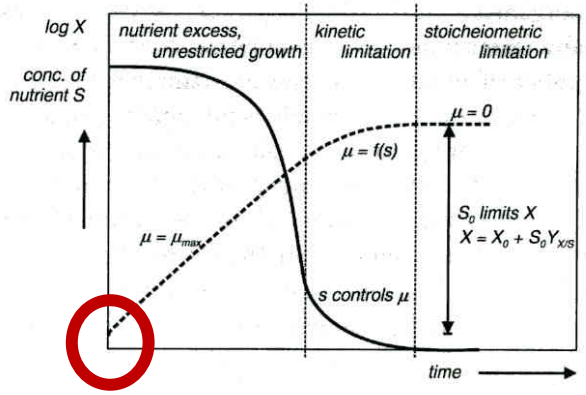
$$Y_{X/S} = \frac{r_X}{-r_S}$$

The yield coefficient is constant under exponential growth conditions.

Typical phases of a batch culture in the lab

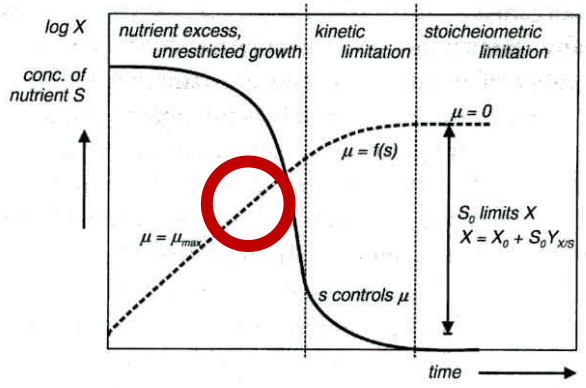


The start of a batch culture in the lab



- A good start of a batch culture is important!
- Lag time is influenced by:
  - Quality of preculture (still growing, density)
  - Medium composition of preculture (change from complex to minimal medium)
  - Growth state of preculture (still growing?)
  - Concentration of medium components (toxic concentration of substrates and inhibiting products in preculture)
  - Oxygen limitation
- Lag time can be determined graphically and mathematically
- Inoculum amount: Ideally 1-2% of batch culture but less than 10%
- Ideal start concentration of a batch culture: OD(600nm) = ca. 0.1

The exponential phase of a batch culture



- The exponential growth phase is an autocatalytic process (no change of growth rate when cells are «unrestricted» in growth)

- During exponential growth phase:
  - Growth rate μ is maximal (= μ_{max}) and is constant
  - Specific uptake rate q_s is maximal and constant
  - Biomass yield $Y_{X/S}$ is constant
  - Average biomass composition is constant
  - Specific CO_2 production rate q_{CO2} is constant

- Average cell size is largest compared to all other growth phases.

- Duration of exp. growth phase can be controlled by inoculum concentration and available nutrient concentrations.

- Identification of exponential growth phase by linear regression:

$$\ln(x) = \ln(x_0) + \mu^*\Delta t$$

Describing exponential growth based on biomass

$$\frac{dX}{dt} = \mu X$$

Separation of variables

$$\mu = \frac{1}{X} \cdot \frac{dX}{dt}$$

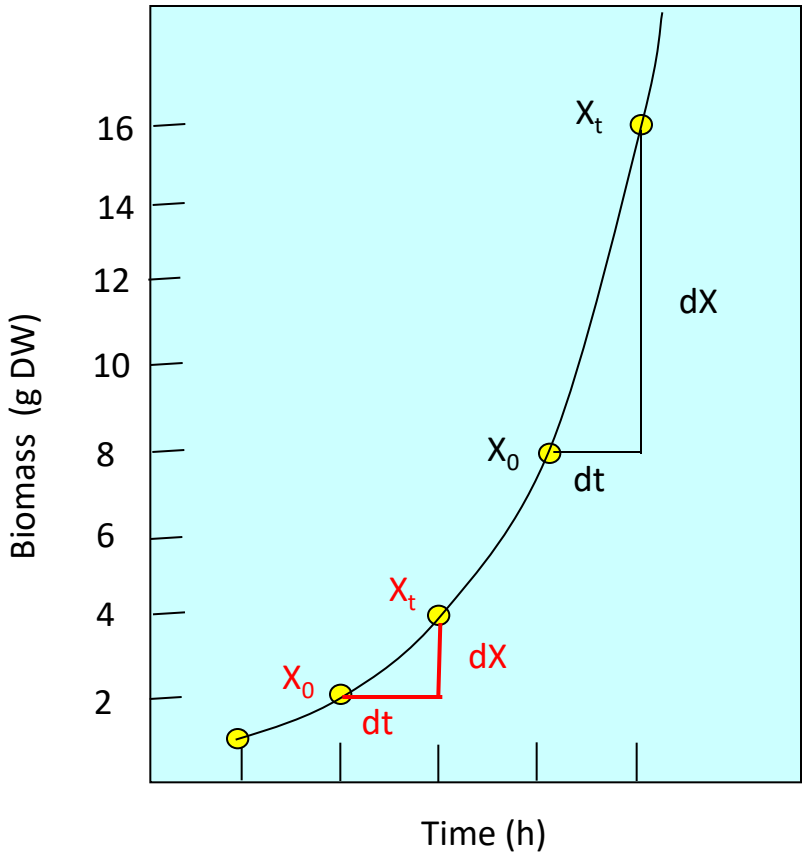
Integration

$$X(t) = X_0 \cdot e^{\mu t}$$

$$\mu = \frac{\ln X - \ln X_0}{\Delta t}$$

Doubling time

$$\mu = \frac{\ln 2}{t_d} = k \ln 2$$



μ = „specific growth rate“ (usually in h^{-1}); in Brock and also other text books abbreviated as „k“

μ_{max} is the maximum specific growth rate (a constant for the conditions given (T, pH, medium composition, etc.)

Stoichiometry according to Monod model

There is almost a constant relationship between the growth of biomass and its consumption of substrate.

$$Y_{x/s} = -\frac{r_x}{r_s} = const.$$

$$r_x = \frac{dx}{dt}$$

$$r_s = \frac{ds}{dt}$$

} constant ratio only in exponentially growing cultures

Generally, all $Y_{X/E}$ are considered to be a constant value under exponential growth conditions.

$$Y_{x/s} = \frac{\Delta x}{\Delta s} = \frac{x - x_0}{s_0 - s} = \frac{\mu}{q_s}$$

[g g^{-1}] or [g mol^{-1}]

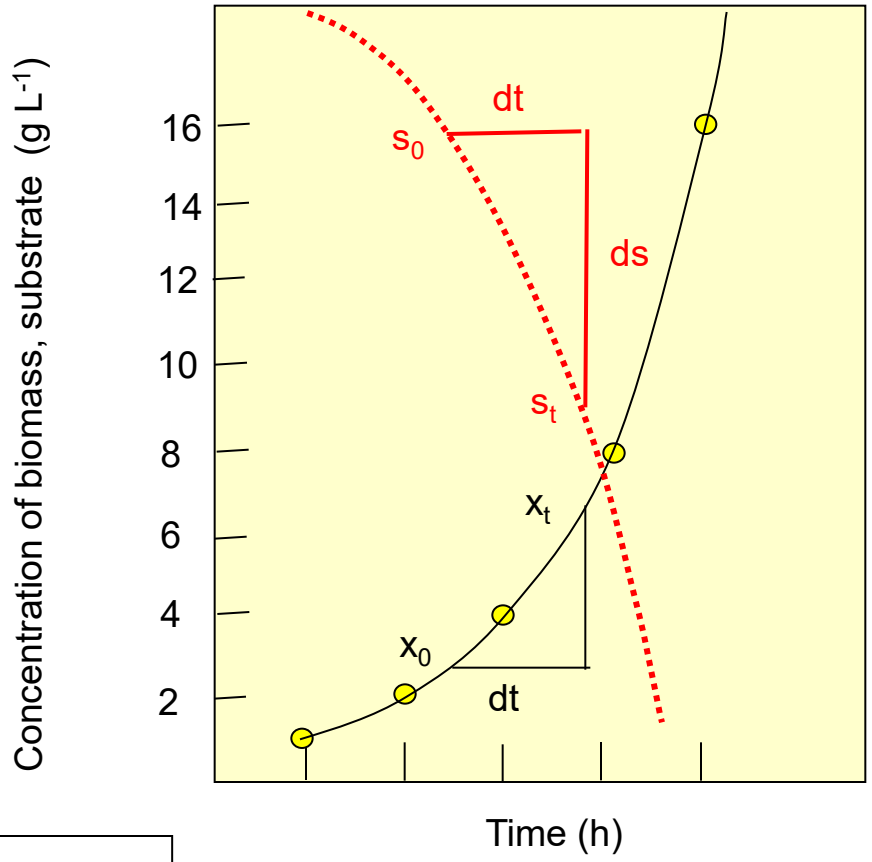
Substrate consumption during batch

$$Y_{X/S} = \frac{x - x_0}{s_0 - s} = \frac{\Delta x}{\Delta s}$$

$$q_s = \frac{ds}{dt} \cdot \frac{1}{x}$$

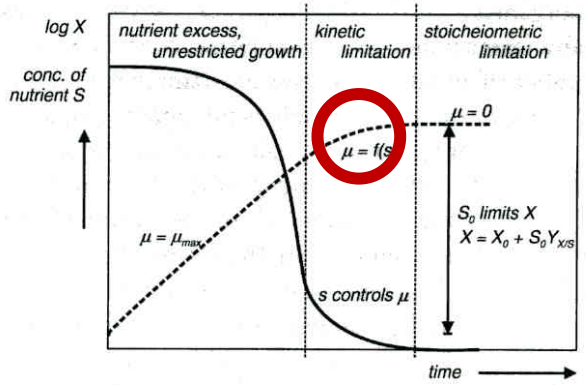
$$q_s = \frac{\mu}{Y_{X/S}}$$

$$-\frac{dS}{dt} = q_s \cdot X$$



$Y_{x/s}$ = growth yield [g g^{-1}]
q_s = specific substrate consumption rate [g g^{-1} h^{-1}]

The decelaration phase of a batch culture



- During the deceleration growth phase the cells are restricted in their growth: One nutrient **kinetically** limits growth.

- During kinetic limitation:
  - Growth rate μ is not maximal (< μ_{max}) and is constantly changing
  - Specific uptake rate q_s is getting smaller
  - Biomass yield $Y_{X/S}$ may get smaller due to maintenance energy consumption
  - Average biomass composition is changing
  - Specific CO_2 production rate q_{CO2} is slowly decreasing

- Average cell size is getting smaller.

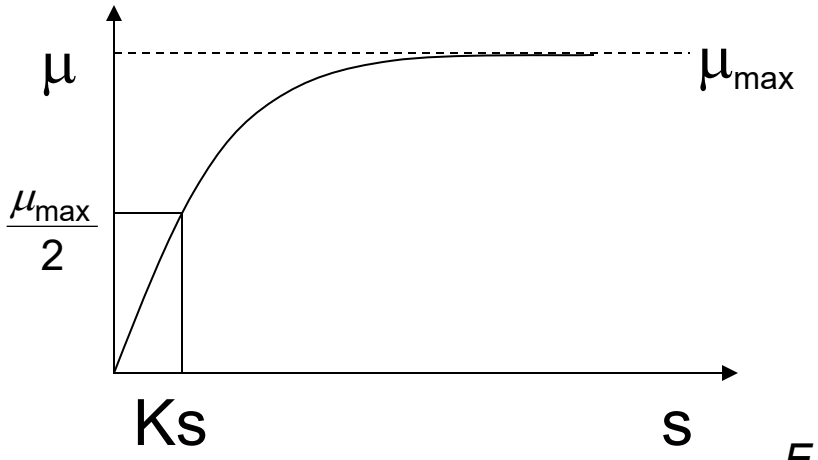
- Cells start to express high affinity uptake systems and start to prepare for stationary phase (synthesis of «alarmones»).

Kinetics according to the Monod model

The growth rate becomes proportional to the concentration of one nutrient when this concentration is getting small. **Only one** nutrient is growth limiting.

$$\mu = \mu_{max} \frac{S}{S + K_S}$$

μ: specific growth rate [h^{-1}]
Ks: saturation or *Monod* constant [g L^{-1}]
s: actual concentration of substrate s [g L^{-1}]



$$\mu = \frac{\mu_{max}}{2} = \mu_{max} \frac{s}{s + Ks}$$

$$\Rightarrow \frac{s + Ks}{s} = 2$$

$$\Rightarrow s + Ks = 2s$$

$$\Rightarrow Ks = s$$

E. coli on glucose: Ks: $7*10^{-7}$ - $4*10^{-3}$ g L^{-1}

K_s and μ_{max} for different MO

Organism	Substrate	K_s [g L^{-1}]
E. coli	Glucose	0.068
E. coli	Lactose	20.0
E. coli	Mannitol	2.0
S. cerevisiae	Glucose	25.0
Trichoderma viride	Glucose	9300
Xanthomonas campestris	Glucose	1.99 (at 27°C)

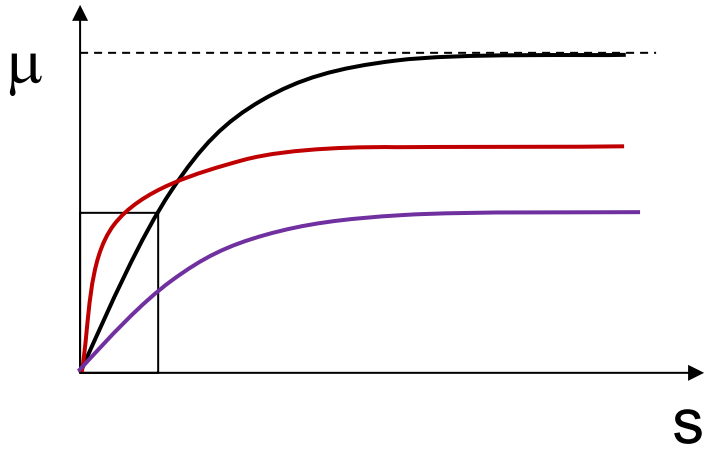
Organism	Substrate	μ_{max} [h^{-1}]
Aspergillus niger	Glucose	0.2
S. cerevisiae	Glucose	0.41
Trichoderma viride	Glucose	0.104
Xanthomonas campestris	Glucose	0.29 (at 27°C)

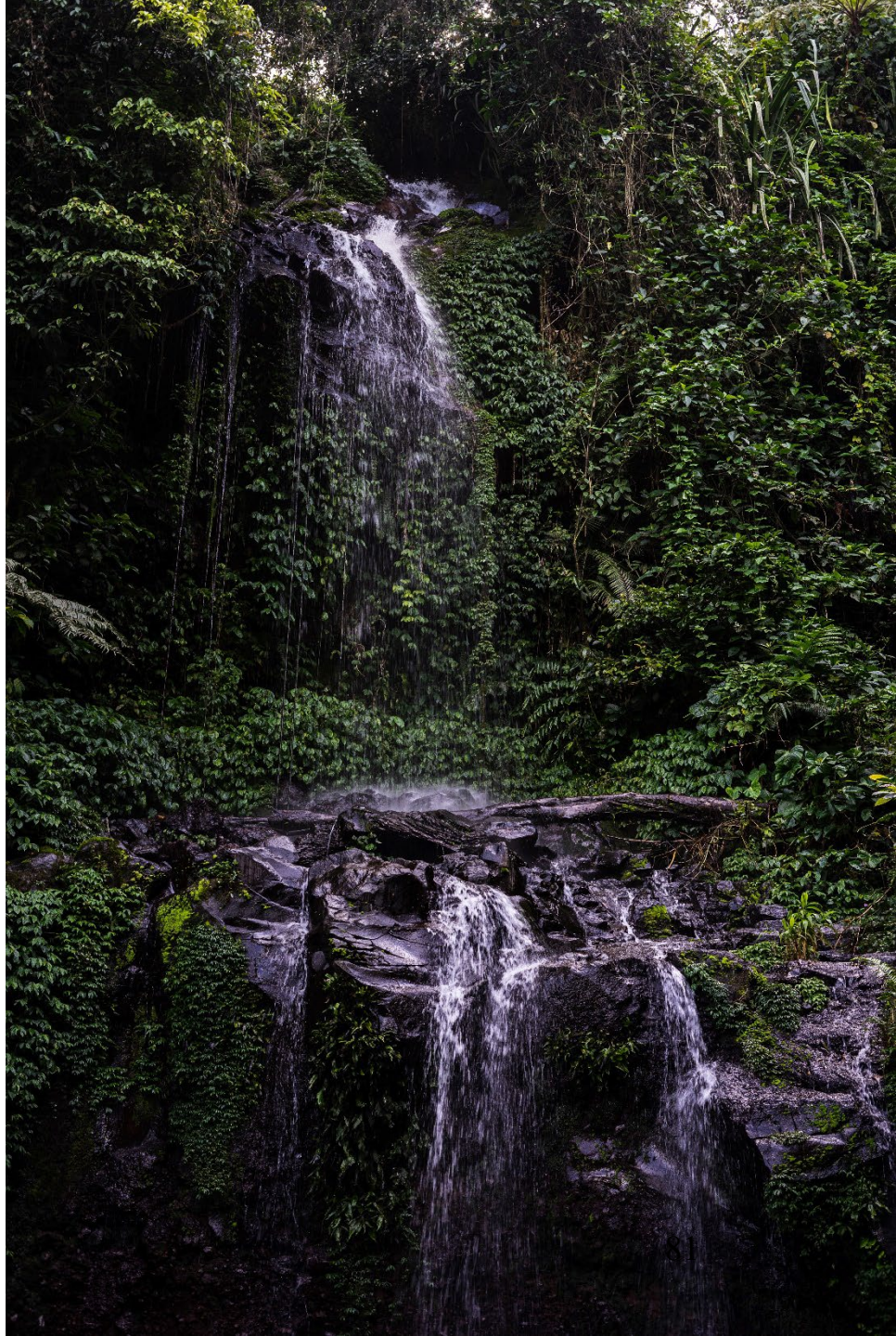
Ks value is important in nature

A low Ks value means:

- High affinity to a particular substrate => faster growth at lower concentration
- Most uptake systems for essential substrates are supported by complex systems (see next slide).

A high μ_{max} is only of competitive advantage in nutrient rich systems (*e.g., E. coli* in bowel).





Nutrient uptake

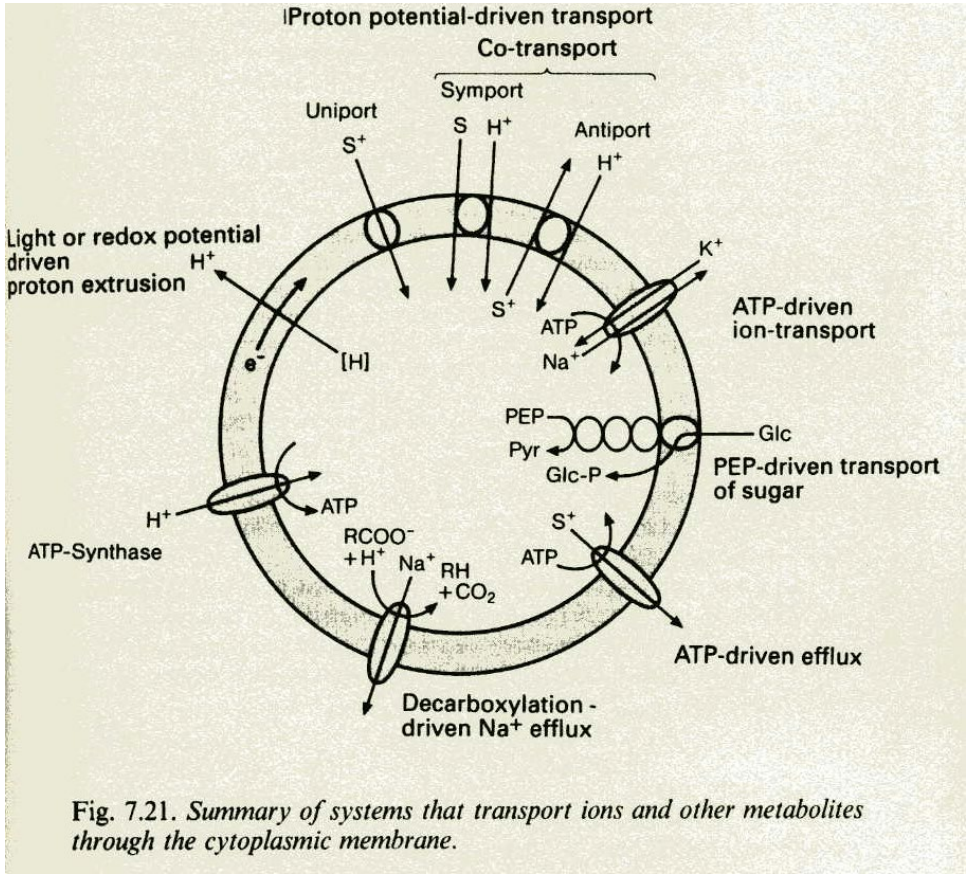


Fig. 7.21. *Summary of systems that transport ions and other metabolites through the cytoplasmic membrane.*

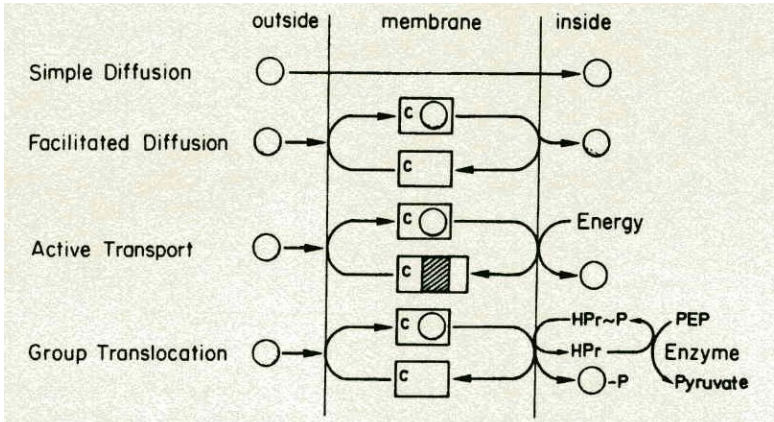


Fig. 7.19. *Schematic representation of the four mechanisms for transport of materials into the cell.*

Circle, substrate to be transported; C, permease(carrier)-protein; C with grey hatching, energised carrier; PEP, phosphoenolpyruvate; HPr, heat stable protein. See text for details.

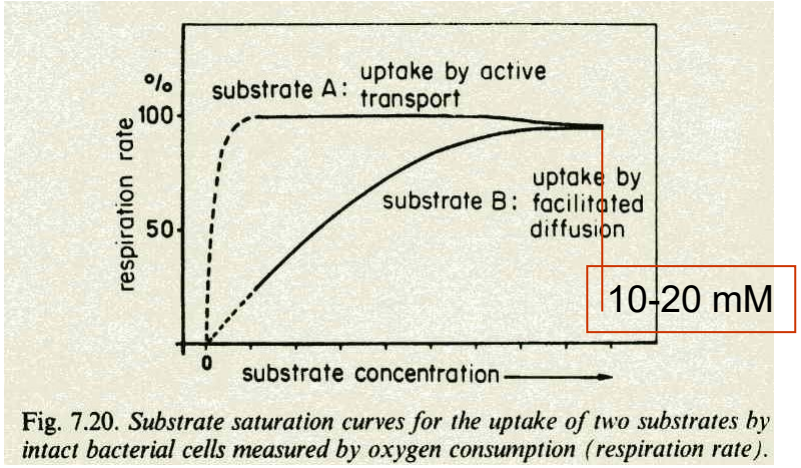


Fig. 7.20. *Substrate saturation curves for the uptake of two substrates by intact bacterial cells measured by oxygen consumption (respiration rate).*

Rule of thumb:
Up to C6 fatty acids may pass the membrane by diffusion. Larger molecules require an active transport sytem.

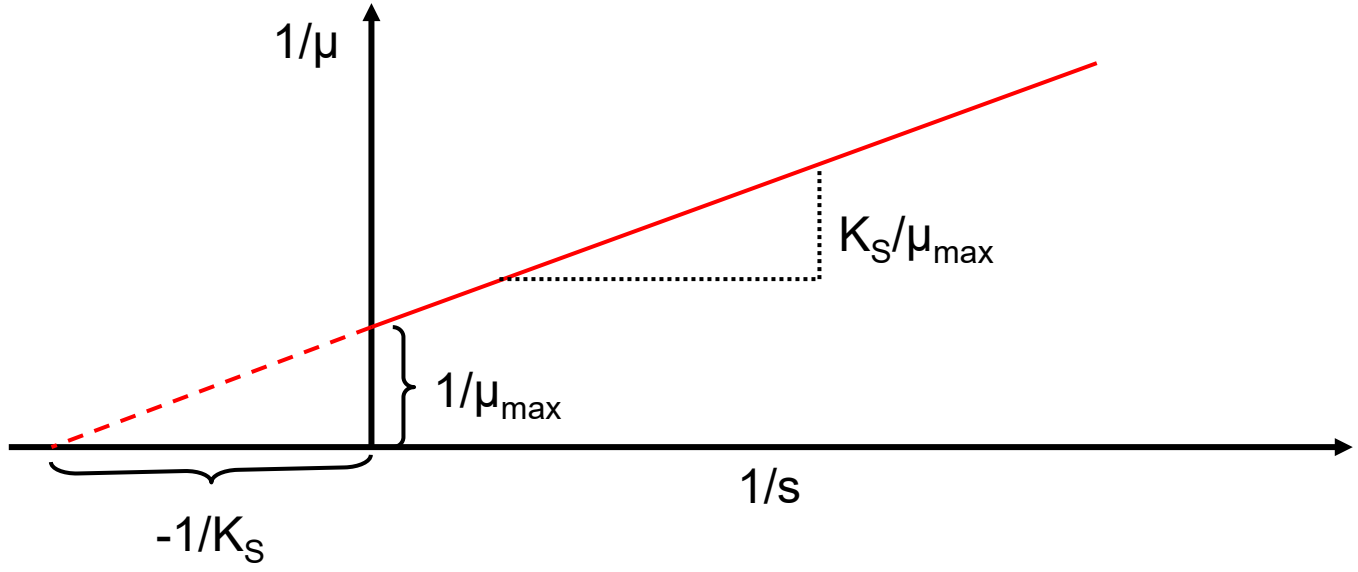
Lineweaver-Burk Plot

Kinetic constants are usually determined in chemostat culture by varying μ (= D at steady-state) and mesuring s. However, they may also be determined by using several batch cultures with different s_0 concentrations. The Lineweaver- Burk plot (see graph) can be used to determine the growth constants Ks and μ_{max}:

$$\frac{1}{\mu} = \frac{1}{s} \bullet \frac{K_S}{\mu_{max}} + \frac{1}{\mu_{max}}$$

When $1/s \approx 0$ then $1/\mu \approx 1/\mu_m$

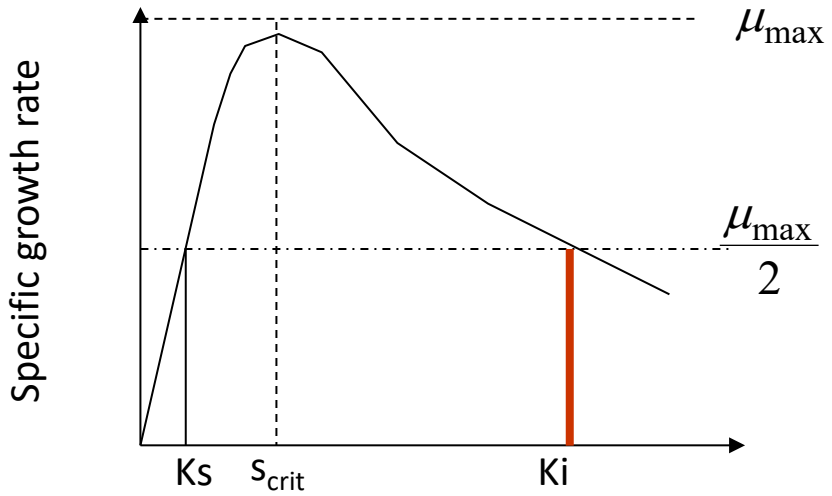
When $1/\mu \approx 0$ then $1/s \approx -1/K_S$



Too much of the good: Growth inhibition by the substrate

Some substrates, for example alcohols, phenol, and hydrocarbons, if present in excess, also inhibit growth.

In batch cultures the specific growth rate increases up to a critical substrate concentration s_{crit}, thereafter the inhibitory effect becomes dominant. In case the substrate was initially above s_{crit} but did not result in complete inhibition, the specific growth rate will increase with consumption of s until $s<s_{crit}$.



$$\mu = \mu_{max} \frac{s}{K_s + s + \frac{s^2}{K_i}}$$

Andrews (1968)

K_i is usually in the range of 10x K_s

Too much of the good: Growth inhibition by the substrate

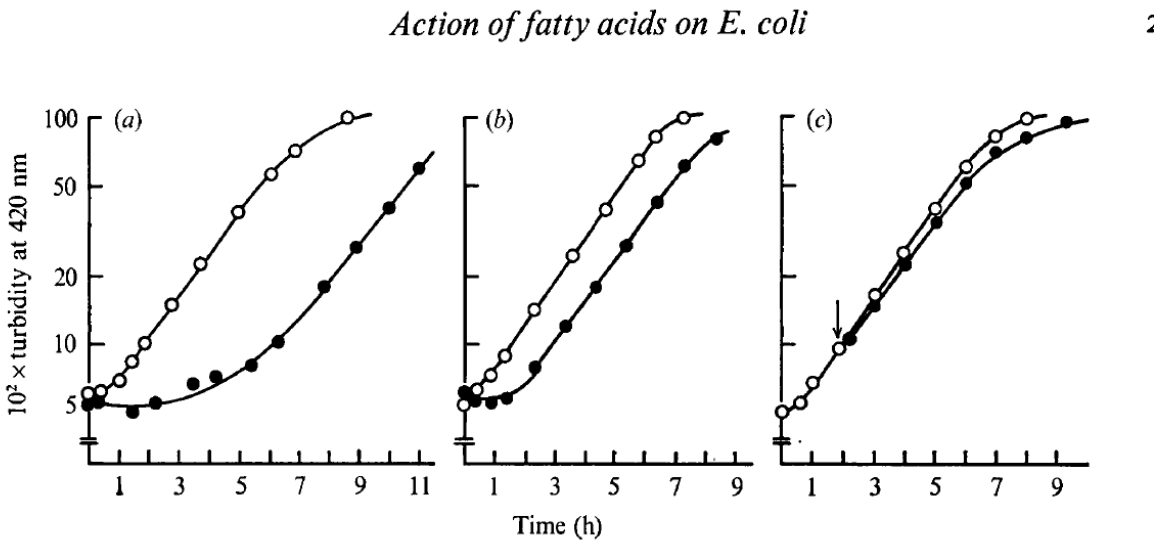


Fig. 1. Effect of lauric acid on the growth of *E. coli* K12/154 in a glucose minimal medium. Growth was measured as turbidity. Media were inoculated with: (*a*) a 2 h stationary-phase culture, (*b*) a mid-exponential phase culture, (*c*) an exponentially-growing culture. In (*c*) 0·1 % lauric acid was added (arrow). ○, Glucose minimal medium; ●, glucose minimal medium+0·1 % lauric acid.

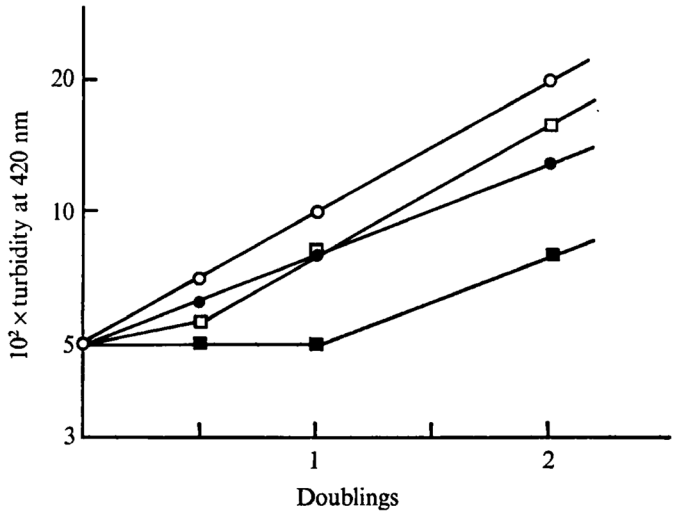


Fig. 2. Effect of a change in carbon source on the growth inhibition of *E. coli* K12/154 by nonanoic acid. Mid-exponential-phase cultures in succinate medium were used as the inoculum for the new media. The dilution was 1:25. The curves from different media were normalized to allow visual comparisons. One doubling time means the time which would be taken for a culture without fatty acid addition to undergo a doubling, whatever the medium. The control curves (open symbols) were theoretically determined from the doubling times of the bacterium in different media and in the absence of fatty acids (for values in minutes see footnote *, Table 1). ○, Succinate; ●, succinate +0·1 % nonanoic acid; □, acetate; ■, acetate+0·1 % nonanoic acid.

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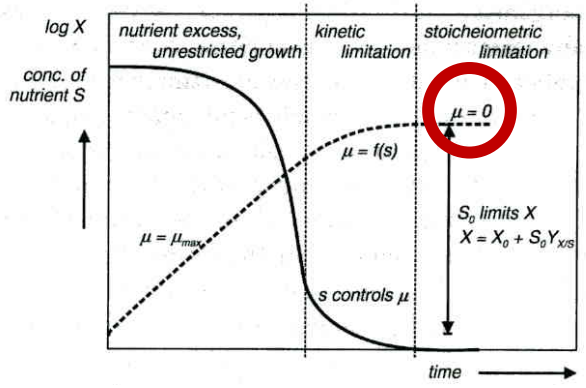
The stationary phase of a batch culture



- During the stationary growth phase the cells are not growing anymore.
- During stoichiometric limitation following points are important:
  - Growth (μ) is not measurable ($\mu = 0\ h^{-1}$)
  - Growth and cell lysis are balanced out ($\mu = k_d$)
  - Biomass concentration reached its maximum
  - Average biomass composition is changing (optimization to starvation)
  - Oxygen uptake rate is slowly decreasing
- Average cell size is small, Gram$^+$ strains tend to sporulate.
- Non-carbon limited cultures are synthetizing overflow metabolites (*e.g.,* acetate or EtOH).

12. 5. 1803 – 18. 4. 1873

Law of the minimum

One nutrient limits the amount of biomass that can be produced in a system. All other nutrients are in excess.

Justus von Liebig, 1840

Production kinetics

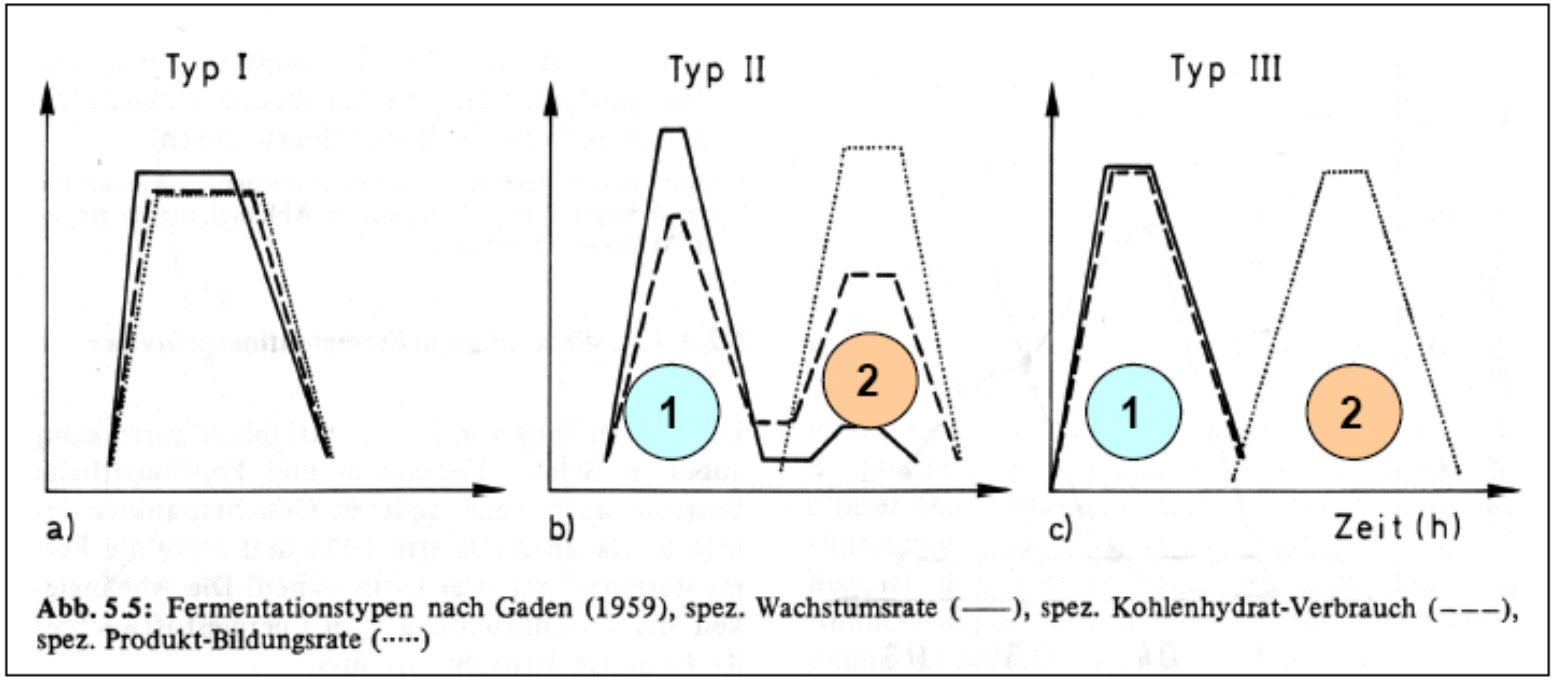


Abb. 5.5: Fermentationstypen nach Gaden (1959), spez. Wachstumsrate (——), spez. Kohlenhydrat-Verbrauch (– – –), spez. Produkt-Bildungsrate (·····)

Products directly associated with generation of energy in the cell (ethanol, acetic acid, acetone, butanol, lactic acid, other products of anaerobic fermentation)

Products indirectly associated with energy generation (amino acids and their products, citric acid, nucleotides, polyhydroxyalkanoates)

Products for which there is no clear direct or indirect coupling to energy generation (penicillin, streptomycin, vitamins)

Growth-linked specific product formation

Biomass related productivity

$$dp = Y_{P/X} * dx$$

$$\frac{dp}{dt} = Y_{P/X} * \frac{dx}{dt} = Y_{P/X} * \mu * x$$

Substrate related productivity

$$dp = Y_{P/S} * ds$$

$$\frac{dp}{dt} = Y_{P/S} * \frac{ds}{dt} = Y_{P/S} * \frac{\mu * x}{Y_{X/S}}$$

$$\frac{Y_{P/S}}{Y_{X/S}} = Y_{P/X}$$

For growth-linked product formation

$$q_P = Y_{P/X} * \mu$$

Non-growth-linked product formation

The **non-growth-linked product** can be a complex function of the specific growth rate. An example of this type is **melanin** formation by *Aspergillus niger*, which is represented by

$$q_P = q_P^{\max} - k * \mu$$

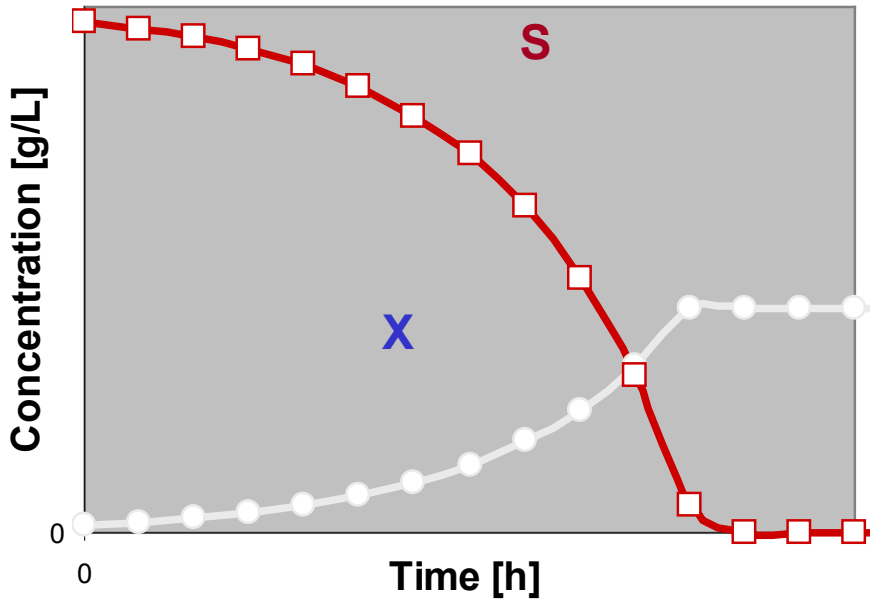
where q_P^{max} and k are constants.
The formation of **cyclodextrin** from starch by *Bacillus macerans* and spore production by *Bacillus subtilis* are similar.

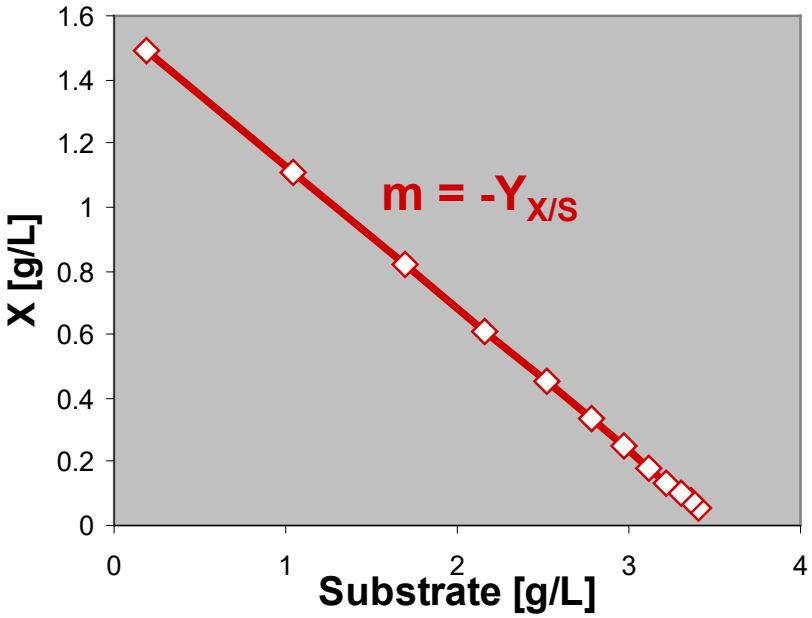
When product formation is **partly growth-linked** and partly independent of growth rate, we have:

$$q_P = Y_{P/X} * \mu + \beta$$

Formation of end products of energy metabolism follow this relation, where β includes the production which results from either the maintenance energy requirement or uncoupling of ATP production. **Lactic acid** production from sugar by *Lactobacillus* species follows this model.

Microbial growth - The Yield coefficient Y





Definition:

$$Y_{P/S} = \frac{r_P}{-r_S} = \frac{q_P}{q_S}$$

Nomenclature:

P = Product

X = Cell mass

ATP

S = Substrate

C = Carbon source

O = Oxygen

N = Nitrogen

Questions

- What is the benefit of models and what are their limitations?
- What is the Monod model not describing?
- How can you modify the specific growth rate?
- What is the meaning of a high Ks value?
- What is the influence of the Ks value on μ_{max}?

And finally a few useful equations to simulate batches

V = const.; many times μ = μ_{max} is used

$$\frac{dx}{dt} = \mu * x \qquad x = x_0 * e^{\mu * t}$$

$$\frac{ds}{dt} = \frac{ds}{dx} * \frac{dx}{dt} = \frac{\mu * x}{Y_{\frac{X}{S}}} = \frac{\mu}{Y_{\frac{X}{S}}} * x_0 * e^{\mu * t} \qquad s = s_0 - \frac{x_0}{Y_{X/S}} * (e^{\mu * t} - 1)$$

$$\frac{dp}{dt} = \frac{dp}{ds} * \frac{ds}{dx} * \frac{dx}{dt} = \frac{Y_{\frac{P}{S}}}{Y_{\frac{X}{S}}} * \mu * x_0 * e^{\mu * t} \qquad p = p_0 + \frac{Y_{\frac{P}{S}}}{Y_{\frac{X}{S}}} * x_0 * (e^{\mu * t} - 1)$$

This 2nd chapter should help you to understand the functioning of a batch culture and its different growth phases.

Very important are correct applications of Monod equations: Learn how to use them using the exercises.

Try to simulate a batch culture using Excel/Matlab/Phyton and understand the differences to the reality.

5. Medium design

Microbial growth media

Media	Purpose
Complex	Grow most heterotrophic organisms
Defined	Grow specific heterotrophs; often mandatory for chemoauto-trophs, photoautotrophs and for microbiological assays
Selective	Suppress unwanted microbes, or encourage desired microbes
Differential	Distinguish colonies of specific microbes from others
Enrichment	Similar to selective media but designed to increase the numbers of desired microorganisms to a detectable level without stimulating the rest of the bacterial population
Reducing	Growth of obligate anaerobes

Five main functions of media

1) Supply all essential nutrients necessary for growth and allow production of biomass of defined composition.

2) Ensure “optimum” and “constant” growth conditions over a certain period of time.

3) Control the physiological performance of a microbial culture, e.g.
   - aerobic versus denitrifying growth
   - glucose versus acetate as carbon/energy source
   - nitrate versus ammonia as N-source...

4) Control the maximum specific growth rate.

5) Control growth limitation by specific nutrient.

pH optimum for growth

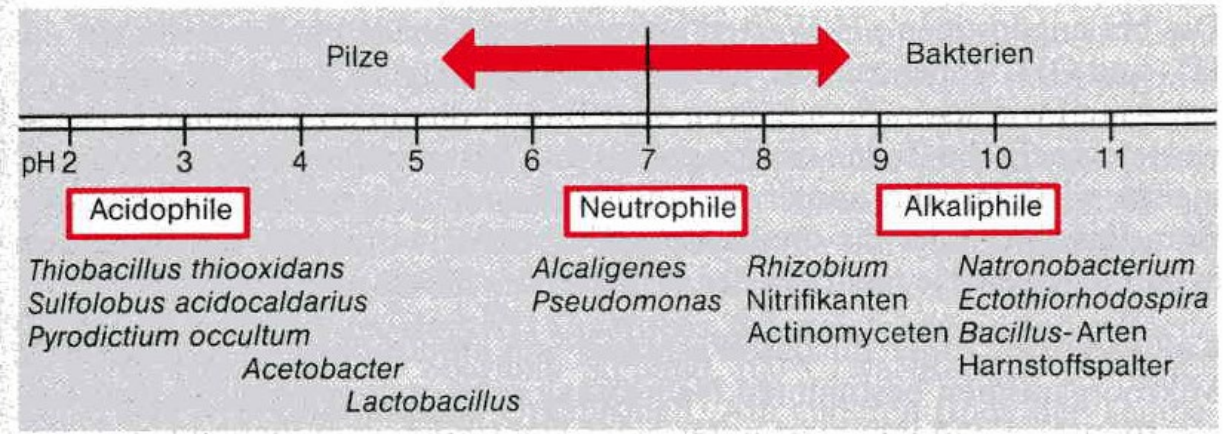


Abb. 6.1 Von Pilzen und verschiedenen Bakterien bevorzugte oder tolerierte pH-Bereiche

Organism	Minimum	Optimum	Maximum
Bacteria	2-5	6.5-7.5	8-11
Yeasts	2-3	4.5-5.5	7-8
Molds	1-2	4.5-5.5	7-8

Aspergillus niger: pH 7: oxalic acid, pH 2: citric acid

Typical medium composition

Factors	Range of concentration [% w/v]	Examples
Carbon source	0.5 - 20	Glucose, sucrose, starch, molasses, dextrins, alcohols,corn meal, glycerol, lipids
Nitrogen source	0.1 - 10	Ammonia gas, ammonium salts, casein hydrolysates, glutamic acid, nitrates, peptones, urea, yeast extract
Phosphorus	0.1 - 2	Corn steep liquor, phosphates
Sulfur	0.1 - 1	Methionine, proteins, sulfates
Other nutrients	<1	Iron salts, magnesium salts, oxygen, potassium, trace elements, vitamins

Defined medium

The defined medium consists of many substrates that are all - **without exception**- well-described and their chemical structure is known.

Table 6.1. *Example of a simple synthetic nutrient solution*

K_2HPO	0.5 g
NH_4Cl	1.0 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$FeSO_4 \cdot 7H_2O$	0.01 g
$CaCl_2 \cdot 2H_2O$	0.01 g
Glucose	10.0 g
Water	1000 ml
Trace element stock solution	1 ml

Table 6.2. *Trace element stock solution*

$ZnCl_2$	70 mg
$MnCl_2 \cdot 4H_2O$	100 mg
$CoCl_2 \cdot 6H_2O$	200 mg
$NiCl_2 \cdot 6H_2O$	100 mg
$CuCl_2 \cdot 2H_2O$	20 mg
$NaMoO_4 \cdot 2H_2O$	50 mg
$Na_2SeO_3 \cdot 5H_2O$	26 mg
[$NaVO_3 \cdot H_2O$	10 mg]
[$Na_2WO_4 \cdot 2H_2O$	30 mg]
HCl (25%) 1.0 ml	
Distilled water	to 1000 ml

[ ], required by only a few organisms.

Table 6.3. *Well-established solution of vitamins for soil and water bacteria*

Biotin	0.2 mg
Nicotinic acid	2.0 mg
Thiamine	1.0 mg
4-Aminobenzoate	1.0 mg
Pantothenate	0.5 mg
Pyridoxamine	5.0 mg
Cyanocobalamine	2.0 mg
Distilled water	100 ml

2–3 ml of the solution are added per 1000 ml nutrient solution

Note:
It is well-known that all trace metals easily form highly insoluble phosphate salts and precipitate in growth media. This can be avoided by the addition of metal-chelating agents such as EDTA, NTA, or sometimes also carboxylic acids such as citrate or tartrate.

Microbial nutrition: Growth factors

Organic compounds required by some bacteria (particularly pathogens)

E.g.: Streptoccus, Lactobacillus, Leuconostoc (lactic acid bacterium):
complex vitamin requirements

Vitamin	Function
p-Aminobenzoic acid	Precursor of folic acid
Folic acid	One-carbon metabolism; methyl group transfer
Biotin	Fatty acid biosynthesis; β-decarboxylations; some CO_2 fixation reactions
Cobalamin (B_{12})	Reduction of and transfer of single carbon fragments; synthesis of deoxyribose
Lipoic acid	Transfer of acyl groups in decarboxylation of pyruvate and α-ketoglutarate
Nicotinic acid (niacin)	Precursor of NAD^+; electron transfer in oxidation–reduction reactions
Pantothenic acid	Precursor of coenzyme A; activation of acetyl and other acyl derivatives
Riboflavin	Precursor of FMN, FAD in flavoproteins involved in electron transport
Thiamine (B_1)	α-Decarboxylations; transketolase
Vitamins B_6 (pyridoxal-pyridoxamine group)	Amino acid and keto acid transformations
Vitamin K group; quinones	Electron transport; synthesis of sphingolipids
Hydroxamates	Iron-binding compounds; solubilization of iron and transport into cell

Questions

- What is auxotrophic growth?
- What is autotrophic growth?
- How can you determine whether a medium has i) a too low or ii) a too high calcium concentration?
- What is the Maillard reaction?

Medium check

TABLE 4.4 Examples of cu

Defined culture medium for *Escherichia coli*

K_2HPO_4 7 g
KH_2PO_4 2 g

$(NH_4)_2SO_4$ 1 g
$MgSO_4$ 0.1 g
$CaCl_2$ 0.02 g
Glucose 4–10 g
Trace elements (Fe, Co, Mn, Zn, Cu, Ni, Mo) 2–10 μg each
Distilled water 1000 ml
pH 7

(a)

Concentration of DW that can be produced from N = [conc. N in medium] x $Y_{X/N}$:

1 g $(NH_4)_2SO_4$ contains ~ 0.21 gN

0.21 gN x 8 gDW/gN = 1.7 gDW

Concentration of DW that can be produced from C = [conc. C in medium] x $Y_{X/C}$:

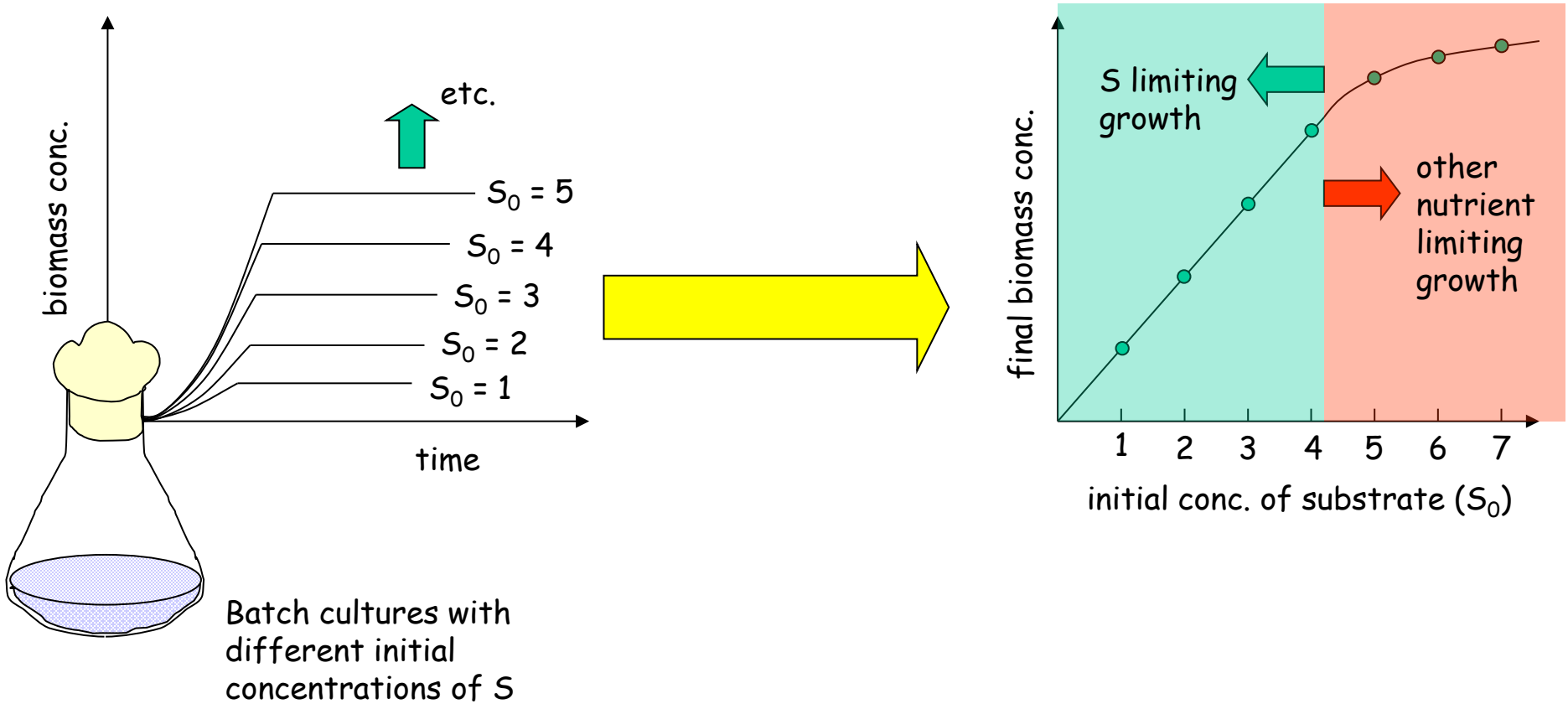
4-10 g Glucose contains ~ 1.6-4 gC

1.6-4 gC x 1 gDW/gC = 1.6-4 gDW

* **With 4 g glucose there is just enough nitrogen in the medium,**

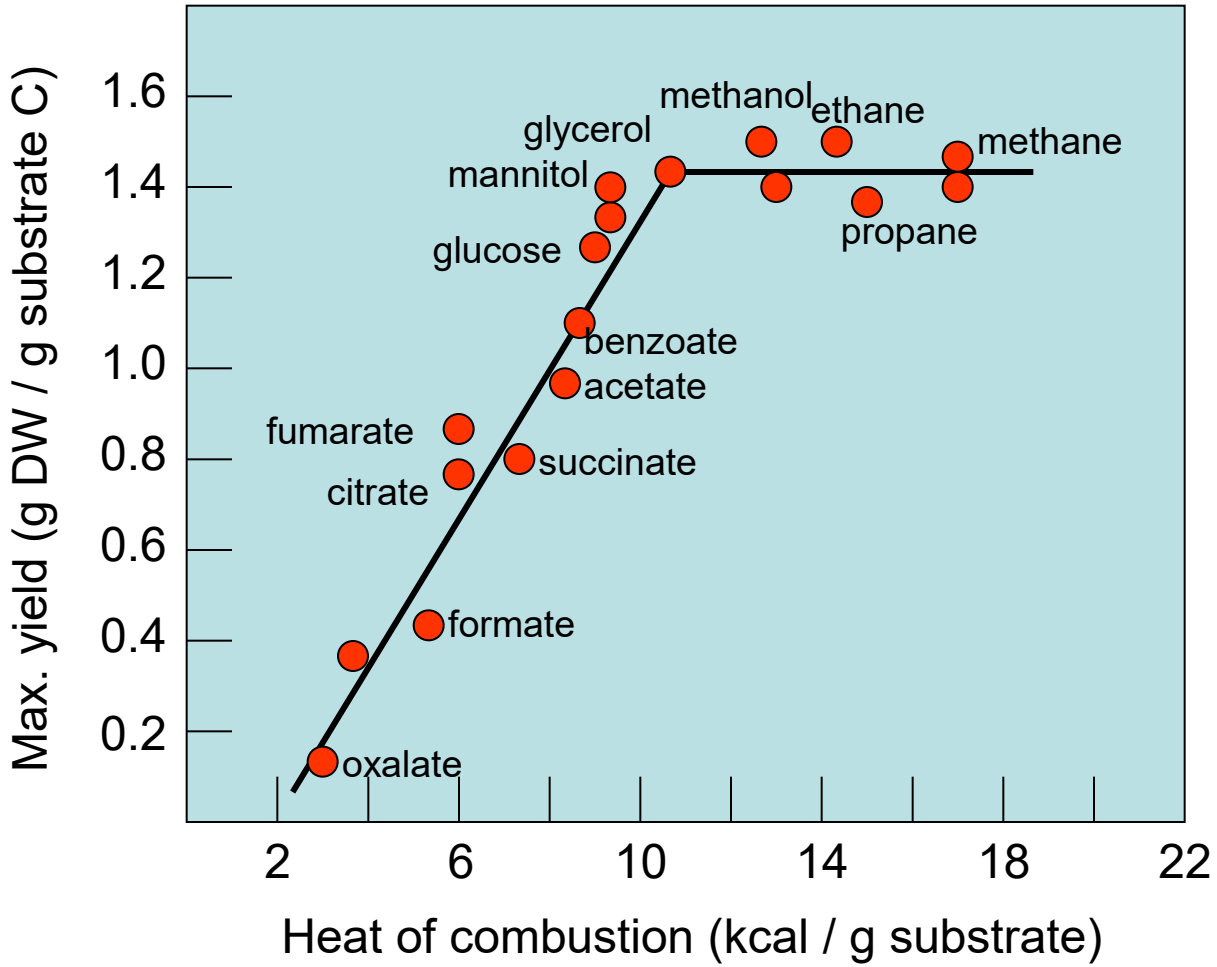
but if 10 g glucose is supplied, there is not enough nitrogen in this medium!

Testing a medium for the growth-limiting substrate

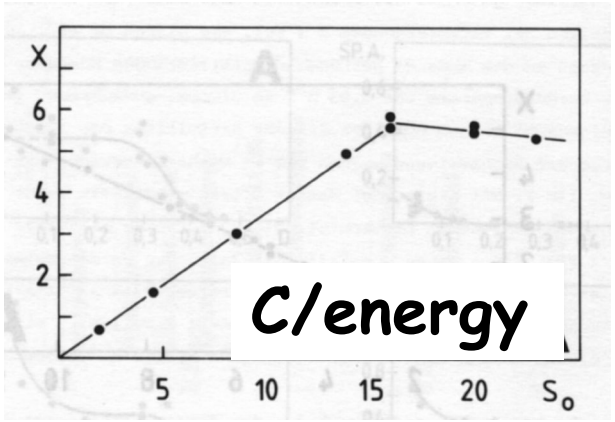


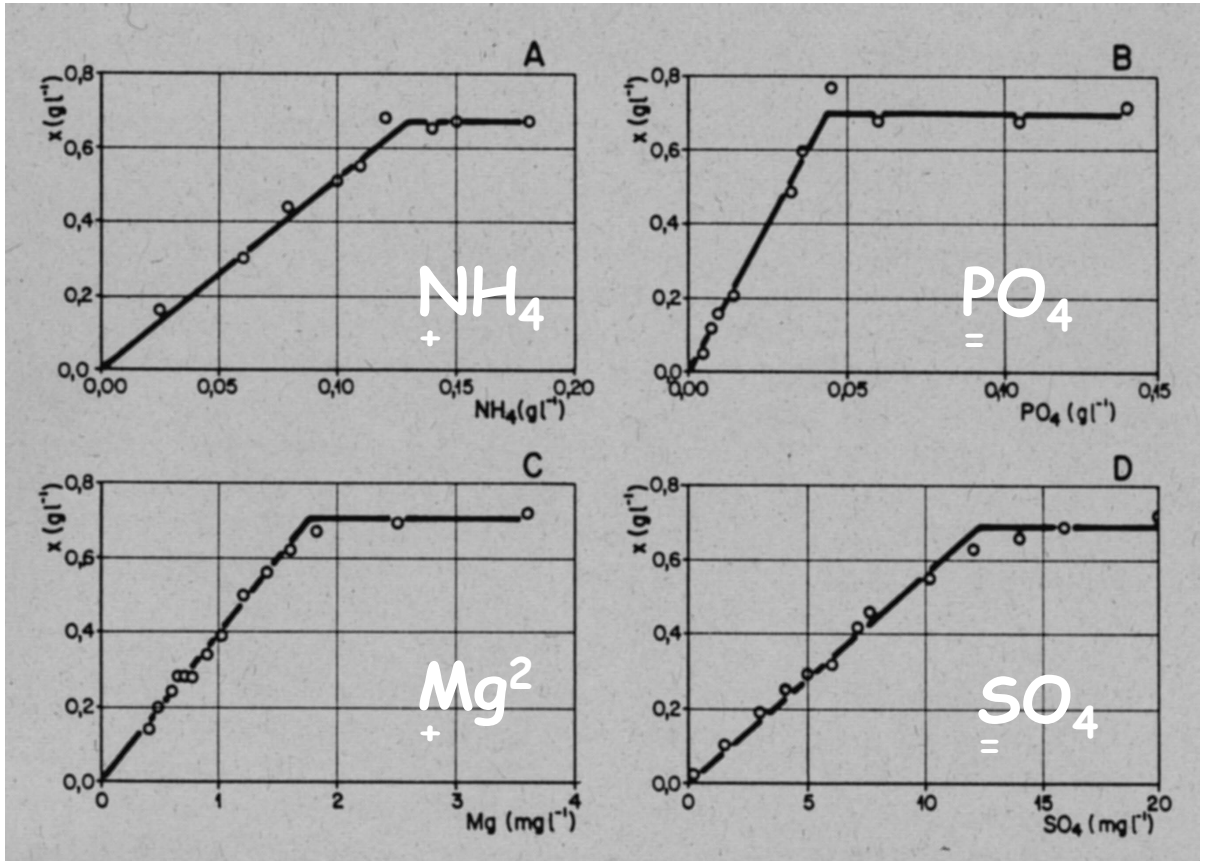
(see also slides medium design)

Growth yields for different carbon/energy sources



Examples of nutrient limitations in continuous cultivations

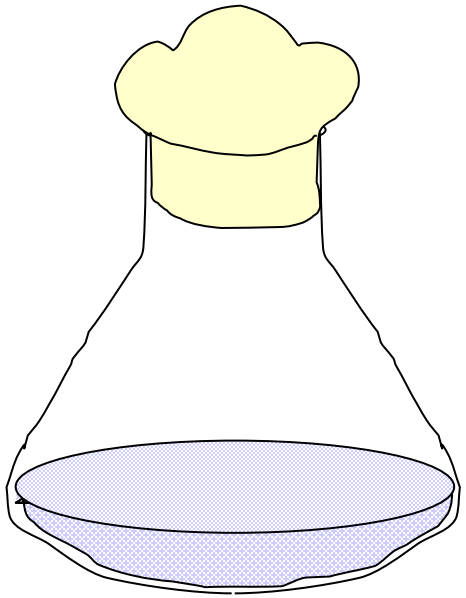




Growth of *Thermus aquaticus* with different conc. of N-, P-, Mg- and S-source in the feed medium, D = 0.63 h^{-1}. (Cometta et al., 1982)

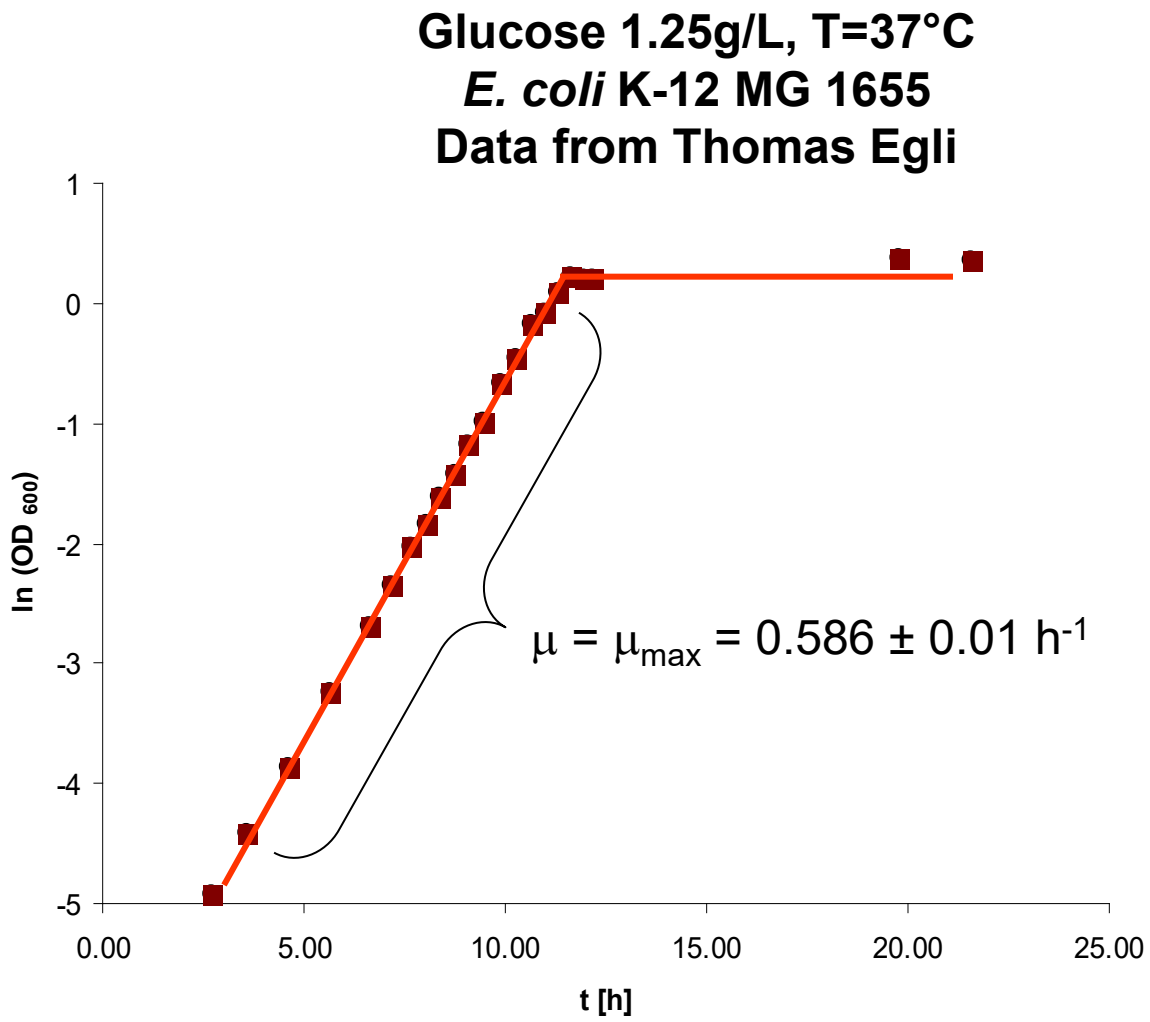
Growth kinetics in mineral medium

Designed medium:



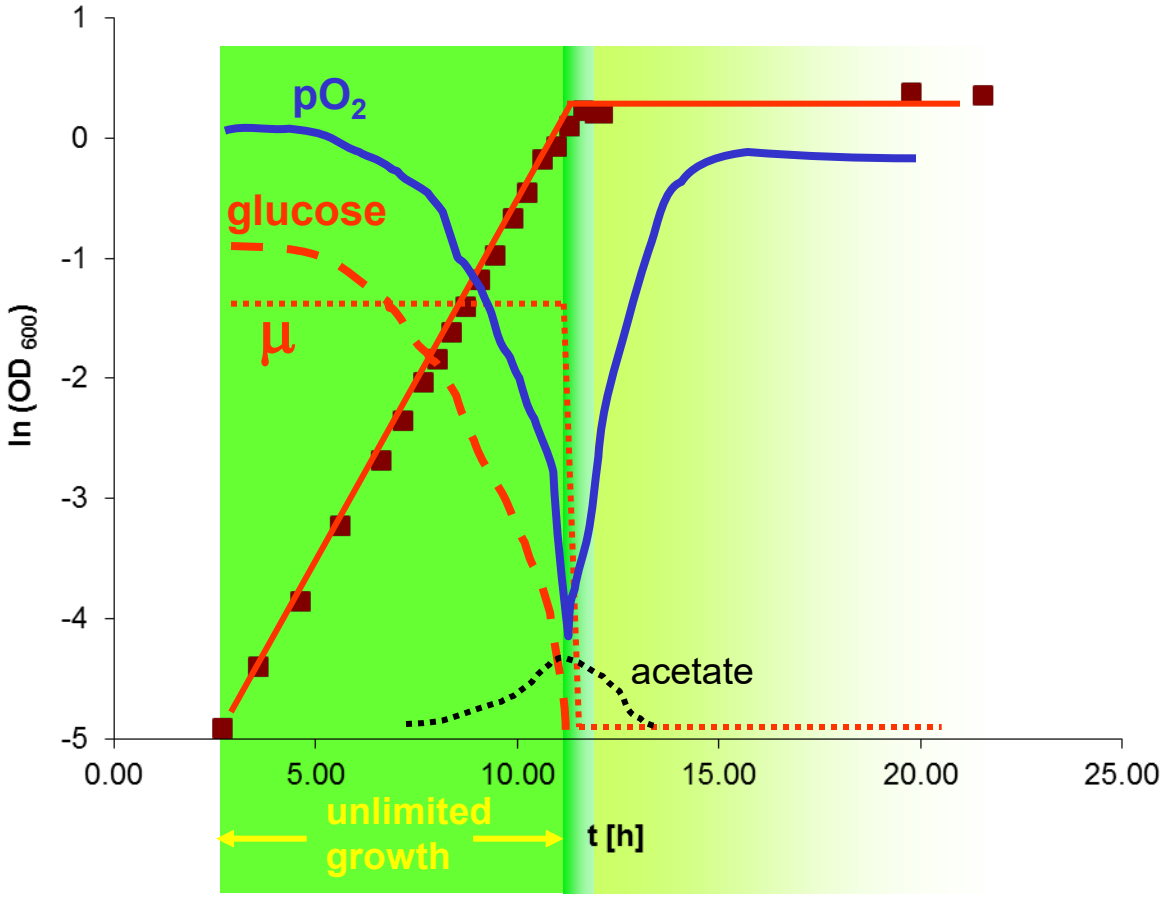
	per liter:
Glucose	**< 4 g**
$Na_2HPO_4 \cdot 2H_2O$	12.8 g
KH_2PO_4	3 g
$(NH_4)_2SO_4$	1.8 g
$CaCO_3$	80 mg
$FeCl_3 \cdot 6\ H_2O$	77 mg
$MnCl_2 \cdot 4\ H_2O$	11.5 mg
$CuSO_4 \cdot 5\ H_2O$	1.5 mg
$CoCl_2 \cdot 6\ H_2O$	1.3 mg
ZnO	4 mg
H_3BO_3	1.2 mg
$NaMoO_4 \cdot 2\ H_2O$	10.4 mg
$MgCl_2 \cdot 6\ H_2O$	113 mg
EDTA $Na_4 \cdot 2\ H_2O$	790 mg

Growth kinetics in mineral medium



Batch growth in mineral medium

More defined than complex medium growth!

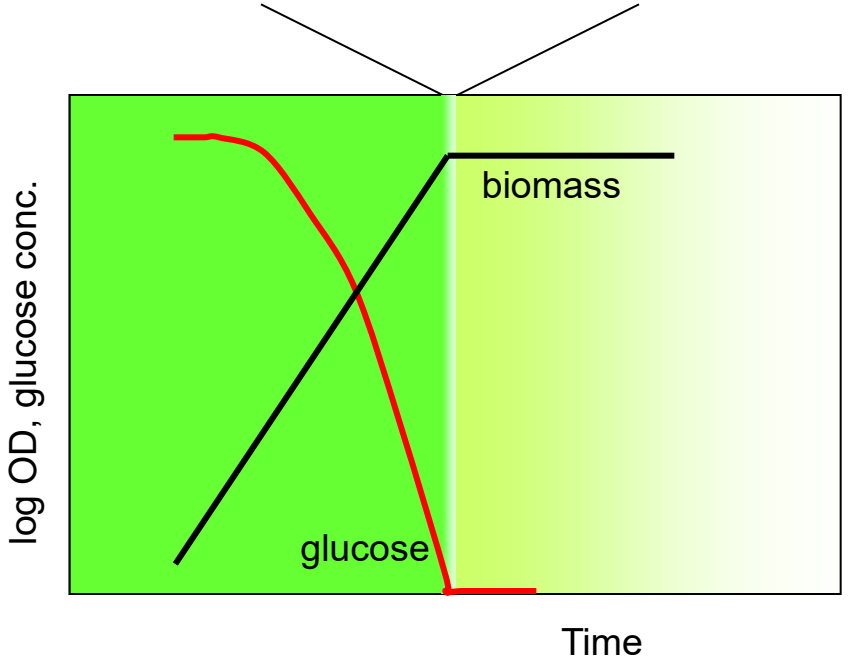


Batch growth in mineral medium

Transition from unlimited to stationary phase

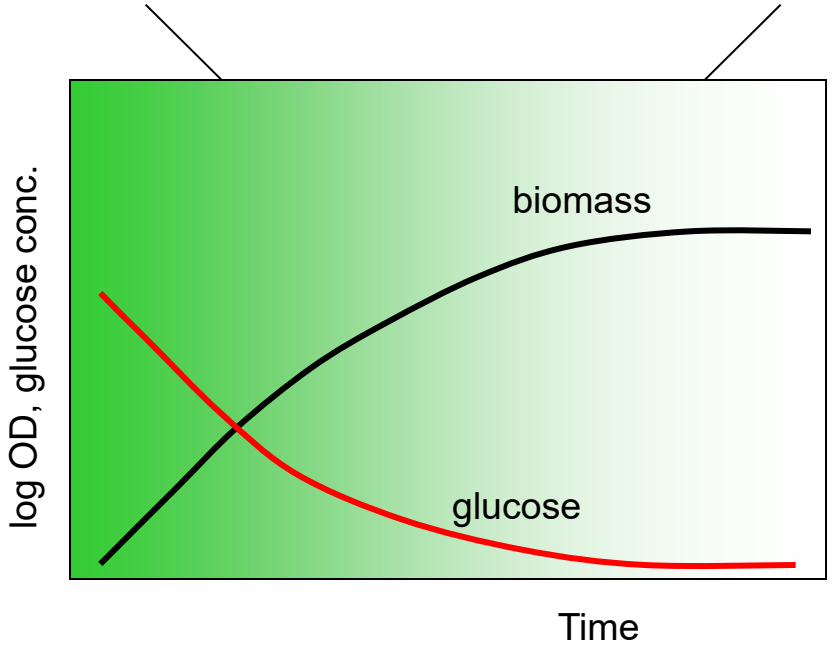
At **high** initial conc. of limiting substrate:

Abrupt transition (sec.-min) from growth to starvation



At **low** (< 10 x Ks) initial conc. of limiting substrate:

Slow transition (hours)



Complex media

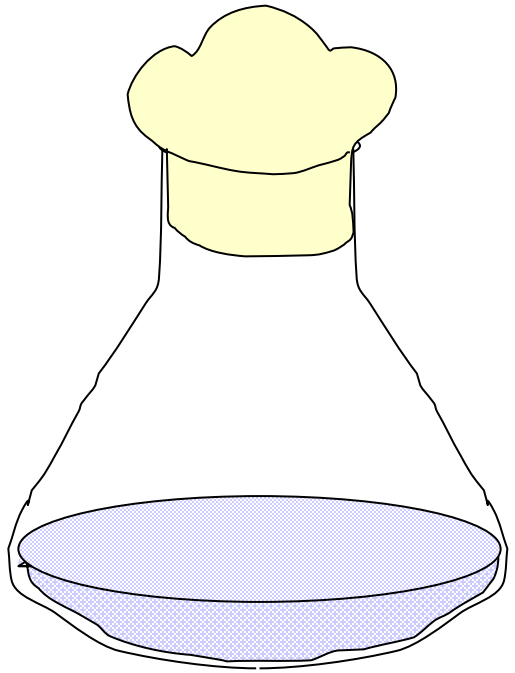
The medium contains ingredients that are not well defined and their composition is not known. Among those there are extracts and hydrolysates (peptones) that can be gained from cheap waste compounds. These media are especially suitable for microorganisms that are difficult to cultivate.

Samples for complex media:

Nutrient broth (peptone from gelatine, beef extract)
Tryptic soy broth (tryptone (pancreatic digestion of casein), peptone from soy beans, glucose, NaCl, K_2HPO_4)

Malt extracts, hay infusions, plum juice, carrot juice. For coprophilic fungi, even horse dung infusions may be used.

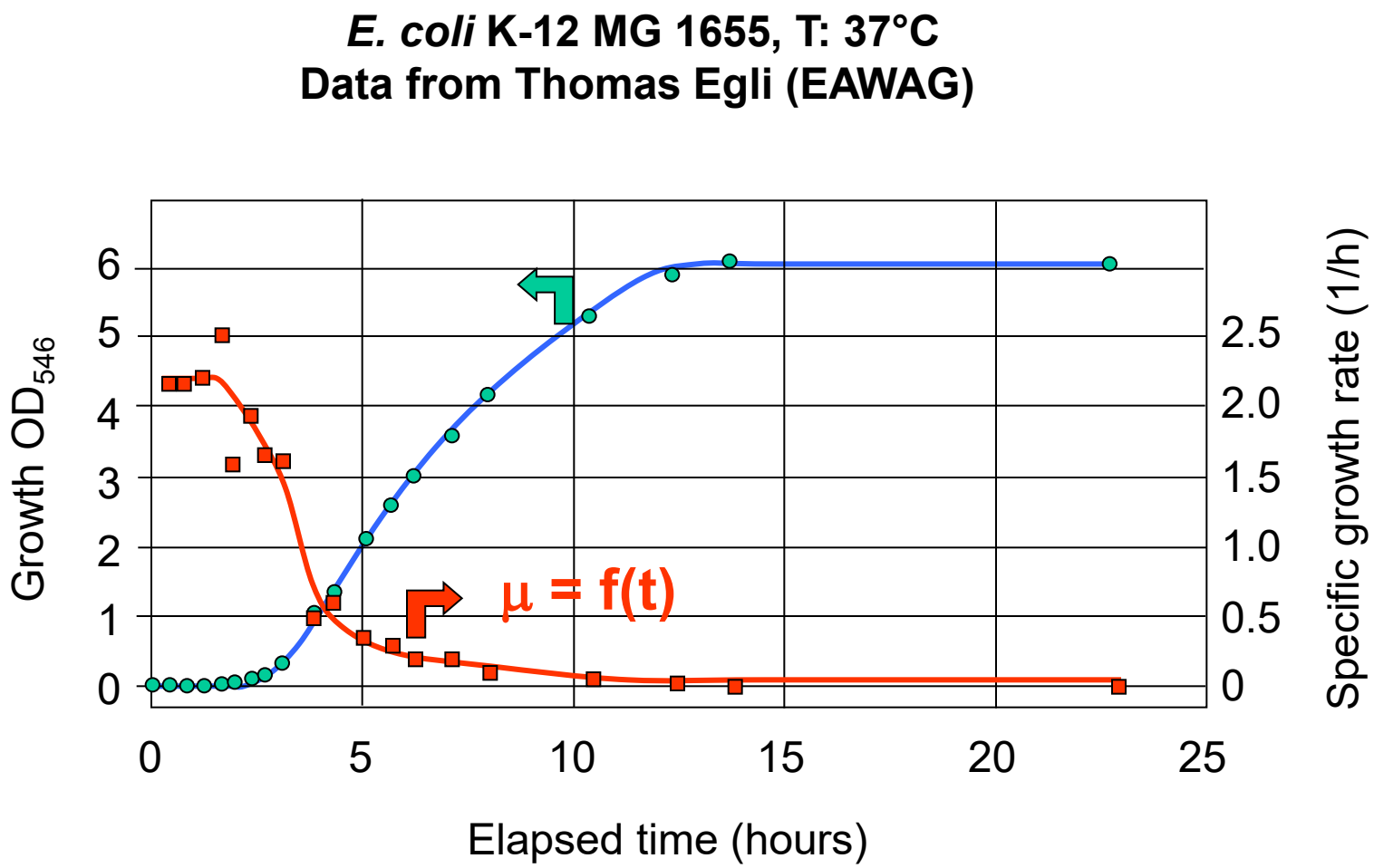
Growth kinetics in complex medium



Luria - Bertani (LB) broth

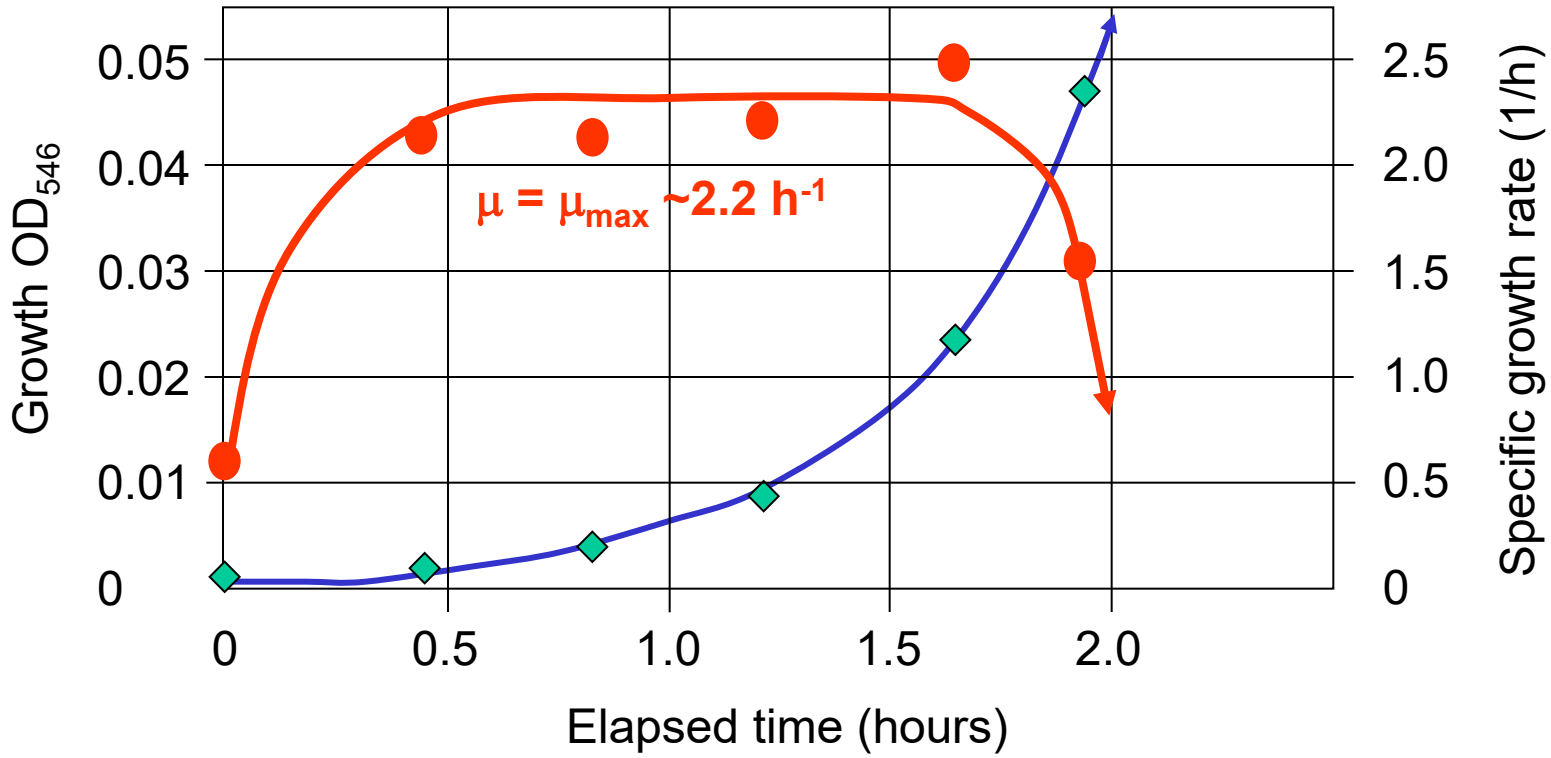
Glucose	0-5 g
Tryptone	10 g
Yeast extract	5-10 g
NaCl	0-10 g
Distilled water add	1 Liter

Batch growth of *Escherichia coli* in LB



A quick start!

***E. coli* K-12 MG 1655, T: 37°C**
Data from Thomas Egli (EAWAG)



Physiological state I

unlimited growth,
sequential utilization or substrates,
maltose and maltodextrins as main substrates,
acceleration to achieve μ_{max}

Physiological state II

preferred C-sources run out,
depletion of maltose,
switch to simultaneous utilization of C-sources,
massive acetate excretion,
transient N-limitation, proteases induced,
strong *rpoS*-expression indicates stress

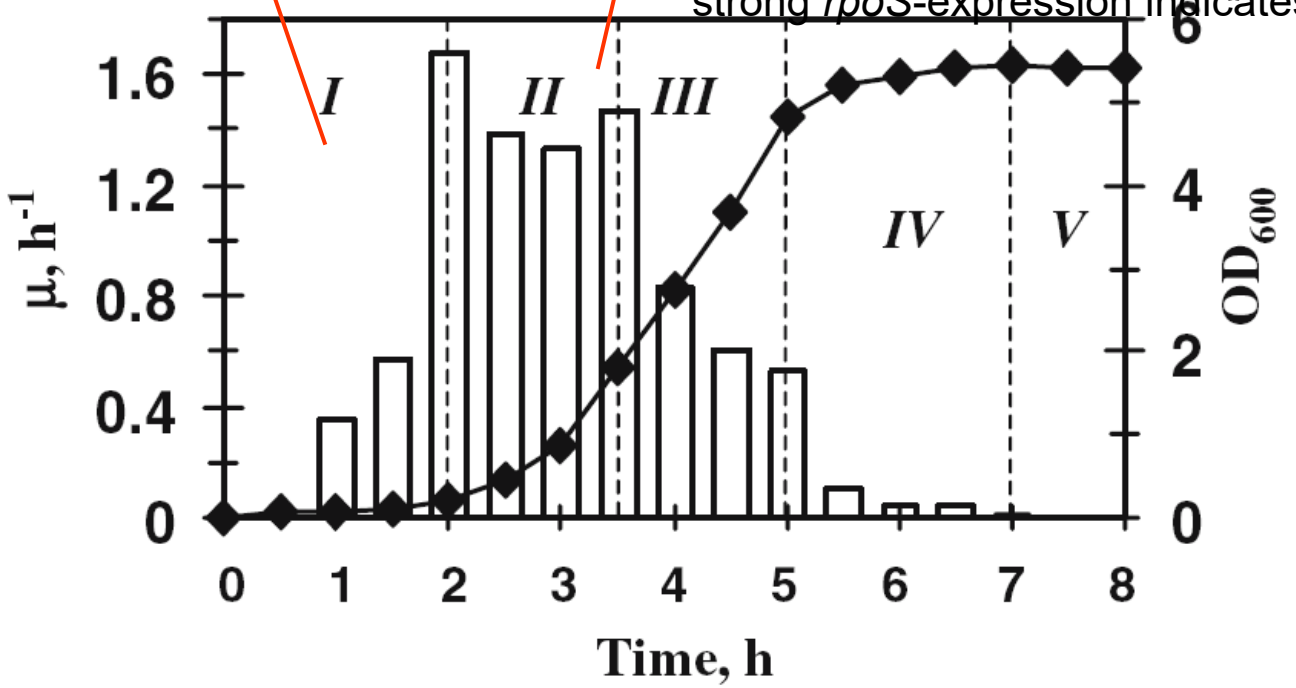


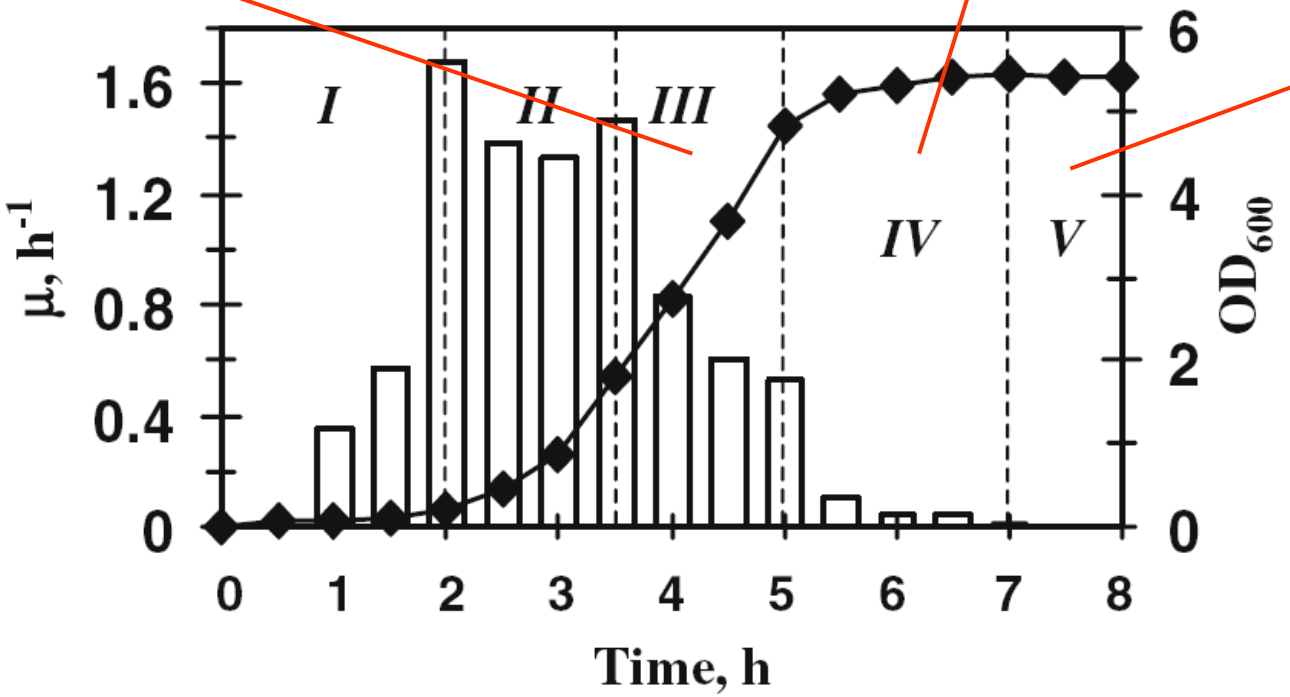
Fig. 3 Physiologic states of *E. coli* MG1655 growing in LB medium. OD_{600} (♦) and specific growth rate μ (*bars*). Discrete growth phases are marked with *Roman numerals* corresponding to the discussion in the text

Physiological state III

massive decrease in μ_{max}
simultaneous utilization of “bad” substrates
porins for peptide transport induced
acetate reutilized
relA and *spoT* expression: nutrient limitation

Physiological state IV

“Hunger” phase
most catabolic pathways induced maximally
biosynthetic pathways activated
transition into stat. phase (*relA, spoT*)



Physiological state V

stat. phase complex,
more than 2/3 of all
mRNAs increase

Fig. 3 Physiologic states of *E. coli* MG1655 growing in LB medium. OD_{600} (♦) and specific growth rate μ (*bars*). Discrete growth phases are marked with *Roman numerals* corresponding to the discussion in the text

Mark V. Baev · Dmitry Baev · Agnes Jancso Radek · John W. Campbell

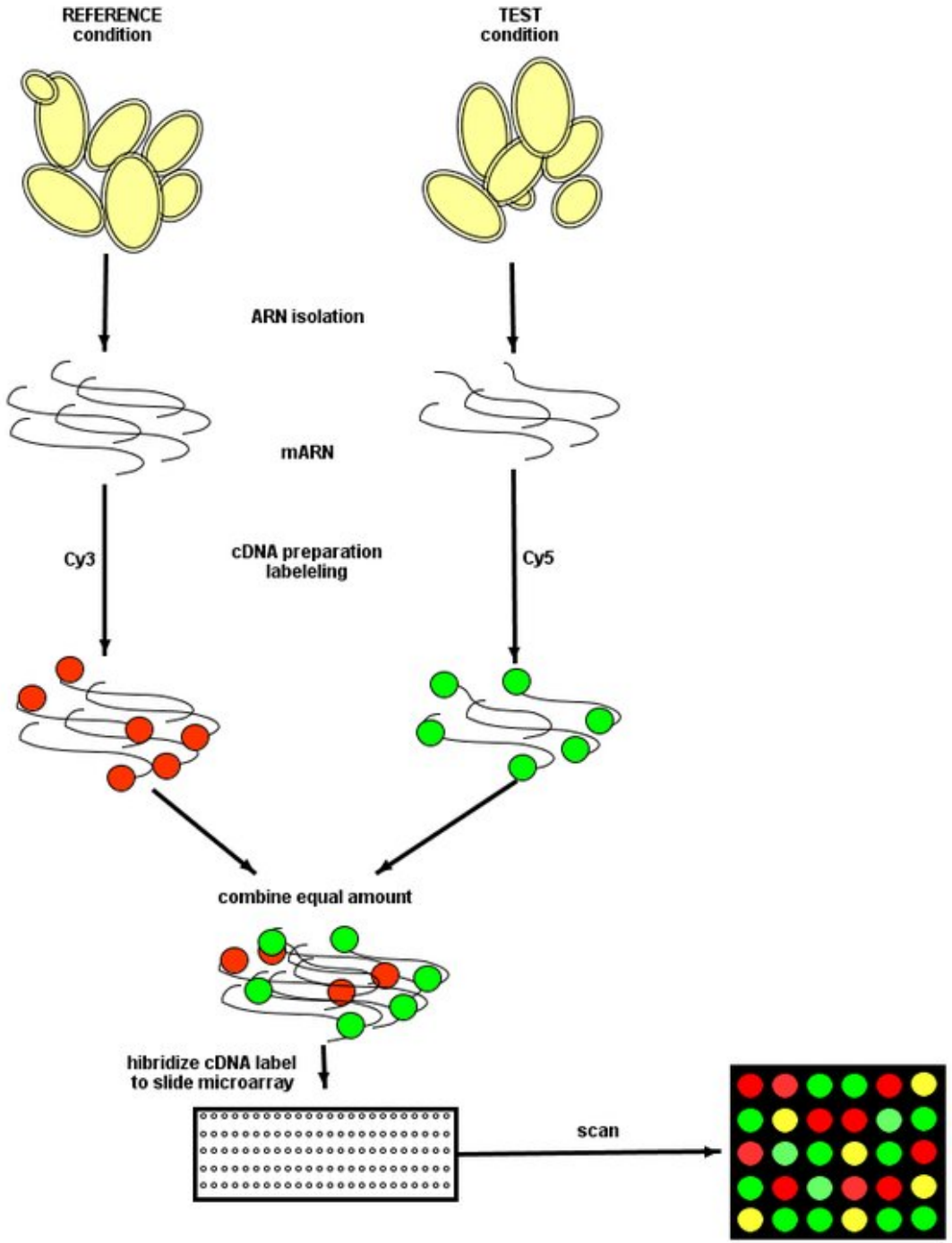
Growth of *Escherichia coli* MG1655 on LB medium: monitoring utilization of sugars, alcohols, and organic acids with transcriptional microarrays Appl Microbiol Biotechnol (2006) 71: 310–316

Growth of *Escherichia coli* MG1655 on LB medium: monitoring utilization of amino acids, peptides. and nucleotides with transcriptional microarrays Appl Microbiol Biotechnol (2006) 71: 317–322

Growth of *Escherichia coli* MG1655 on LB medium: determining metabolic strategy with transcriptional microarrays

Abstract Expression profiles of genes related to stress responses, substrate assimilation, acetate metabolism, and biosynthesis were obtained by monitoring growth of *Escherichia coli* MG1655 in Luria–Bertani (LB) medium with transcriptional microarrays. Superimposing gene expression profiles on a plot of specific growth rate demonstrates that the cells pass through four distinct physiological states during fermentation before entering stationary phase. Each of these states can be characterized by specific patterns of substrate utilization and cellular biosynthesis corresponding to the nutrient status of the medium. These data allow the growth phases of the classical microbial growth curve to be redefined in terms of the physiological states and environmental changes commonly occurring during bacterial growth in batch culture on LB medium.

Appl Microbiol Biotechnol (2006) 71: 323–328



Materials and methods

Bacterial growth conditions

Escherichia coli strain MG1655 (CGSC 6300) was used in this study. Batch cultivation was carried out in LB medium at 37°C in a laboratory-scale Bioflo 2000 (New Brunswick Scientific) fermentor with a 2-l working volume. LB medium was from the recipe of Miller (5 g yeast extract, 10 g peptone tryptone, and 10 g NaCl) (Miller 1972). The pH was maintained at 6.95 automatically by titration with 5% H_2SO_4 or 5% NaOH. Peptone tryptone and yeast extract were from Difco, and NaCl was from Sigma. The medium was made in distilled water and autoclaved under standard conditions. Dissolved oxygen in the culture was maintained at 40% saturation automatically by varying speed of impeller rotation. Culture growth (OD_{600}) was monitored with a DU640 spectrophotometer (Beckman). OD_{600} immediately after inoculation was 0.05.

Samples of cell culture were centrifuged for 2 min at 13,000×*g*, 4 °C. The supernatant was filtered through a 0.22-μm filter and stored at −80 °C until further use.

E. coli genomic DNA from MG1655 was used as template for generating the microarray probe library. The ERGO database (Integrated Genomics) identifies 4,485 open reading frames (ORFs) within the *E. coli* genome; primers were designed using proprietary software such that the most unique 300–500 bp region of 4,442 of these ORFs was amplified with two consecutive rounds of PCR.

Questions

- What kind of media should be used in science?
- What is typical for growth on a complex medium?
- What could be a problem in preparation of a medium?

How to prepare a medium for an unknown organism?

Four different methods can be used for the design of a defined medium:

-Analogy method

-Differential method

-Integral method

-Statistical approach

Analogy method

Starting from an elemental analyses of the biomass, one can estimate the minimal requirements for an organism and also the biomass that can be produced by the medium. It has to be taken care that products that are excreted from the cell can be included in the mass balance.

As a rule of thumb one can postulate:

Anaerobic growth conditions: about **10% of C** go into biomass
Aerobic growth conditions: about **50% of C** go into biomass

Check also information with strain collections!
https://www.dsmz.de/collection/catalogue/microorganisms/culture-technology/list-of-media-for-microorganisms

Cell composition of *Escherichia coli*

			C	H	N	O	P	S
Total [wt%]	Average	Range	47.82	6.38	14.36	24.49	2.74	0.75
Protein	55.0	15-75	61.17	61.55	67.93	46.35	0.00	100.00
RNA	20.5	5-30	15.17	10.38	24.29	28.62	71.25	0.00
DNA	3.1	1-5	2.45	1.70	3.66	3.92	11.31	0.00
Lipid	9.1	0-15	12.38	16.07	0.95	7.16	14.59	0.00
LPS	3.4	0-4	3.48	4.44	0.41	5.32	2.85	0.00
Peptidoglycan	2.5	0-20	2.57	2.57	1.88	3.61	0.00	0.00
Glycogen	2.5	0-50	2.32	2.43	0.00	5.03	0.00	0.00
Polyamines	0.4	0-10	0.47	0.86	0.87	0.00	0.00	0.00

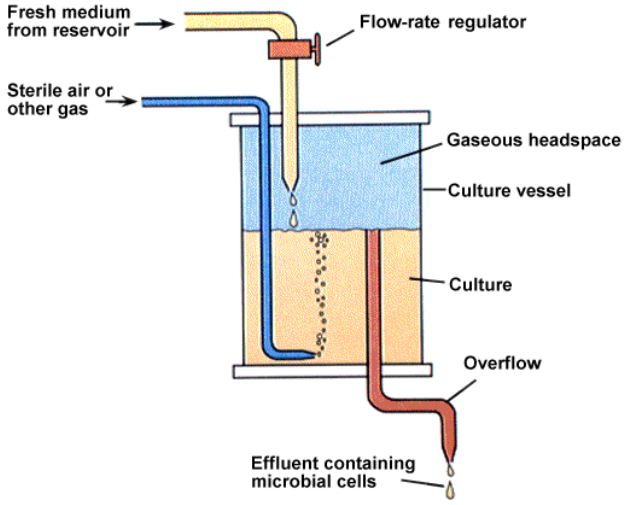
(Composition of *E. coli* in exponential growth phase)

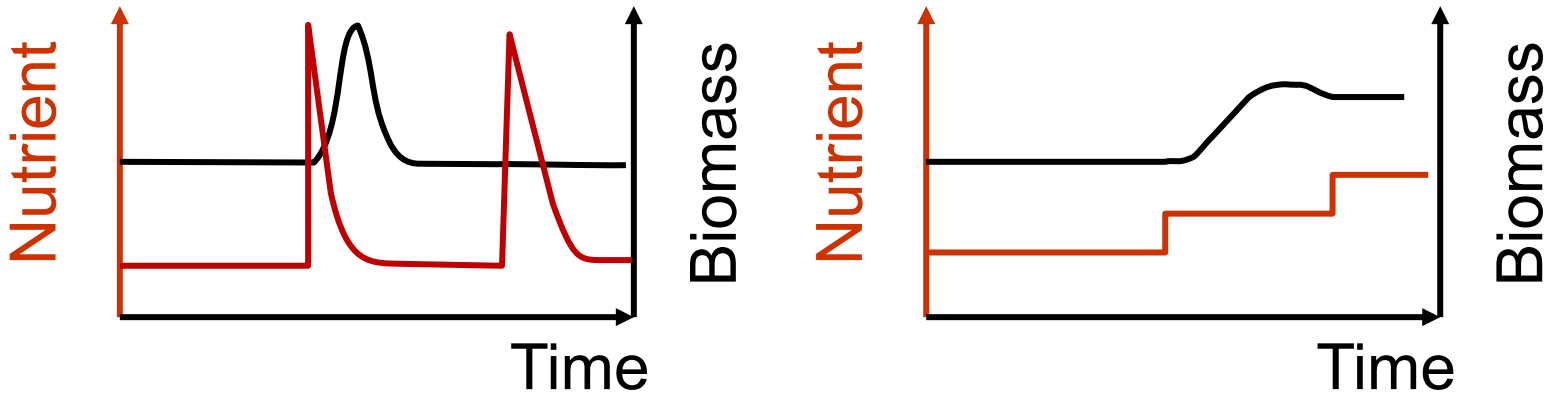
The total weight of an average cell is 9.5×10^{-13} g (containing 70% water). The total dry weight of *E. coli* is therefore 2.8×10^{-13} g.

Differential method

The influence of substrate pulses on the time courses of biomass, substrate or product concentrations as responses to the change of the medium composition.

The most suitable tool is the **chemostat** cultivation to assess the influence of hidden limitations using **pulse** and **shift experiments**. As a benefit of the method can be seen that the minimal nutrient concentration can be determined (reduction of waste of nutrients).





Integral method

1 Choose the growth limiting nutrient

For heterotrophs most often the carbon/energy source because it has to be added in highest quantities

2 Choose the maximum amount of biomass you want to produce in your system

Depends on cultivation system and goal (shake flask or bioreactor; high cell density cultivation or drinking water study, etc.

3 Choose how much other nutrients should be in excess

Depends on safety factor you want, in biotechnological production you do not want excess because this will be lost

4 Calculate for each nutrient how much you have to add using established average growth yield factors

$Y_{X/S}$ = g biomass formed / g substrate utilized
$Y_{X/C}$ = g biomass formed / g substrate carbon utilized
$Y_{X/S}$ = g biomass formed / mol substrate utilized...

Medium design

How much carbon and nitrogen source do I have to add to a medium to obtain 10 g of dry biomass (DW)?

$$\text{Gram element consumed} = \frac{\text{Gram dry biomass formed}}{Y_{X/E}}$$

Carbon: 10 gCDW / 1 gDW/gC = 10 g C

Nitrogen: 10 gCDW / 8 gDW/gN = 1.25 g N

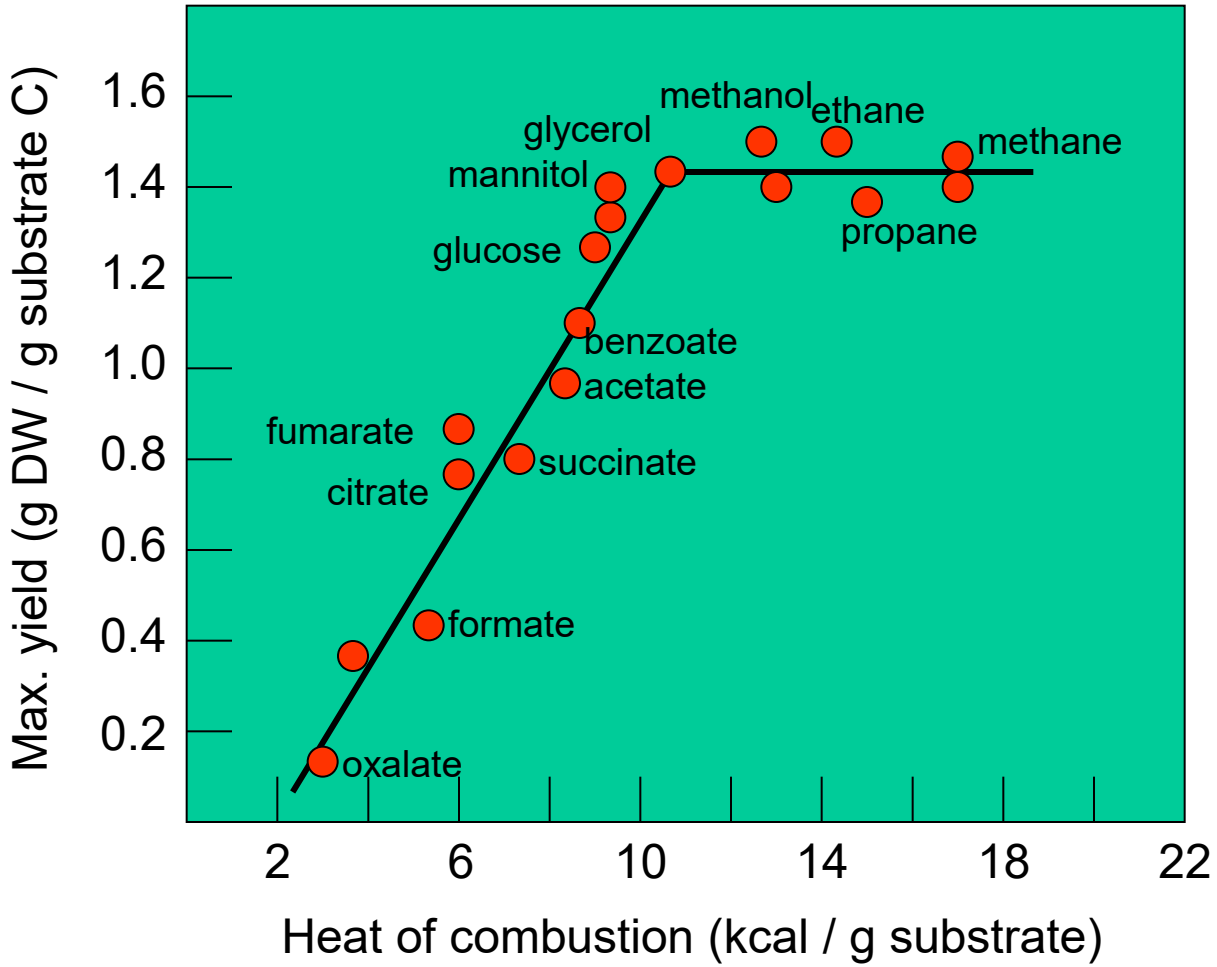
Design of a mineral medium

Design of a carbon-limited minimal medium allowing the production of 10 g L^{-1} of dry biomass, based on elemental growth yields obtained from the composition of dry biomass.

Medium constituent	Source of, function	Growth yield assumed (g dry bio-mass/g ele-ment)	Excess factor assumed with respect to carbon	Mass of element (g/L)	Mass of constituent (g/L)
Glucose	C, energy	1	1	10	25.0
NH_4Cl	N	8	3	3.75	14.33
NaH_2PO_4	P	33	5	1.52	5.88
KCl	K	100	5	0.5	0.95
$NaHSO_4$	S	100	5	0.5	1.87
$MgCl_2$	Mg	200	5	0.25	0.98
$CaCl_2$	Ca	100	10	1.0	2.77
$FeCl_2$	Fe	200	10	0.5	1.13
$MnCl_2$	Mn	10^4	20	0.02	0.046
$ZnCl_2$	Zn	10^4	20	0.02	0.042
$CuCl_2$	Cu	10^5	20	0.002	0.0042
$CoCl_2$	Co	10^5	20	0.002	0.0044

Based on Pirt (1975) and Egli & Fiechter (1981). Elemental growth yields for C and the trace elements Zn, Cu, Mo, and Mn were taken from Pirt (1975). Excess factors were chosen taking into account their variation observed in dry biomass.

Growth yields for different carbon/energy sources



Oxygen requirements

Organism	Substrate	Maximum observed yield [g g^{-1}]		
		$Y_{X/S}$	$Y_{X/C}$	O_2 used
Candida utilis	glucose	0.51	1.28	1.3
Aerobacter cloacae	glucose	0.44	1.1	1.03
Candida utilis	acetic acid	0.36	0.9	0.62
Candida utilis	ethanol	0.68	1.3	0.58
Pseudomonas sp.	methanol	0.41	1.09	0.44
Methylococcus sp.	methane	1.01	1.34	0.29

The biomass growth rate (dx/dt) is constant and the specific growth rate is decreasing over time when oxygen is limiting growth.

Optimization of existing media for high cell density cultivations in micro-titer plates using statistical design-of-experiments (DoE)

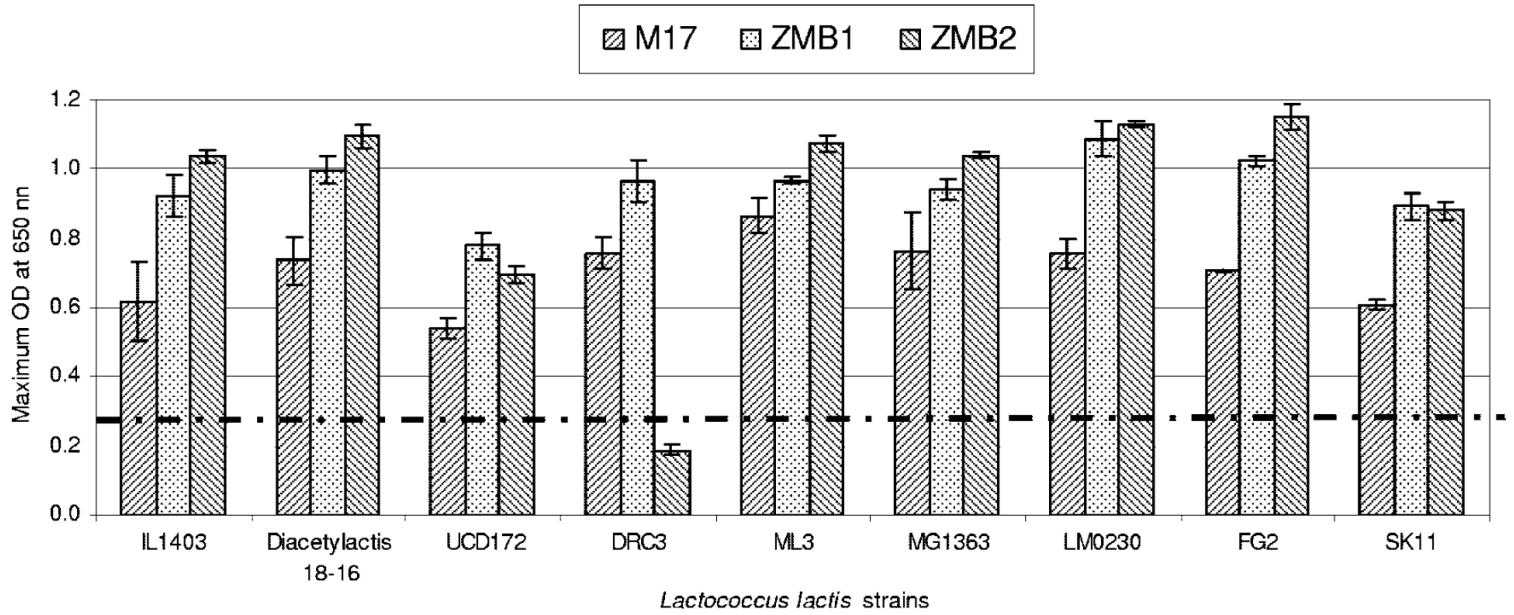


FIG. 4. Evaluation of the maximum OD of nine *L. lactis* strains cultivated in three media. The dashed and dotted line indicates the maximum biomass of *L. lactis* IL1403 growing in the best previously described CDM, SA. Cultures were grown in 96-well plates with a working volume of 200 μl for 20 h at set incubation temperatures (ZMB1 at 27.5°C, ZMB2 at 24.1°C, M17 at 30°C, and SA at 30°C). Optical densities were determined at a wavelength of 650 nm.

G. Zhang, D.A. Mills, D.E. Block (2009). Development of chemically defined media supporting high-cell-density growth of Lactococci, Entereococci, and Streopcocci. *Appl. Environ. Microbiol.* 75(4): 1080-1087.

(see also: https://www.isixsigma.com/tools-templates/design-of-experiments-doe/design-experiments-%E2%90%93-primer/)

Questions

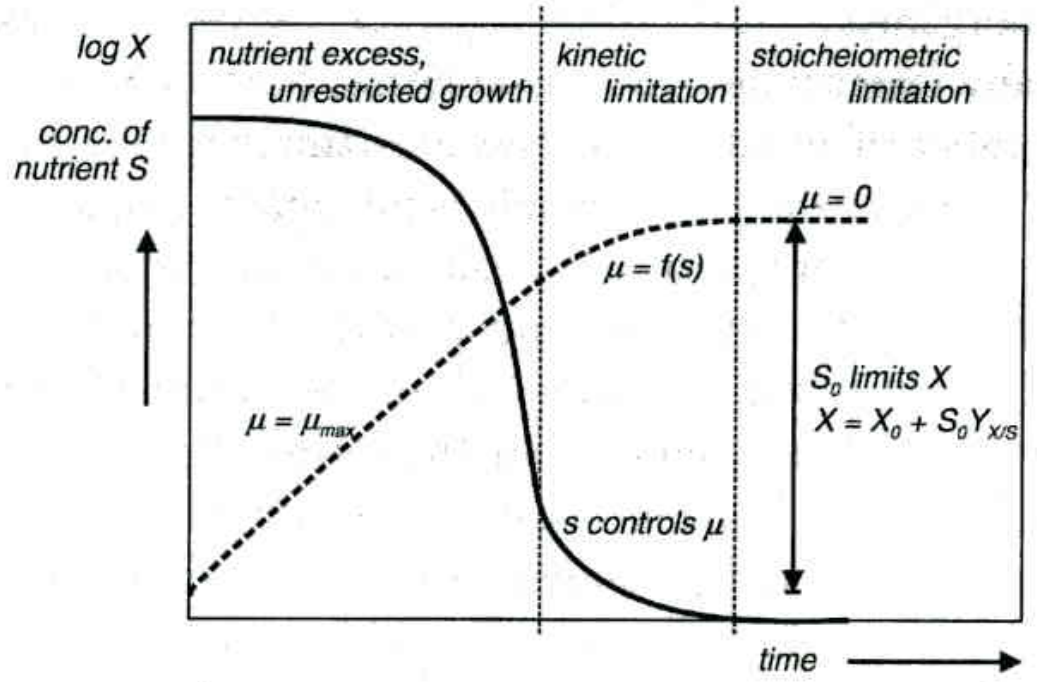
What are the advantages and disadvantages of following methods?

-Analogy method
-Differential method
-Integral method
-Statistical approach

Some exercises:

https://www.climbing.com/skills/training-gym-free-climbing-exercises-you-can-do-anywhere/

Remember there are two types of growth limitations



Task 1

You have prepared a preculture (100 mL) in the same medium as the batch medium you plan to use in the bioreactor.

a) How much do you need to inoculate in the bioreactor that contains 2.5 L of sterile medium?
The final OD_{600} of the preculture is 4.1.

b) What is the percentile increase of the volume in the bioreactor because of the inoculation?

Task 2

a) What will be the OD in the inoculated reactor after 10 h assuming you have a strain that finally grows exponentially with a μ_{max} = 0.69 h^{-1} after a lag phase at an OD600 = 0.1 for 4 h.

b) How much glucose would you have used up until 10 h when you know that the yield coefficient $Y_{OD(600)/Glucose}$ is 1.5 g^{-1}.

c) The strain produces propioniate during growth. What would be the propionate concentration when you know the yield coefficient for propionate is $Y_{Propionate/Glucose}$ = 0.1 g g^{-1}? (p_0 = 0 g L^{-1})

Task 3

a) What will be the specific growth rate of your strain (μ_{max} = 0.69 h^{-1}) when you know the Ks value is 0.2 g L^{-1} and you measure a glucose concentration in the supernatant that is 0.35 g L^{-1}?

b) What would be the substrate concentration when you determine a μ = 0.5 h^{-1} ?

Task 4

Stoichiometric limitation:

a) What will be the maximum biomass (=OD600) that you can produce in a 2.5 L bioreactor with 40 g L^{-1} of glucose when you know the yields are $Y_{Propionate/Glucose}$ = 0.1 g g^{-1} and $Y_{OD(600)/Glucose}$ = 1.5 g^{-1}.
b) What would be the yield $Y_{Propionate/OD(600)}$?

Task 5

a) Analyze the graph to the right:
   - Growth phases
   - Limiting and non-limiting nutrients
   - Any products?
   - Where is PHA to be found (= Poly(3-hydroxyalkanoate), a bioplastic)?

b) Determine μ_{max}

c) Determine growth yield $Y_{X/N}$

d) Calculate the specific uptake rate for N (q_N) in g g h^{-1}

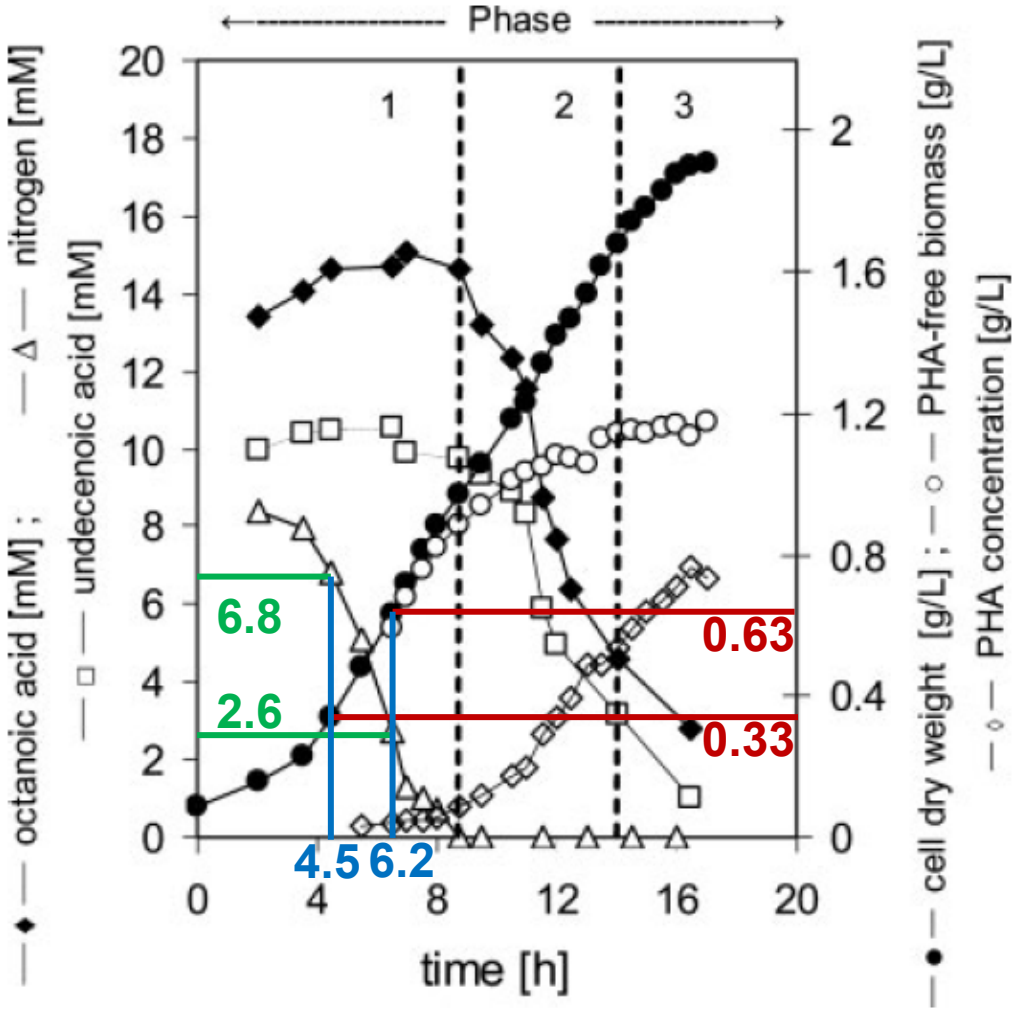


Figure 1. Accumulation of olefinic medium-chain-length polyhydroxyalkanoates (mclPHAs) by *P. putida* GPo1 during batch growth with a mixture of octanoate (15 mM) and 10-undecenoate (11 mM).

Hartmann, R., et al., *Tailor-made olefinic medium-chain-length poly[(R)-3-hydroxyalkanoates] by* Pseudomonas putida *GPo1: Batch versus chemostat production.* Biotechnol. Bioeng., 2006. **93**(4): p. 737-746.

Task 6

a) What will be the cell dry weight in the inoculated reactor after 10 h assuming you have a strain that finally grows exponentially with a μ_{max} = 0.69 h^{-1} i) normally and ii) after a lag phase at an x_0 = 0.1 g for 4 h.

b) How much glucose would you have used up until 10 h when you know that the yield coefficient $Y_{X/Glucose}$ is 0.5 g g^{-1}.

c) The strain produces propioniate during growth. What would be the propionate concentration after 10 h when you know the yield coefficient for propionate is $Y_{Propionate/Glucose}$ = 0.1 g g^{-1}? (p_0 = 0 g L^{-1})

Task 7

a) How were the data obtained for the figure to the right?

b) Determine graphically the Ks and the μ_{max} values.

c) What other method could you use to determine these parameters?

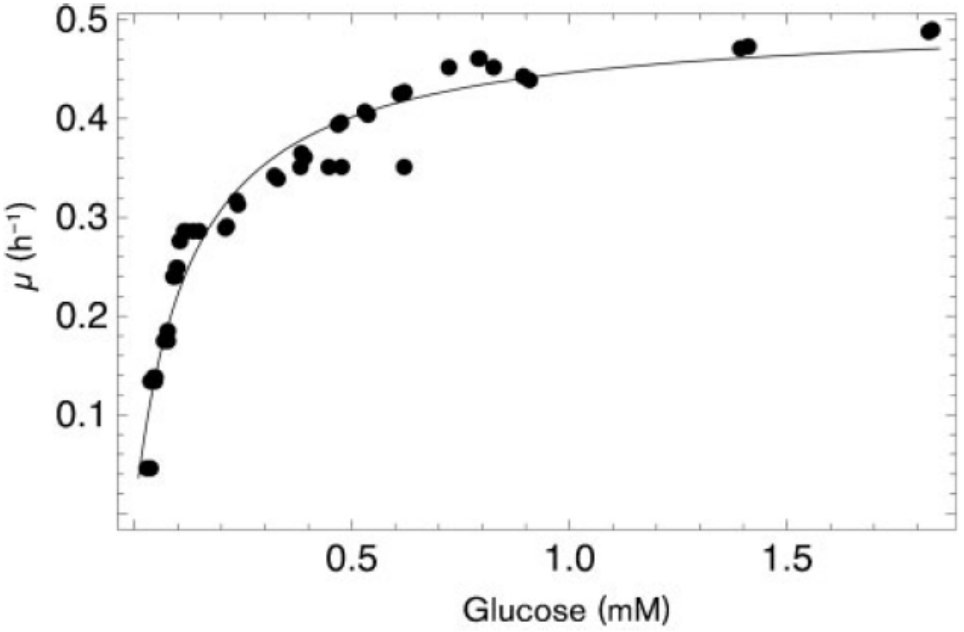


Fig. 2. μ as a function of the residual substrate concentration. *S. cerevisiae* was grown over a range of *D* values and steady-state residual glucose concentrations were determined. The Monod curve is fitted through the data points.

Snoep, J.L., et al., Microbiology (2009), 155, 1699–1707

Additional information for the medium exercises

Molecular weights and growth yields

Element	[Da]	$C_{X/E}$ [g g^{-1}]	Element	[Da]	$C_{X/E}$ [g g^{-1}]
C	12	1	Cu	63.5	10^5
Cl	35.5	-	Co	58.9	10^5
H	1	-	Ca	40.1	100
N	14	8	K	39.1	100
O	16	1	Mn	54.9	10^4
P	31	33	Na	23	-
S	32	100	Mo	95.9	-
Mg	24.3	200	Ni	58.7	-
Fe	55.8	200	Zn	65.4	10^4

Task 8

How much of the nitrogen source do you have to add to a carbon-limited growth medium when you want to reach a maximum biomass of 50 g L^{-1} cell dry weight and the nitrogen source (NH_4Cl) should only be 1.1-fold in excess? How much nitrogen source do you have to add if you use KNO_3 instead of NH_4Cl?

Task 9

M9 is probably the most widely used mineral medium to grow *Escherichia coli*. It contains per liter:
Glucose 2 g, Na_2HPO_4 6 g, KH_2PO_4 3 g, NaCl 0.5 g, NH_4Cl 1 g, $MgSO_4 \cdot 7H_2O$ 0.25 g, $CaCl_2$ 7.6 mg. Analyse the medium composition and comment.

Task 10

You have to treat wastewater from a fruit juice producing factory. It contains on average 30 g L^{-1} of easily degradable carbon compounds (mainly sugars $C_6H_{12}O_6$). The concentration of easily accessible nitrogen compounds in this wastewater is on average 0.5 g L^{-1}. What are the conclusions you draw from your analysis?

Suggest solutions!

Task 11

You grow an obligate aerobic bacterial strain with a water soluble but volatile carbon source in closed glass bottles (volume 100 mL) with a mineral medium that allows cell densities of up to 5 g CDW L^{-1}. The total amount of carbon supplied is 0.5 g L^{-1} and the total amount of growth medium in the bottle is 20 mL. The headspace of the bottle is filled with air. Is there sufficient oxygen in the headspace such that all carbon will be utilized? Assume a yield factor for molecular oxygen of 30 g CDW $(mol\ O_2)^{-1}$.