Bioprocesses and Downstream Processing

ChE-437

Part 2

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Agenda

- Q & A Exercises
- 6. Nutrient starvation
- 7. Optimization of production
- 8. Fed-batch cultivation

- Calculations concerning fed-batches
- Introduction chemostat

Mathematical expression of Monod's model

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:

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

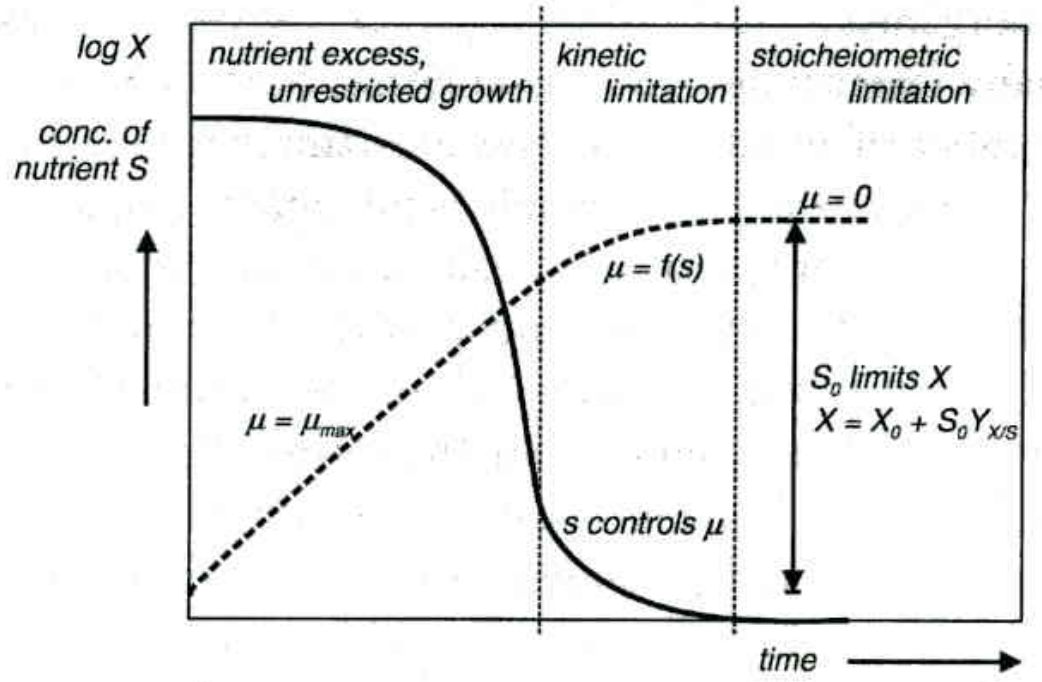
The specific growth rate depends only on the concentration of the limiting substrate according to the Miachaelis-Menten kinetics.

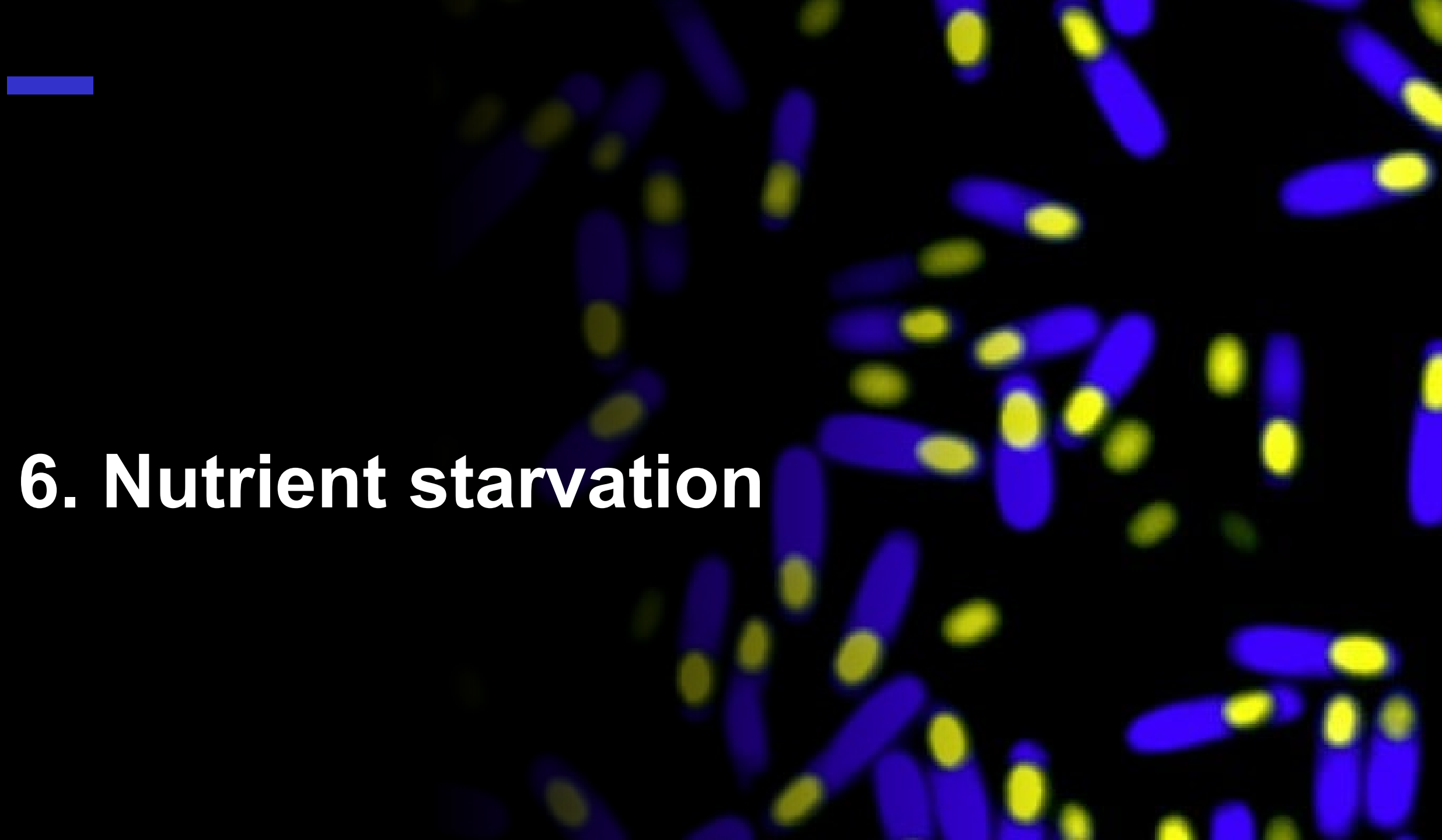
3rd equation of Monod:

$$Y = \frac{r_x}{-r_s}$$

The yield coefficient is constant under exponential growth conditions.

Remember: There are two types of growth limitations:





6. Nutrient starvation

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

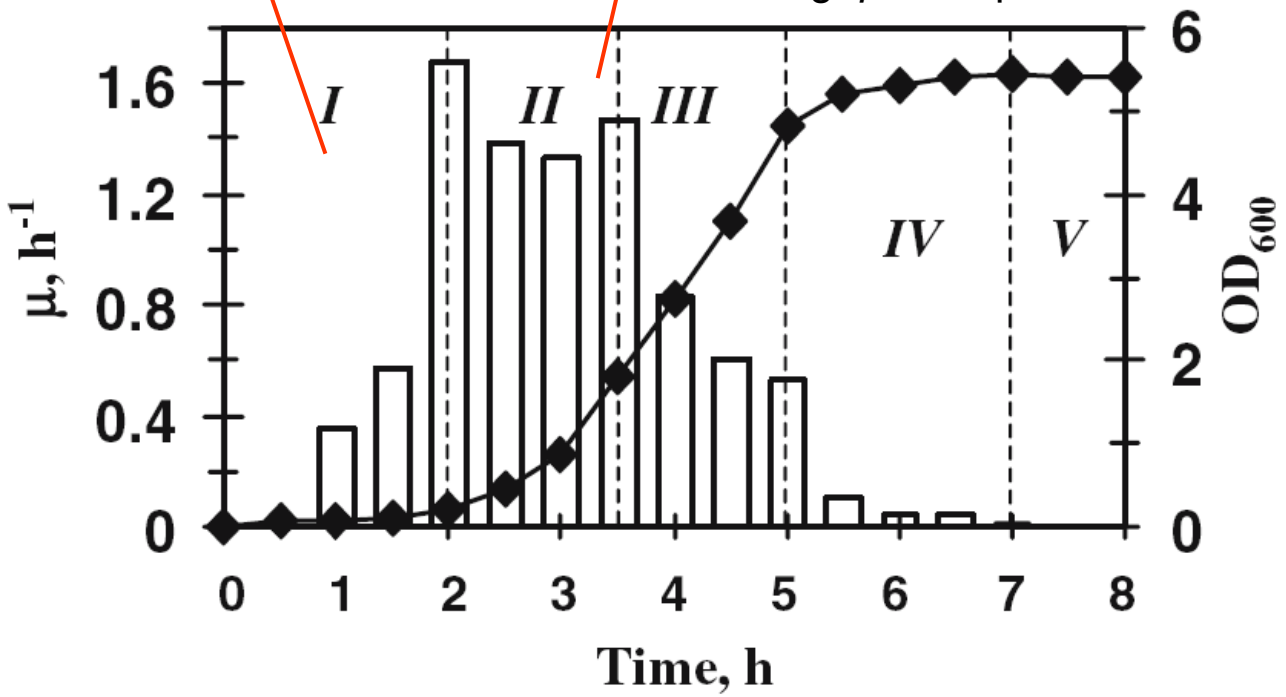


Fig. 3 Physiologic states of *E. coli* MG1655 growing in LB medium. OD_{600} (♦) and specific growth rate μ (*bars*). Discrete growth phases are marked with *Roman numerals* corresponding to the discussion in the text

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*)

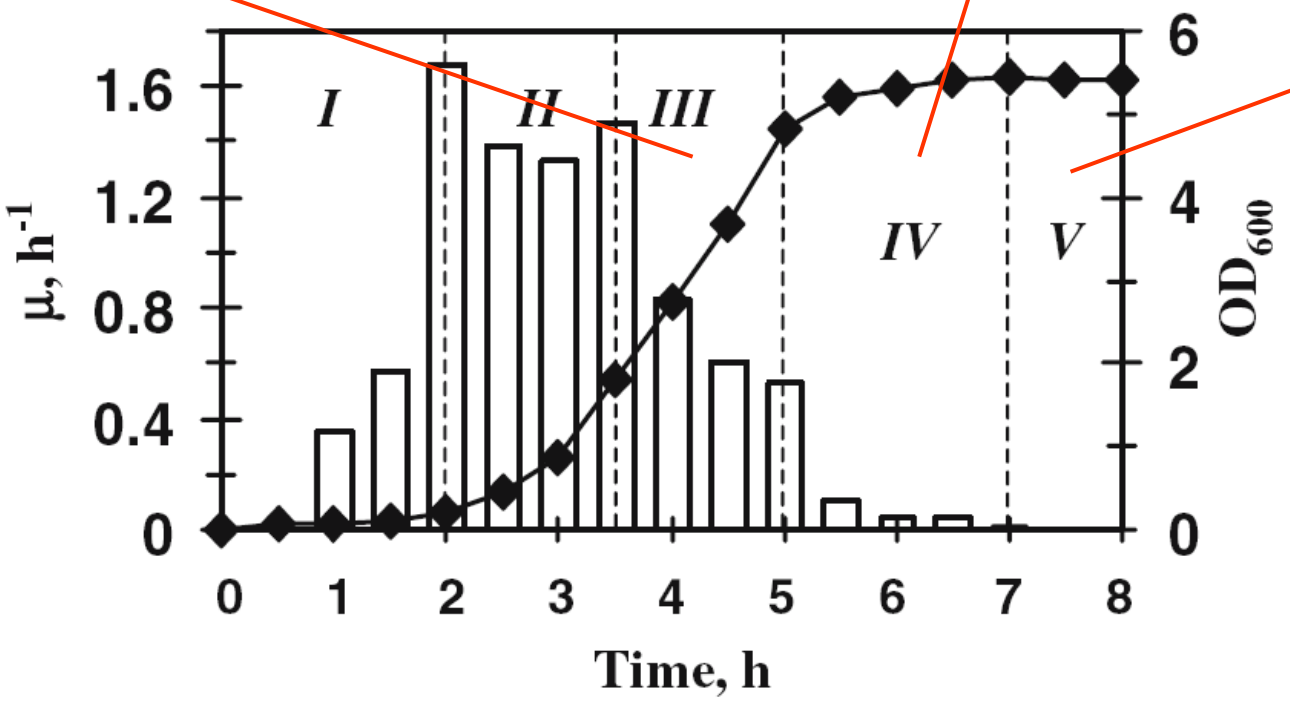


Fig. 3 Physiologic states of *E. coli* MG1655 growing in LB medium. OD_{600} (♦) and specific growth rate μ (*bars*). Discrete growth phases are marked with *Roman numerals* corresponding to the discussion in the text

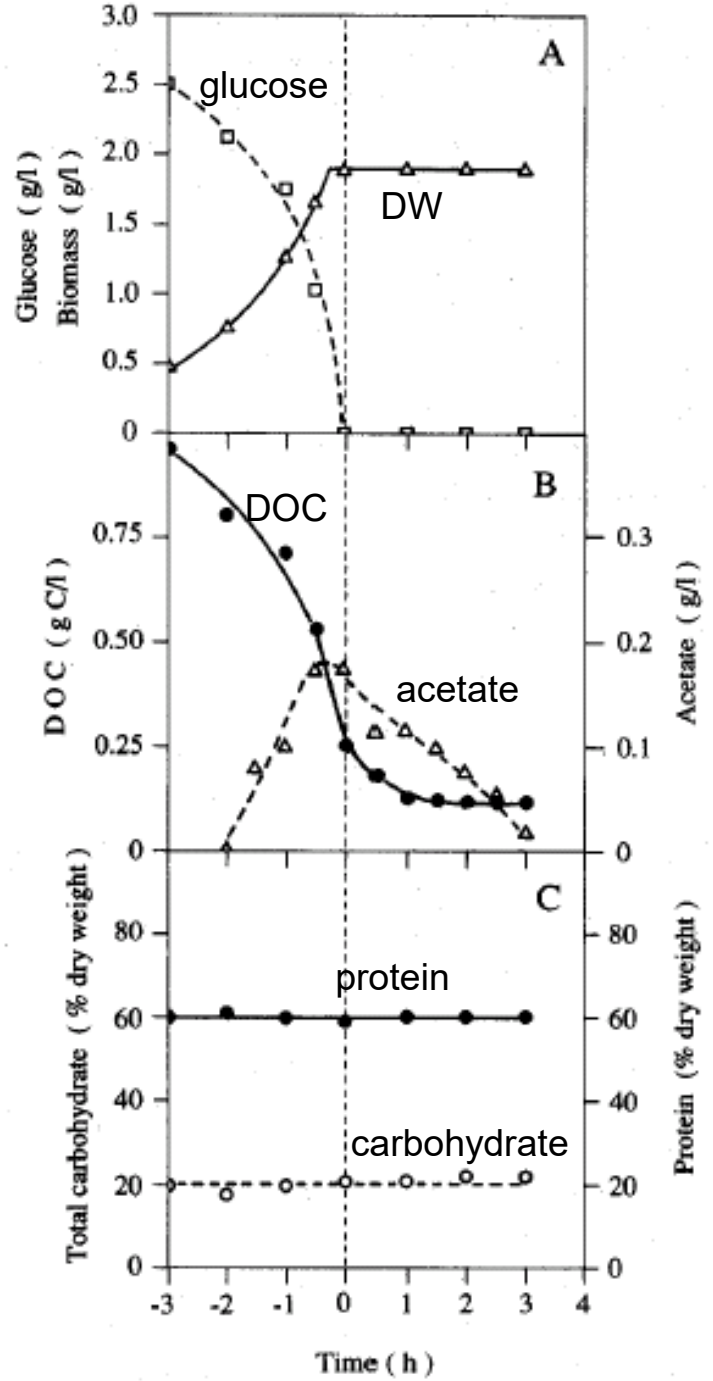
Physiological state V

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

Carbon-limited batch culture

Comments:

- Acetate is produced and consumed again.
- DOC indicates that this is not the only product and that some carbon remains in the medium.
- “Frozen log cells”



Batch growth of *Klebsiella pneumoniae* in synthetic medium with phosphate as limiting substrate.

(from Wanner & Egli, 1990)

Cell rearrangements in C-limited batch culture (1)

Biomass:	Usually constant for several hours; exception cultures that lyse, e.g. Bacillus spp.
Cell number:	Increases, becomes constant after 1-2 hours (DNA replication is finished and cell division is finished whenever possible).
Cell volume:	Decreases, frequently also change in morphology observed (e.g., *K. pneumoniae* changs from 0.6 mm rods to 0.2 mm cocci)
Products:	Products formed in exponential phase are consumed (for yeast forming ethanol during a second growth phase is observed)
Reserve materials:	When accumulated in exp. phase (rarely in large amounts, except *Arthrobacter* spp.) they are consumed.
Cell wall:	Low affinity transport systems replaced by high affinity systems (mostly info from chemostat experiments).
	Lipid composition of membrane changes; replacement of unsaturated fatty acids by saturated ones (tighter lipid layer?).
	Peptidoglycan layer turnover stops (in Gram-positives 30-50%/h in exp. phase). Cross-linking and thickness of murein layer increases in Gram-negative cells.

Cell rearrangements in C-limited batch culture (2)

DNA: Rounds of replication are finished; DNA repair activities are much reduced.

RNA: Stable RNA (rRNA) is partly degraded and used as energy and building material for DNA synthesis (after storage and external products have been consumed). Excretion of surplus phosphate and sometimes also RNA bases. Rate of RNA synthesis reduced to ~10%.

Protein: Protein turnover rates increase from ~2%/h to 5-7%/h.

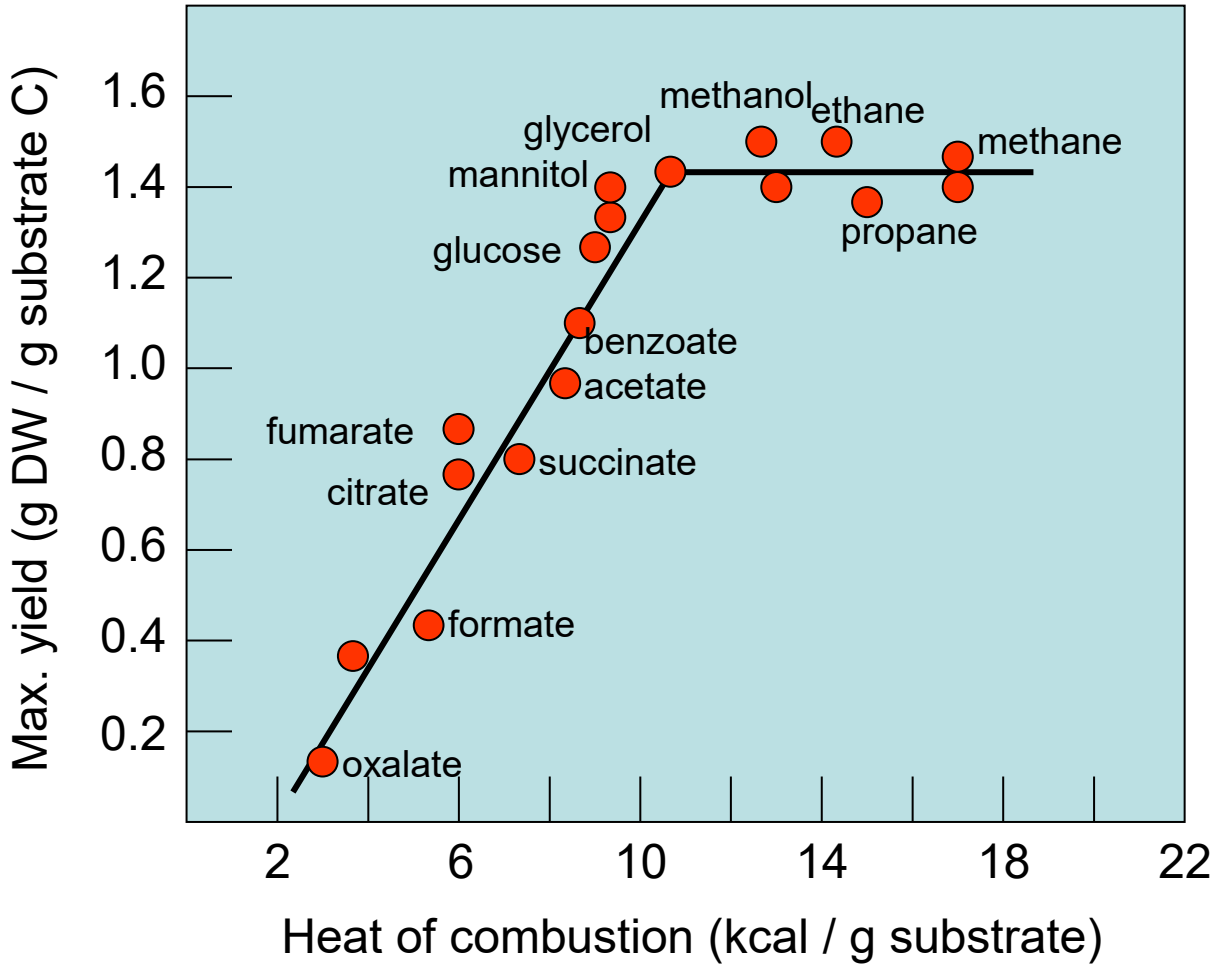
Amino acid pool immediately depleted.

Stringent response induced = ppGpp synthesis, an alarmone that controls expression of many genes; the response is triggered by stalling ribosomes because of unloaded tRNA; the response also includes expression of proteases that are vital for rearrangement.

Protein concentration remains mostly stable, only rearrangement of protein pattern, ca. 30 major new proteins synthesized in *E. coli;* proteins are degraded only after extended starvation periods when rRNA degraded to the minimum required.

ATP: Contradicting results in literature

Growth yields for different carbon/energy sources



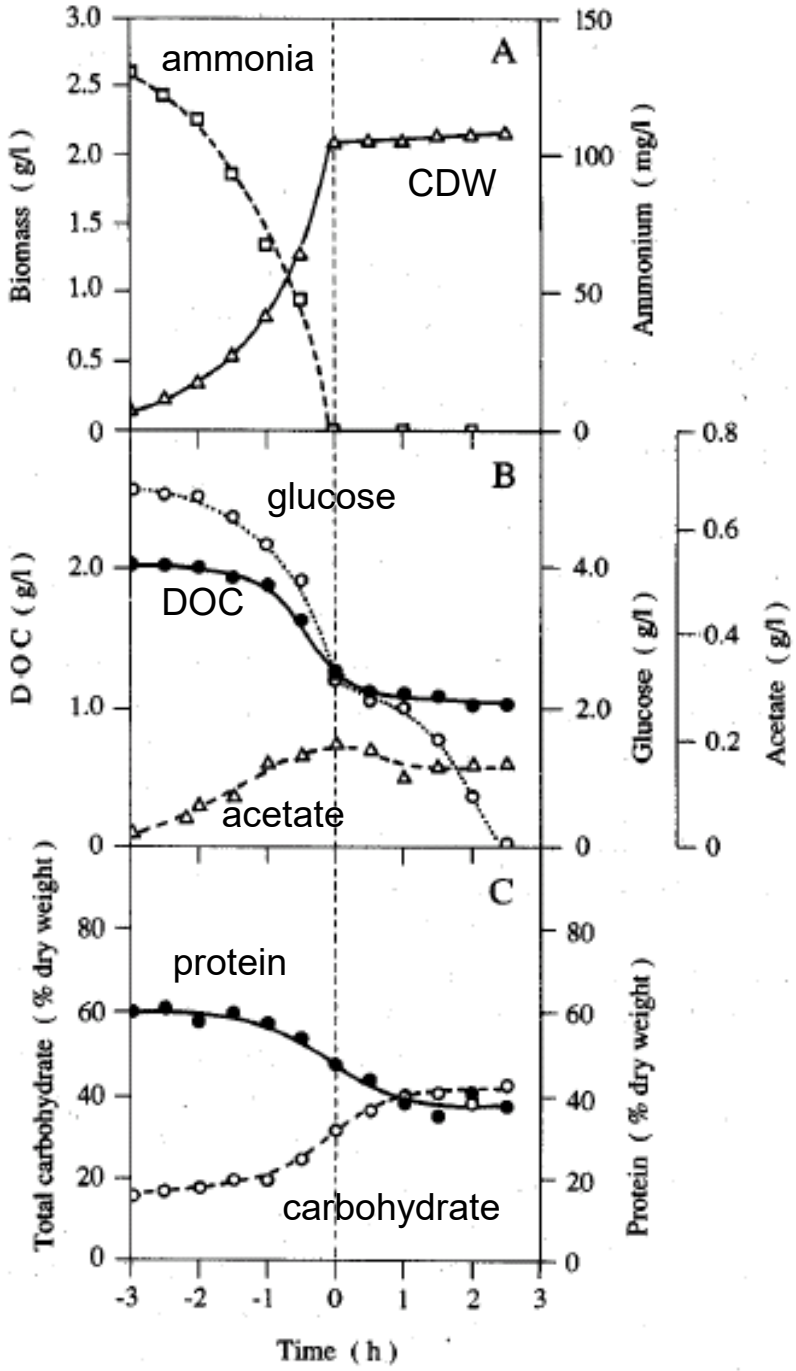
Nitrogen (ammonia)-limited batch culture

Comments:

- Acetate is produced
- All glucose is consumed and the culture is N- plus glucose-limited (residual carbon?)
- DOC indicates that a lot of carbon remains in the medium as products that are only slowly utilized (or not at all?).
- Cell composition changes slightly before limitation is reached

Batch growth of *Klebsiella pneumoniae* in synthetic medium with phosphate as limiting substrate.

(from Wanner & Egli, 1990)



Cell rearrangements in N-limited batch culture

Biomass:	Increases usually, dependent on organism from 10% to 2-300%
Cell number:	Increases also (not only due to finishing DNA replication rounds), up to 4-fold increase observed (*S.typh*.)
Cell volume:	No clear information (should stay constant or even increase)
Products:	Excretion of metabolites observed (depends on C-source)
Reserve materials:	PHB, lipids, glycogen accumulate, sometimes also polyphosphate
Cell wall:	Exopopolysaccharides are formed (slimes, capsules)
DNA:	No clear information
RNA:	RNA degradation was reported but much less than in C-lim. cultures.
Protein:	Observed reduction of protein content results from dilution by incorporated storage compounds.
	Protein turnover increases to 3-5%/h, but no net degradation
	Expression of enzymes of the N-regulon (30-50 new proteins), including proteases, high affinity amino acid transporter and assimilation systems (GS/GOGAT).
Remarks:	Many experiments in literature probably affected also by run out of carbon source (N and C-limitation at the same time).

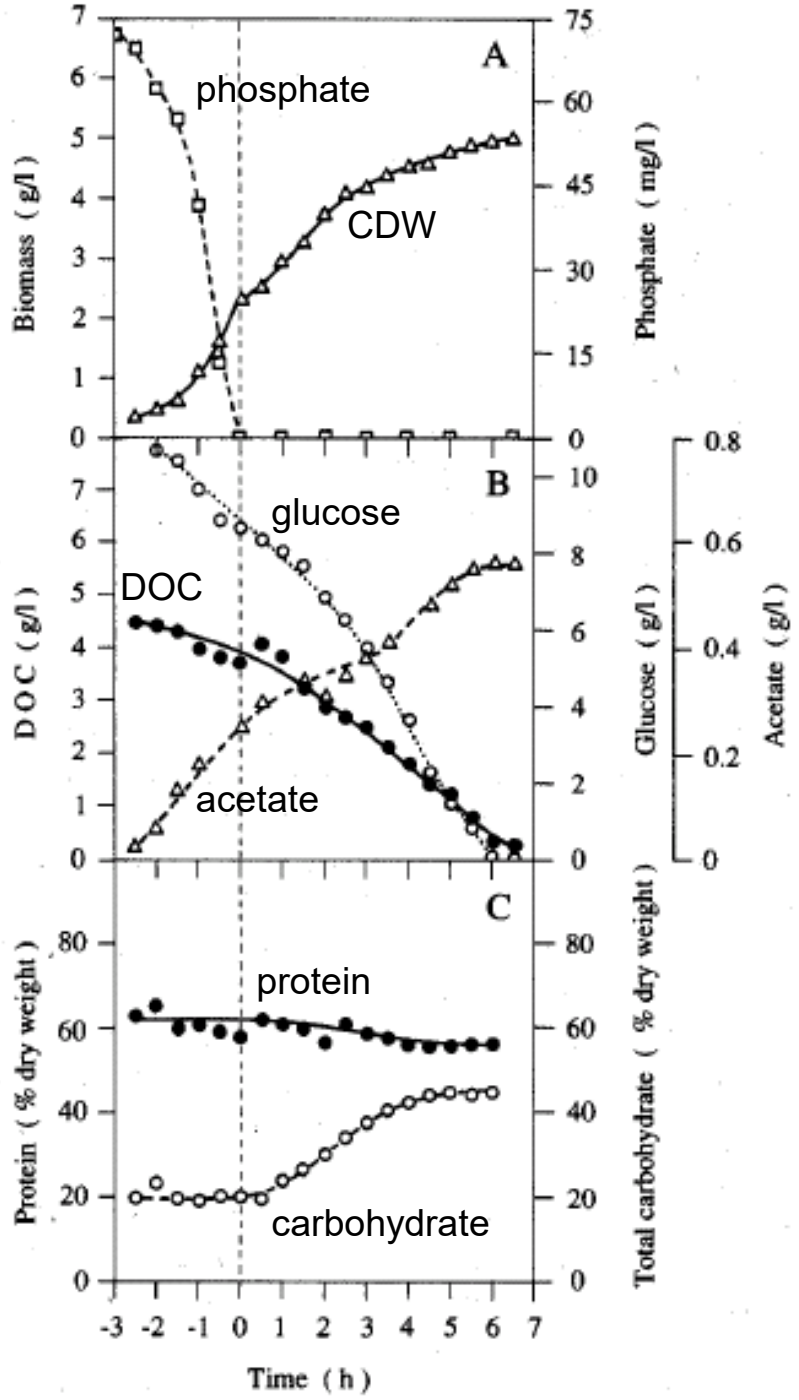
Phosphorus (phosphate)-limited batch culture

Comments:

- Growth continues after P run out; acetate production as well.
- All glucose is consumed and the culture is P- plus glucose-limited (residual carbon?)
- DOC indicates that acetate is probably the main excretion product
- Carbohydrate content changes, why not protein (mistake, interference)?

Batch growth of *Klebsiella pneumoniae* in synthetic medium with phosphate as limiting substrate.

(from Wanner & Egli, 1990)



Cell rearrangements in P-limited batch culture

Biomass:	Increases up to 6-fold (in *K. pneumoniae*).
Cell number:	Increases correspondingly to biomass.
Cell volume:	No data found (should become smaller due to decrease in μ).
Products:	Metabolites excreted, in *K. pneumoniae* primarily acetate.
Reserve materials:	Differing information in literature, probably because the onset of incorporation is sometimes retarded.
Cell wall:	Interestingly, in Gram-positives, replacement of P-containing teichonic acids by P-free components.
	Also, some phospholipids in membrane replaced by P-free analogues.
RNA:	Pi pool immediately reduced to minimum.
	When PPi present this is used for RNA and DNA synthesis.
	After all external P-sources are used up, degradation of RNA starts (10-25% /h); 60% of RNA-^{32}P reappears in DNA.
DNA:	Synthesis continues, P from all available sources appears in DNA.
Protein:	Expression of P-regulon, 20-30 major proteins including high affinity phosphate transporters, phosphatases, porins.
Remark:	Many experiments in literature probably affected also by run out of carbon source (P and C-limitation at the same time).

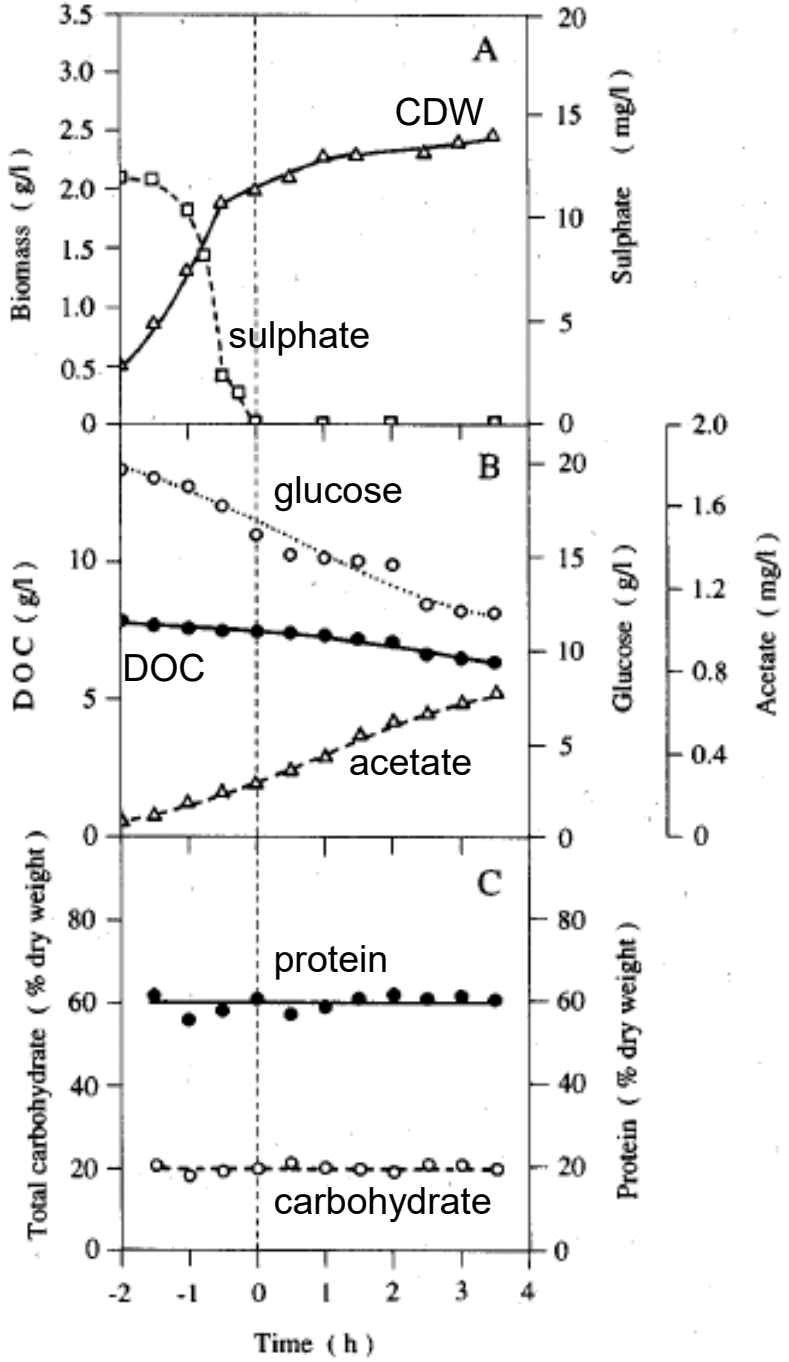
Sulfur (sulphate)-limited batch culture

Comments:

- Growth continues after S run out; acetate production also
- Glucose consumption continues as does acetate production
- DOC indicates that acetate is the major product
- Protein and carbohydrate content remains stable

Batch growth of *Klebsiella pneumoniae* in synthetic medium with phosphate as limiting substrate.

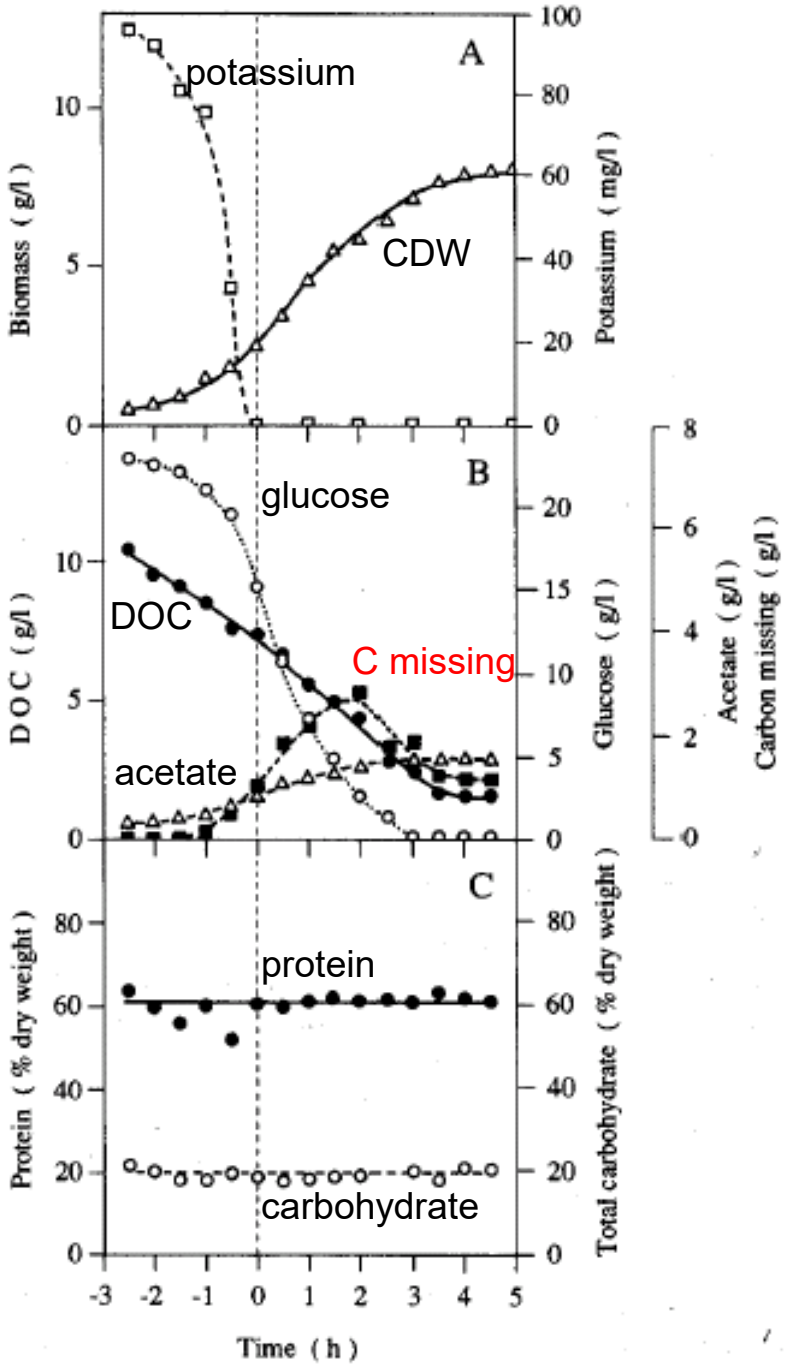
(from Wanner & Egli, 1990)



Potassium-limited batch culture

Comments:

- Growth continues after K^+ run out; acetate production also
- All glucose is consumed and the culture becomes K^+- plus glucose-limited (residual carbon?)
- DOC indicates that not only acetate is excreted, much carbon missing
- Protein and carbohydrate content remains stable



Batch growth of *Klebsiella pneumoniae* in synthetic medium with phosphate as limiting substrate.

(from Wanner & Egli, 1990)

Summary of effects

Changes in parameters of batch culture cells observed within the first few hours after depletion of various nutrients: increase (+), decrease (−), no change (0), no data available in literature (?)

Changes observed after substrate exhaustion	Substrate limiting batch growth:				
	Carbon	Nitrogen	Phosphorus	Sulfur	Potassium
Biomass	0	+	++	+	++
Cell size	−	?	?	?	?
Cell number	+	+	++	+	++
Metabolites	−	++	+	++	++
Carbohydrates	0	++	+	+	0
Polyphosphate	0	+	−	++	0
DNA	+	?	?	?	?
RNA	−	−	−	(0)	?
Protein	0	−	0/−	0	0
ATP pool	−	0/+	?	?	?

(from Wanner & Egli, 1990)

Oxygen requirements

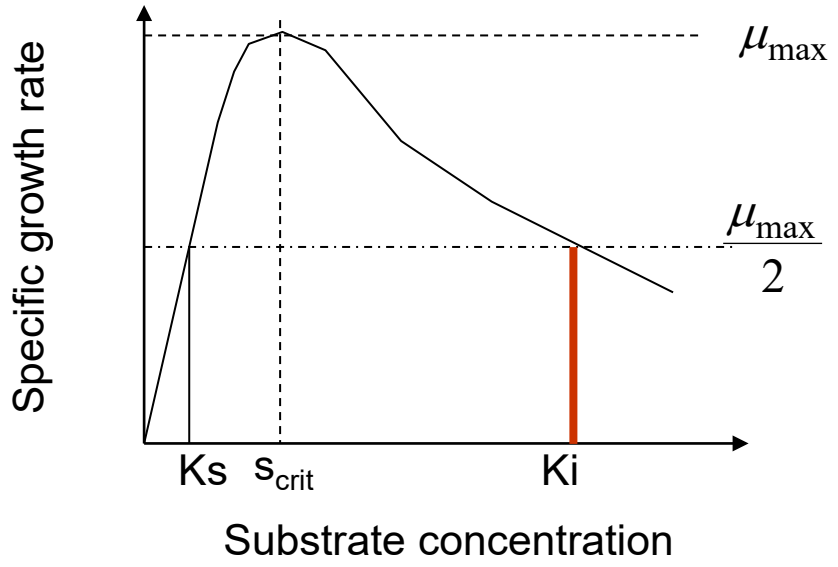
Organism	Substrate	Maximum observed yield [g g^{-1}]		
		$Y_{X/S}$	$Y_{X/C}$	O_2 used
Candida utilis	glucose	0.51	1.28	1.3
Aerobacter cloacae	glucose	0.44	1.1	1.03
Candida utilis	acetic acid	0.36	0.9	0.62
Candida utilis	ethanol	0.68	1.3	0.58
Pseudomonas sp.	methanol	0.41	1.09	0.44
Methylococcus sp.	methane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

Too much of the good: Growth inhibition by substrates

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 $s<s_{crit}$.



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

K_i is usually in the range of 10x K_s

Take home messages

Growth in a closed system (batch) consists generally of two phases:

- **1) unlimited growth phase** (not dependent on “limiting” nutrient as all nutrients present in excess; exception non-covalently bound nutrients)
- **2) phase of limitation**, one nutrient has run out in the medium, redistribution of this nutrient determines the physiology

- **classic (text book) stationary phase** only observed for limitation by carbon/energy source
- **pattern of biomass in limitation phase** depends on lacking nutrient and the ability to get access to and redistribute this nutrient (usually an element):

  usually clear-cut distribution pattern for covalently bound nutrients and fuzzy onset of limitation for non-covalently bound nutrients.
- **availability of (carbon)energy source** (external, internal) is essential for redistribution, adaptation, survival process
- **later phase of limitation** in most experiments affected by multiple limitation (i.e., mostly cultures become limited by carbon/energy source.
- **growth inhibition** can be caused by too high concentrations of a substrate.

7. Optimization of production

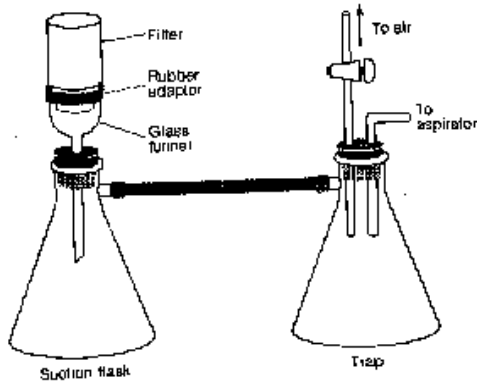
Quantify biomass production

Cell dry weight (CDW):

$$\text{Cell dry weight x [g L}^{-1}\text{]} = \frac{\text{(wt. of dried filter + cells) [g] – wt. of filter [g]} * 10^3}{\text{sample volume [mL]}}$$

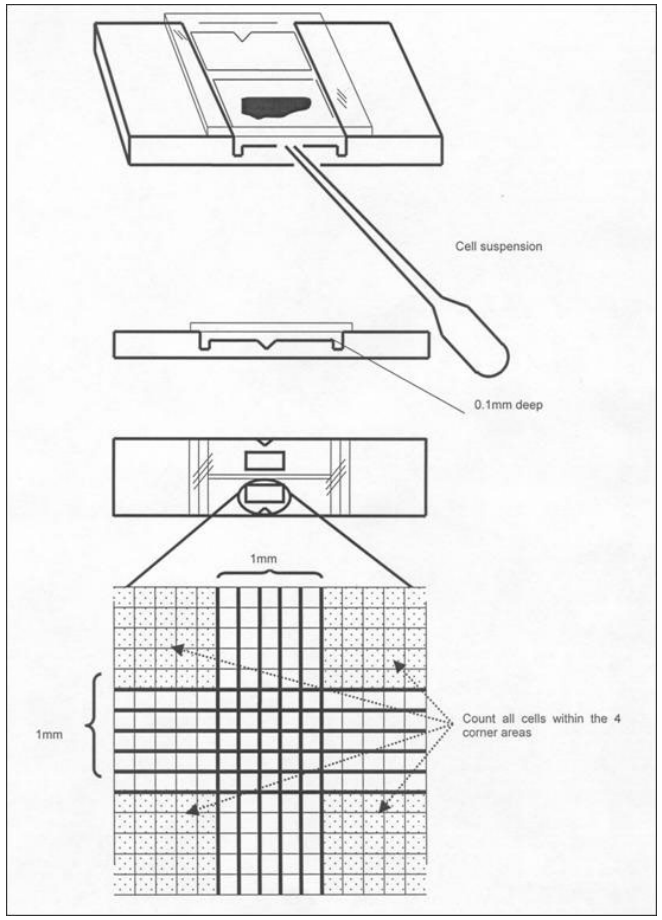
- Use triplicates to improve statistics.
- Wash loaded filters with ca. 10 mL of 10mM $MgSO_4$ or 0.9% NaCl solution.
- Dry loaded filters at 95°C for 20 hours and cool down in a desiccator.

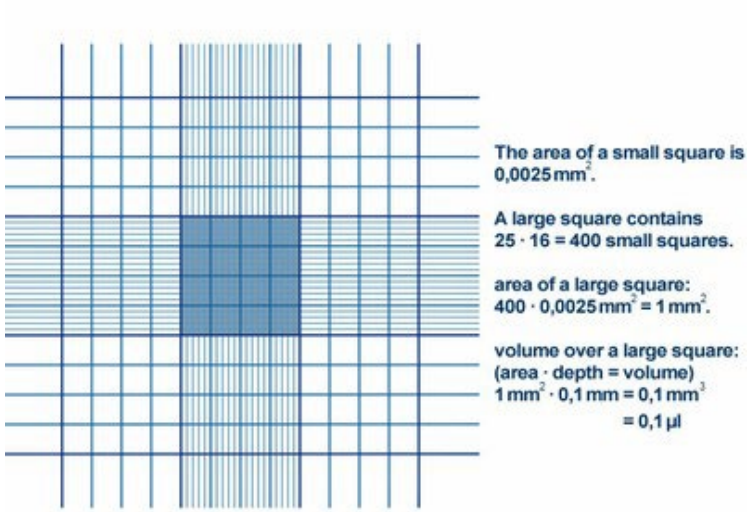
- Alternatively also preweighed and dried Eppendorf or glass tubes can be used.

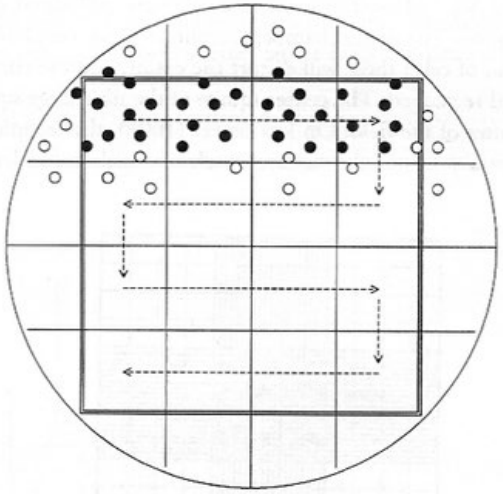


Measurement of cell numbers

Cell number determination using microscope

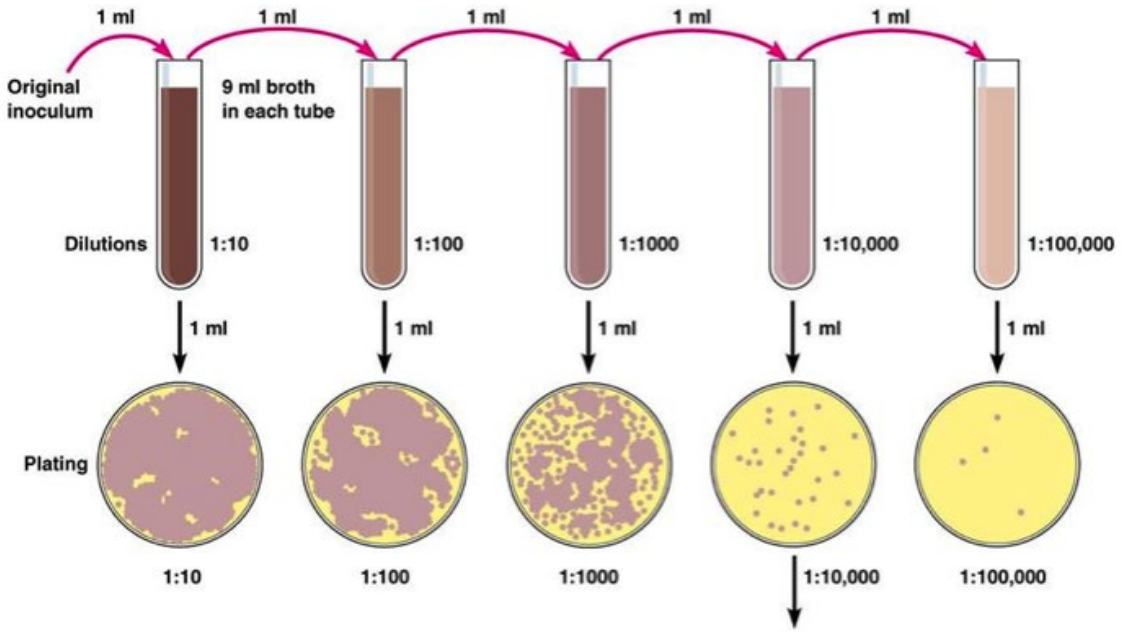






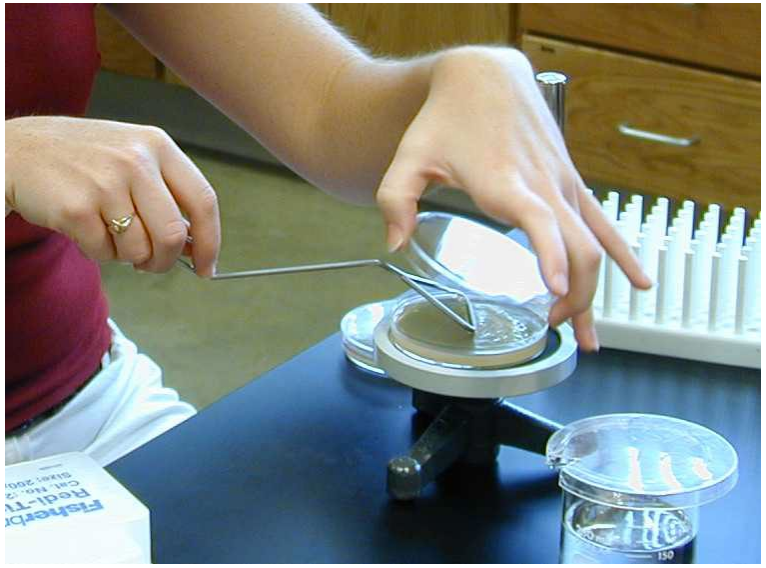
Technique of making total cell counts. The arrows indicate the direction of microscope movement. Filled circles represent cells counted in the first four squares; open circles are cells not counted with the first row.

Cell number determination using plate counts (CFUs)



Calculation: Number of colonies on plate × reciprocal of dilution of sample = number of bacteria/ml
(For example, if 32 colonies are on a plate of $^{1}/_{10,000}$ dilution, then the count is 32 × 10,000 = 320,000 bacteria/ml in sample.)

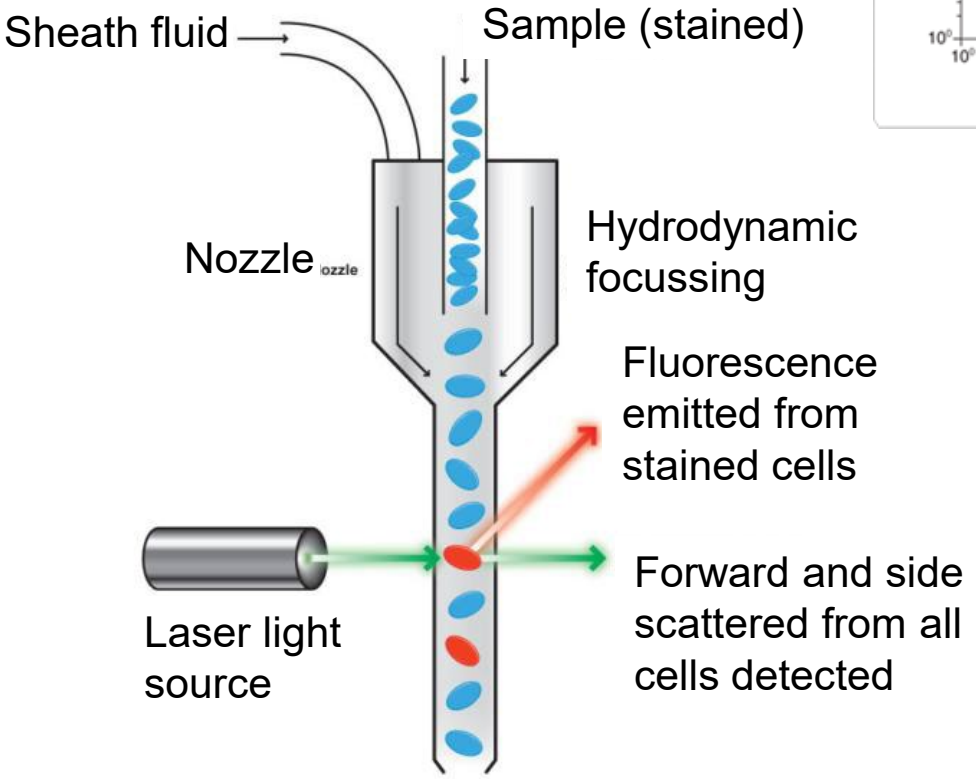
Figure 6.15

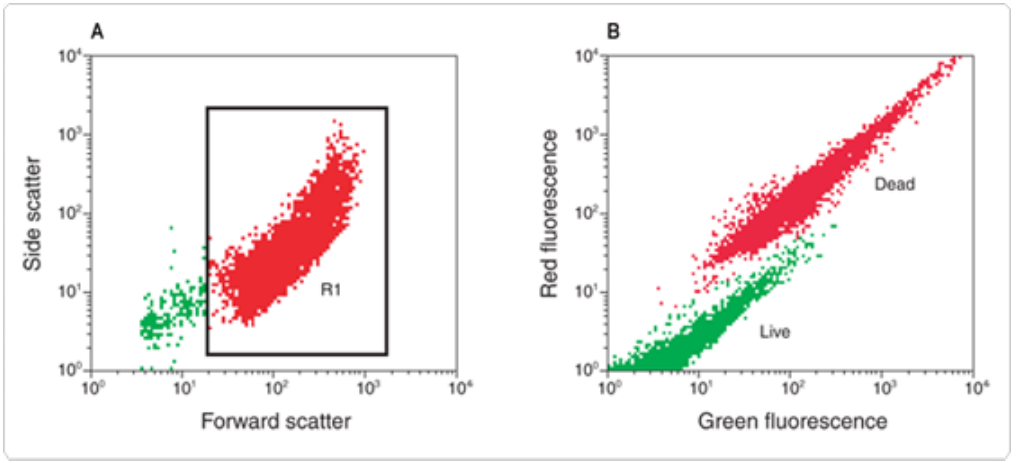


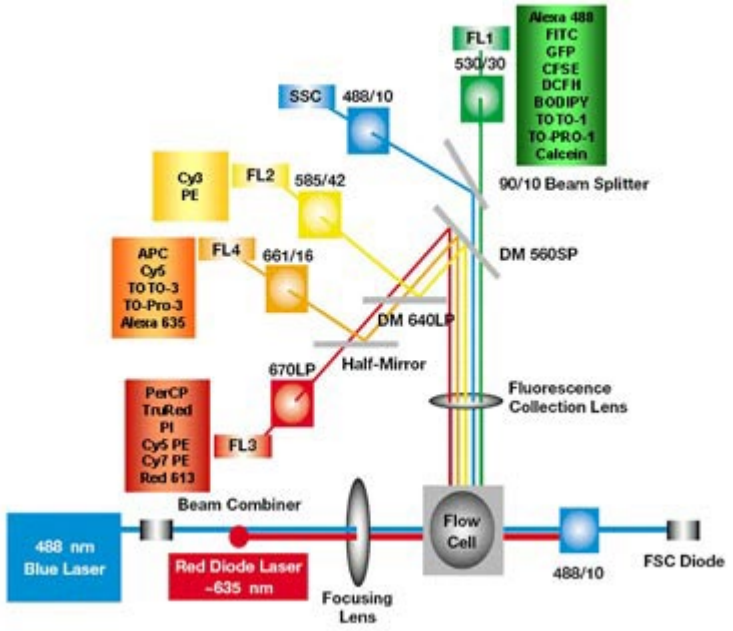
Note:

- Even distribution of cell emulsion is required (see above).
- Optimal volume for aliquots: 150 – 200 μL (not 1 mL as indicated in the figure on the left).
- Each cell forms a colony.
- Optimal number of colonies: ca. 200 per agar plate.
- At least 24 h of incubation needed.

Flow cytometry for quantification and characterization of cells







http://www.invitrogen.com/ http://withfriendship.com/user/mithunss/flow-cytometry.php

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 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.

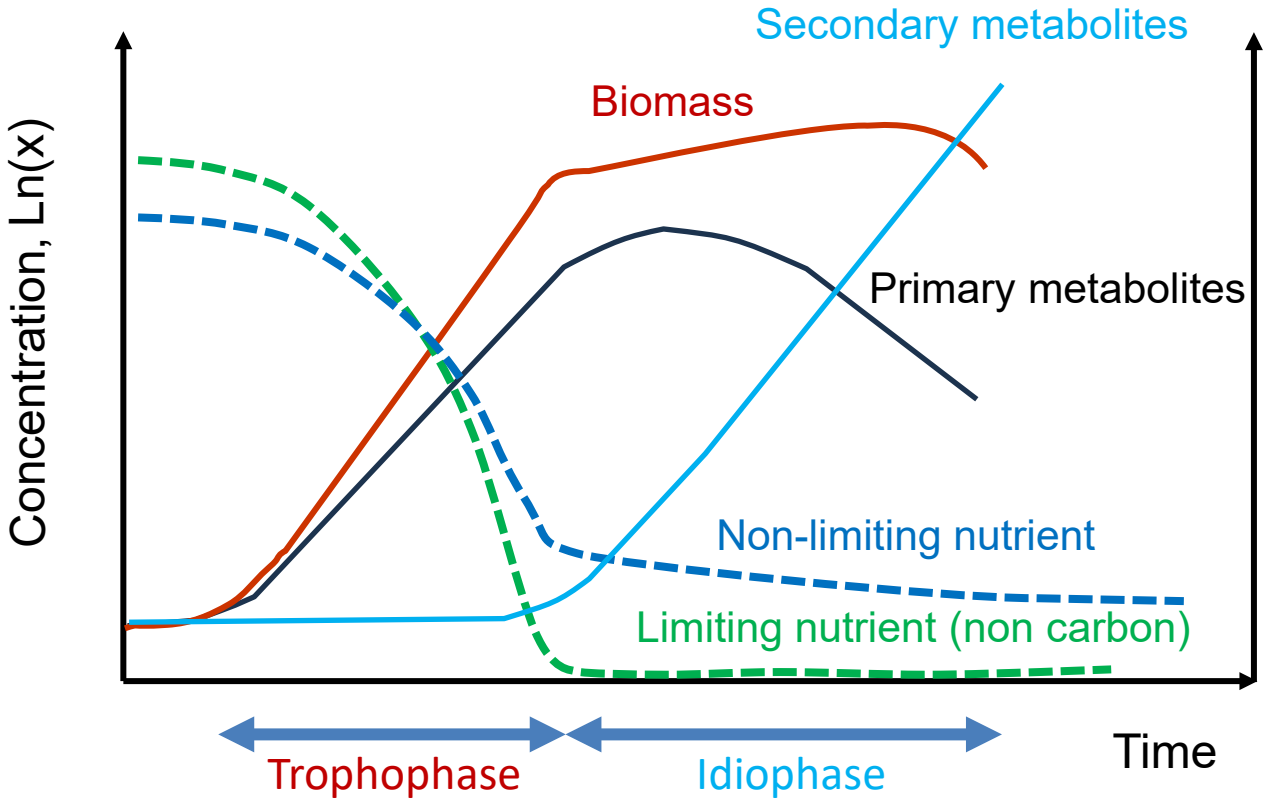
Improvement of cells

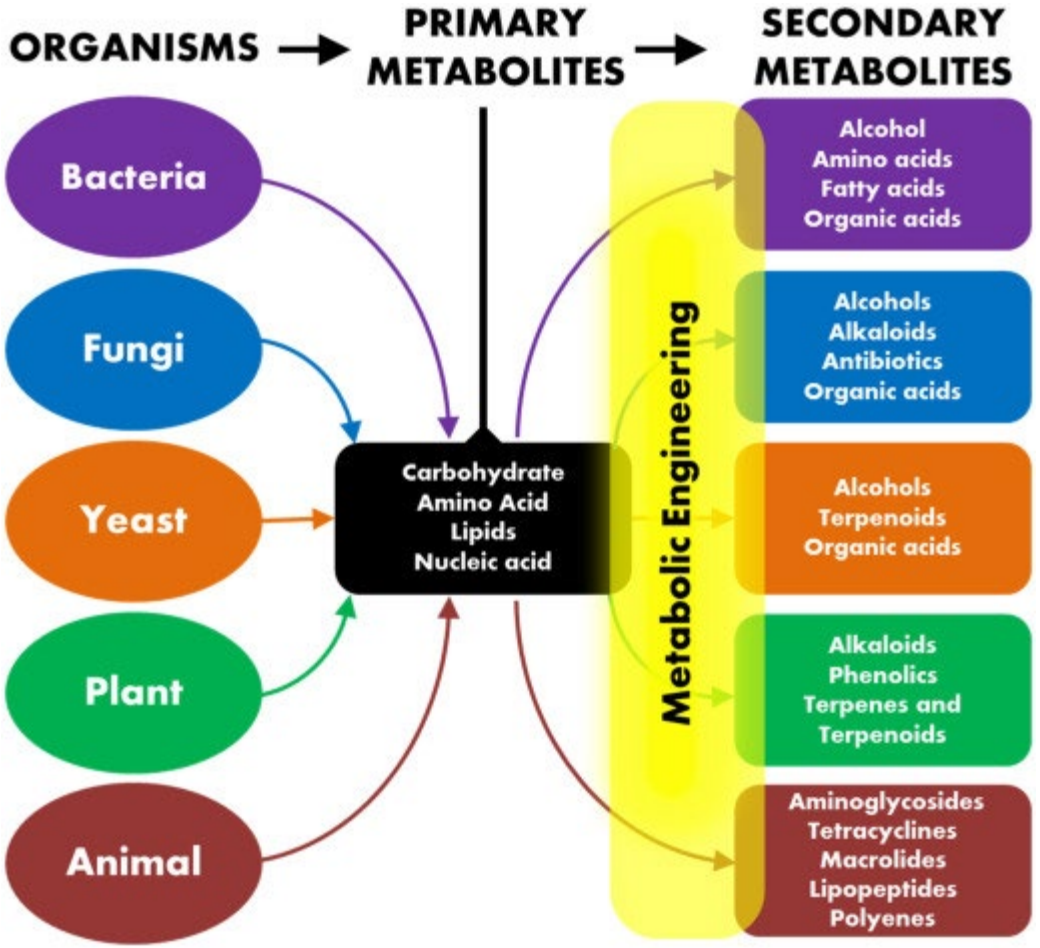
There is a possibility to increase the productivity by genetical engineering. The objectives are:

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

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

Metabolites formed during batch cultivation





Patel, A., N. Patel, A. Ali and H. Alim (2023). Chapter 4 - Metabolic engineering of plant primary–secondary metabolism interface. Genomics, Transcriptomics, Proteomics and Metabolomics of Crop Plants. A. Husen and A. Ahmad, Academic Press**: 69-87.**

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.

Optimization of growth and product formation

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Methods for Intense Aeration, Growth, Storage, and Replication of Bacterial Strains in Microtiter Plates

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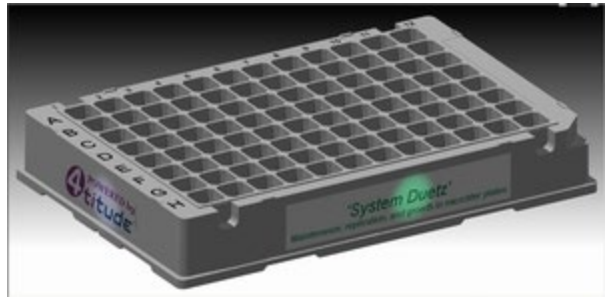
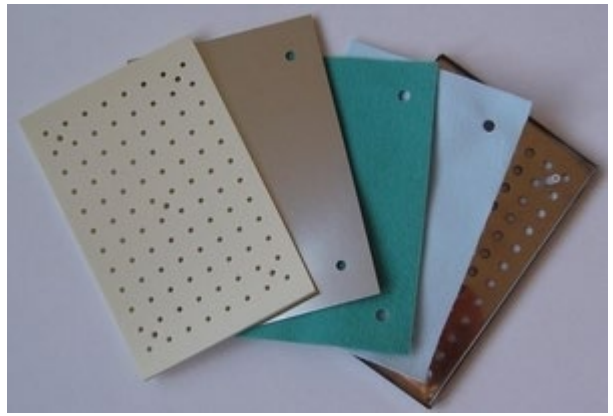
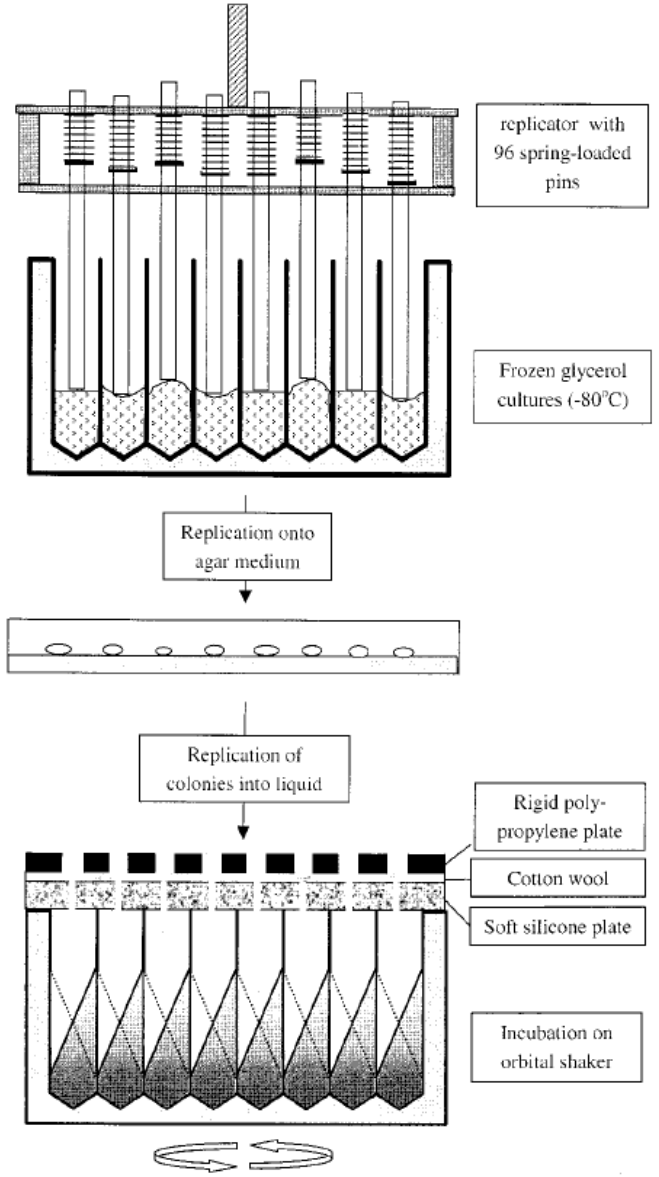
Miniaturized growth systems for heterogeneous culture collections are not only attractive in reducing demands for incubation space and medium but also in making the parallel handling of large numbers of strains more practicable. We report here on the optimization of oxygen transfer rates in deep-well microtiter plates and the development of a replication system allowing the simultaneous and reproducible sampling of 96 frozen glycerol stock cultures while the remaining culture volume remains frozen. Oxygen transfer rates were derived from growth curves of *Pseudomonas putida* and from rates of oxygen disappearance due to the cobalt-catalyzed oxidation of sulfite. Maximum oxygen transfer rates (38 mmol liter^{-1} h^{-1}, corresponding to a mass transfer coefficient of 188 h^{-1}) were measured during orbital shaking at 300 rpm at a shaking diameter of 5 cm and a culture volume of 0.5 ml. A shaking diameter of 2.5 cm resulted in threefold-lower values. These high oxygen transfer rates allowed *P. putida* to reach a cell density of approximately 9 g (dry weight) liter^{-1} during growth on a glucose mineral medium at culture volumes of up to 1 ml. The growth-and-replication system was evaluated for a culture collection consisting of aerobic strains, mainly from the genera *Pseudomonas*, *Rhodococcus*, and *Alcaligenes*, using mineral media and rich media. Cross-contamination and excessive evaporation during vigorous aeration were adequately prevented by the use of a sandwich cover of spongy silicone and cotton wool on top of the microtiter plates.

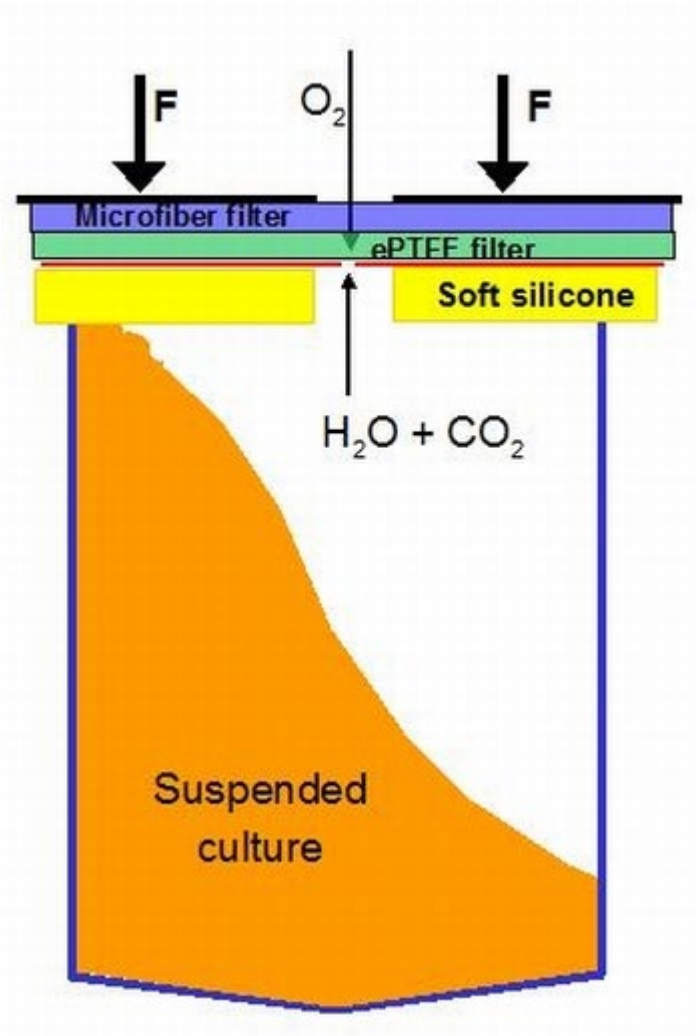
Establishment of a screening system using stock cultures





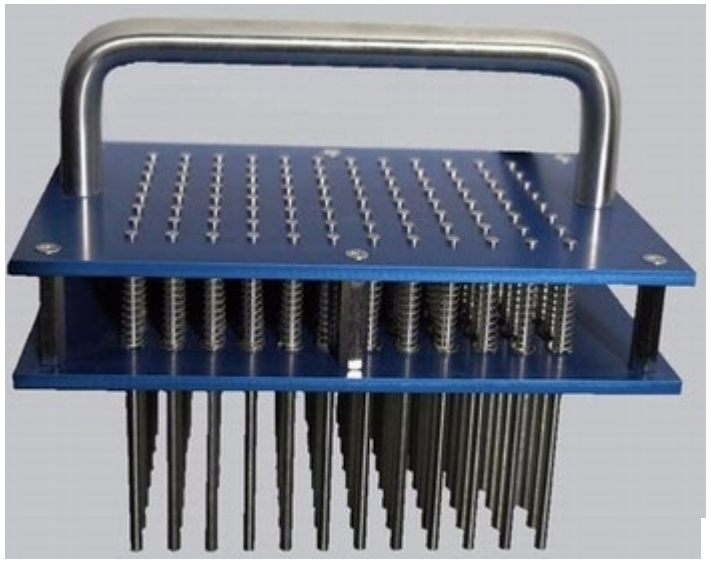


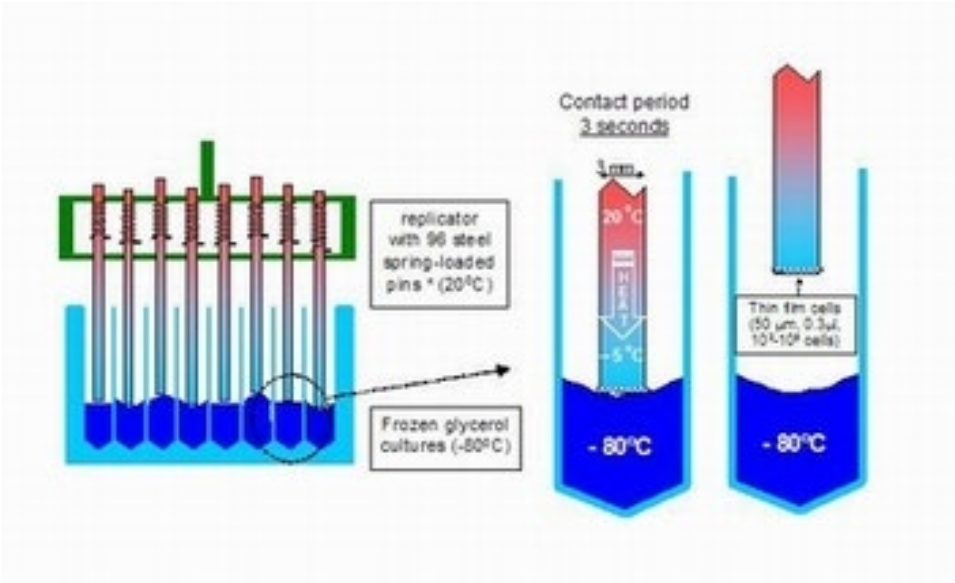
Oxygen transfer is important!



A: a stainless steel lid for rigidity
B: a microfiber filter (blue)
C: an ePTFE filter (0.3 micron) laminated between two polyester/polyamide fabrics (green)
D: a stainless steel foil with pinholes to control the headspace refreshment rate and evaporation (only in covers for 96-lowwell plates)
E: a silicone layer with 96 holes (to hermetically seal the "mini-reactors")

Establish your own strain collection…

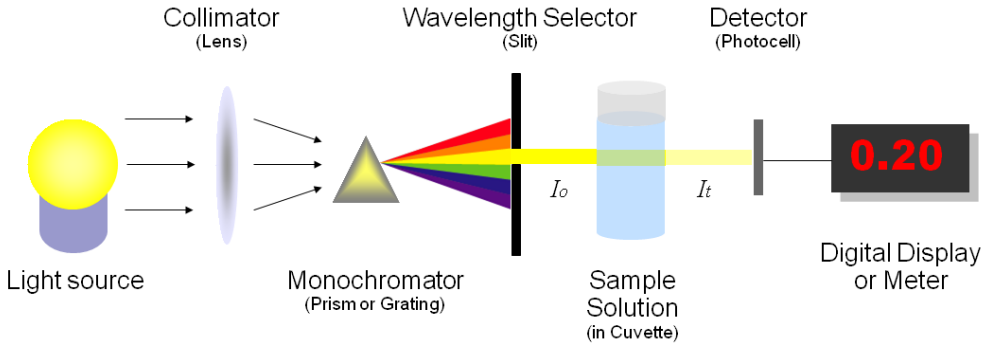




The different strains are stored in 96 well plates at 80°C. Culture plates are inoculated by pins pressed into frozen stocks.

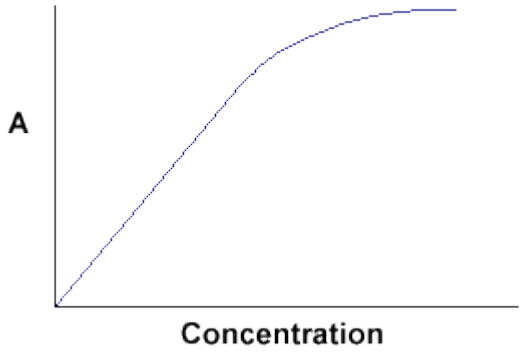
Direct measurement of microbial growth

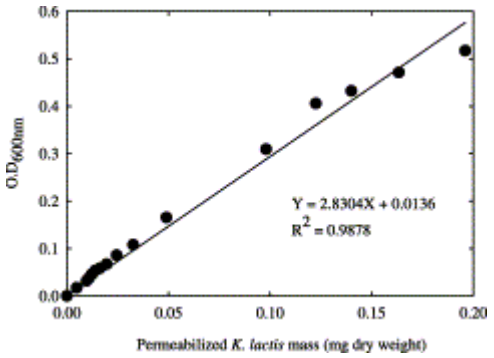
Optical density



Beert Lambert law:

$$A = \log_{10}\left(\frac{I_O}{I}\right) = \log_{10}\left(\%T^{-1}\right) = \varepsilon\, l\, c$$



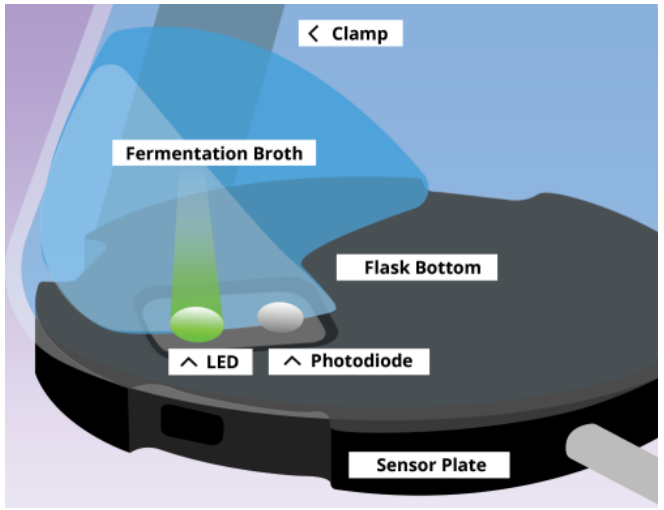


In situ measurement

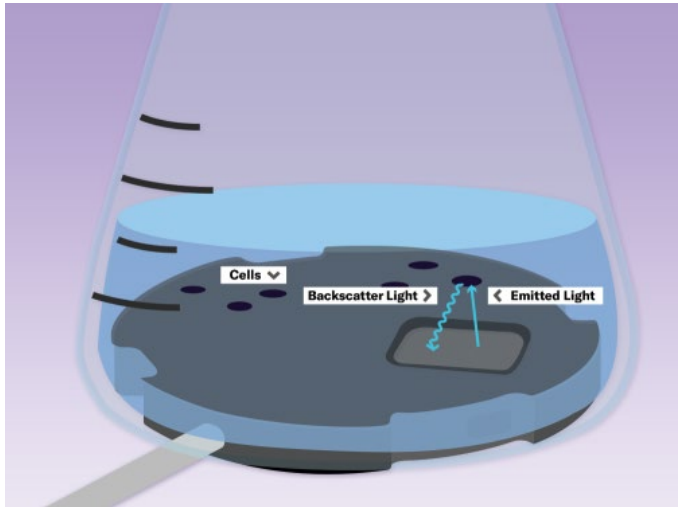
http://www.optek.com/index.asp

Cell growth quantifier (CGQ)

The sensor plate of the CGQ contains an LED light source and a photodiode detector. The CGQ's innovative measuring method treats the signal fluctuations from the moving bulk liquid due to shaking as a valuable information source rather than noise.



Direct monitoring of biomass formed in shake flasks.



Light is emitted from the LED into the culture medium. While most photons go straight through the broth, some are scattered by the cells and return to the photodiode, which measures the scattered light intensity. The higher the cell density, the more light is scattered back to the photodiode.

https://www.infors-ht.com/en/blog/5-ways-to-increase-biomass-in-your-shake-flasks/

https://www.scientificbio.com/cgq/

Micro-24 Microreactor System (PALL)

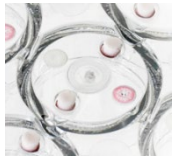


Figure A
Microbial Cassette: Principle of Operation

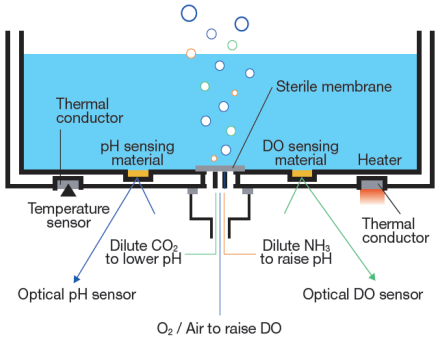
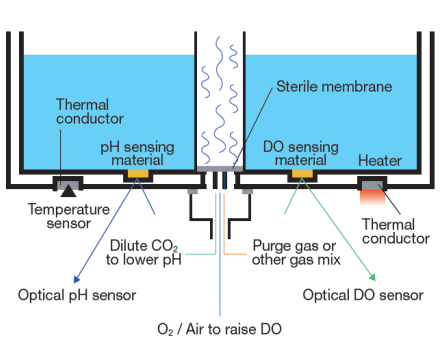
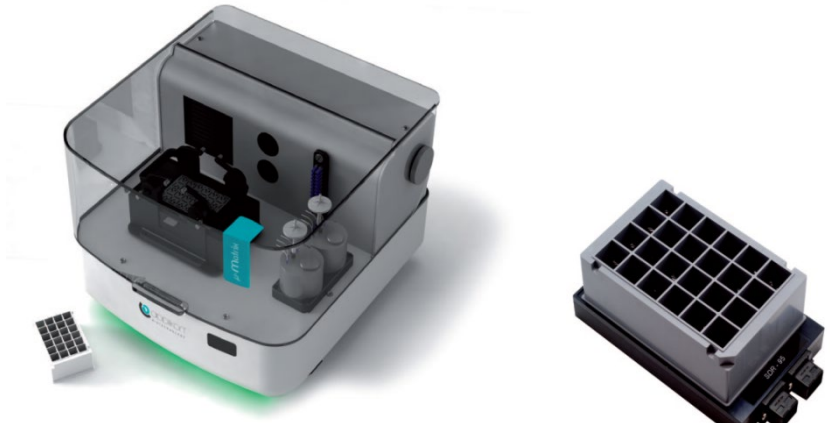


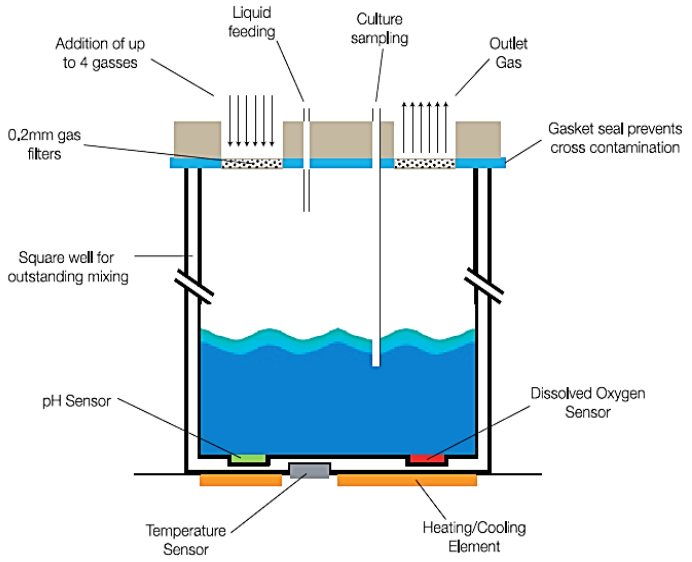
Figure B
Cell Culture Cassette: Principle of Operation



no of wells	24
volume	5 - 7 ml
mixing	shaking
temperature	online measure/control
pH	online measure/control Low pH module
DO	online measure/control
gas addition	bottom
feed	**planned**
cell types	Bacteria, yeast, cells

micro-Matrix (applikon Biosystems)





Schematic diagram illustrating the functioning of the micro-Matrix control loops.

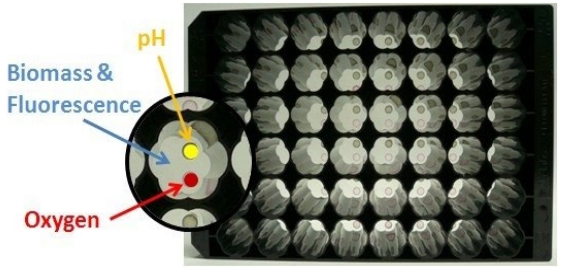
no of wells	24
volume	1 - 7 ml
mixing	shaking
temperature	online measure/control
pH	online measure/control
DO	online measure/control
gas addition	top
feed	yes
cell types	Bacteria, yeast, cells

https://www.vekamaf.cz/documents/52/Applikon%20micro-Matrix%20leaflet.pdf

BioLector pro (m2p labs)

Additional modules:

O_2 upregulation
O_2 downregulation
CO_2 upregulation



anaerobic growth

FRET module
LED module

no of wells	32
volume	0.8 – 2 ml
mixing	shaking
temperature	online measure/control
pH	online measure/control
DO	online measure/control*
OD	online measure
Fluorescence	online measure
gas addition	?
feed	yes
cell types	Bacteria, yeast, cells?

*additional modules

ambr (TAP Biosystems)

no of wells	24/48
volume	10-15 ml
mixing	stirring
lid	no
Automated handling	yes
temperature	no?
pH	online measure/control
DO	online measure/control
gas addition	top
feed	yes
cell types	Bacteria?, yeast?, cells

Fully automated Ambr®250

Ambr® 250 High Throughput pCO_2 Vessel
The new vessel type incorporates a single-use pCO_2 sensor to monitor bioreactor conditions.

The baffled fed-batch vessel contains a new disposable pCO_2 sensor in addition to the standard single-use pH and DO sensors and dual pitched-blade impellers. The vessel configuration allows real-time measurement of bioreactor pCO_2 levels alongside existing bioreactor parameters.

The vessel can be used for:

- Process development and process optimization studies
- Comparison of pCO_2 profiles across bioreactor scales
- Investigation into cell line pCO_2 sensitivity (animal cell cultures)

https://www.sartorius.com/en/products/fermentation-bioreactors/ambr-multi-parallel-bioreactors/ambr-250-high-throughput

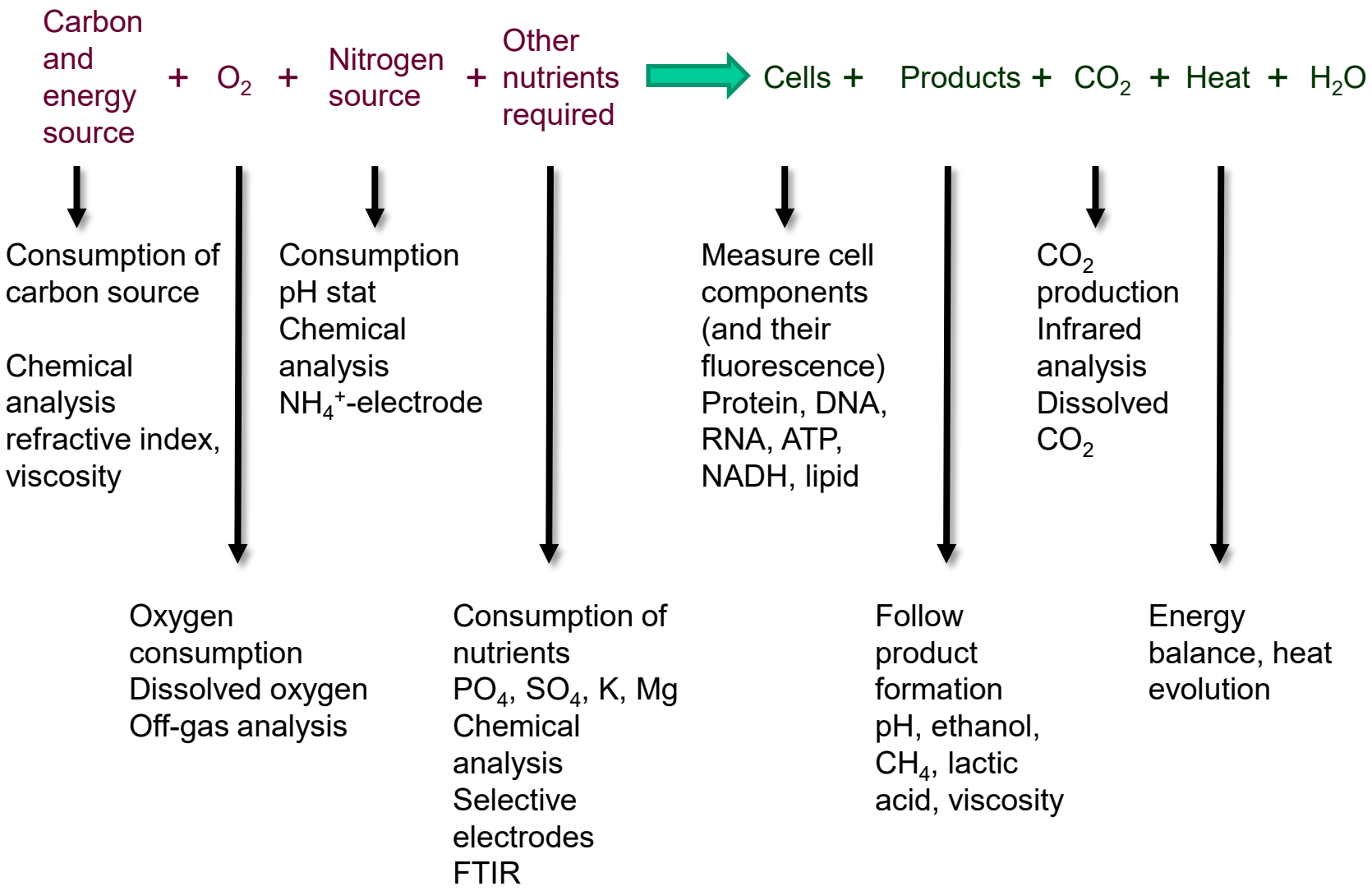
Are microbioreactors worth their money?

Micro 24:	130’000 CHF
plates	333 CHF/plate
per reaction	7-18 CHF

http://wirtschaftsblatt.at/home/nachrichten/oesterreich/burgenland/1558321/Geld-fur-Jungunternehmer-im-Burgenland

8. Process analytical technology

Indirect measurement of growth



The new initiative by FDA

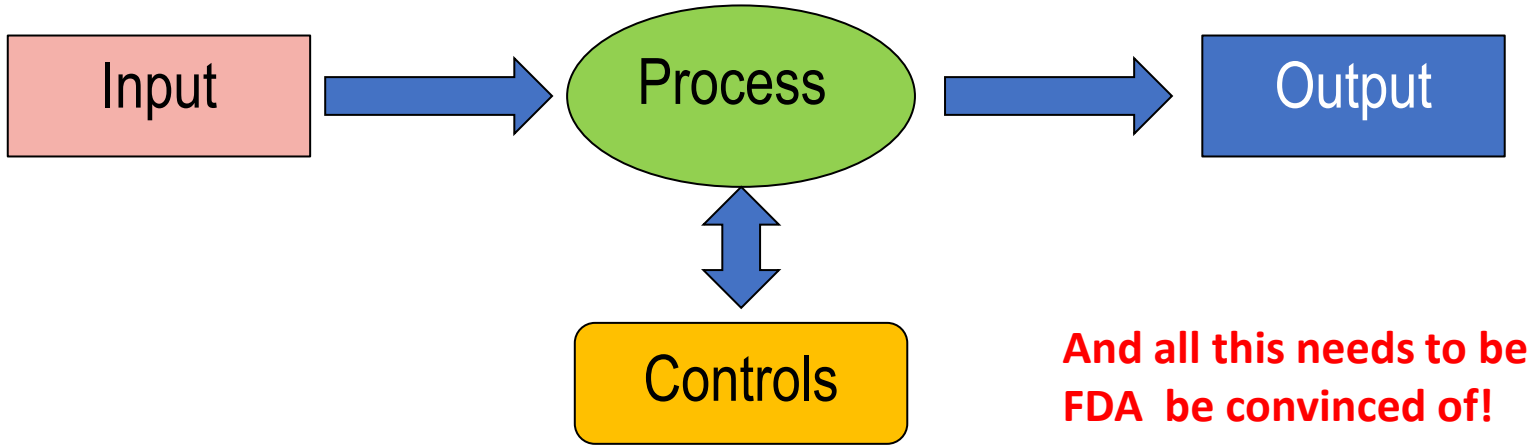
- FDA was viewed by many pharmaceutical companies as a **highly conservative agency**. Modifications of a process resulted in a huge amount of paper work and… binding ressources at FDA!

- Consequently FDA pushed a new concept called **Q**uality **b**y **D**esign (QbD) and was part of their « GMP in the 21st Century » initiative.

- FDA created a new visionnary approach where **product and process understanding** became key issues.

QbD principles

- **Process development** is the key: companies need to make sure that they have understood their process and own capabilities.
- Different starting conditions (e.g., source material) may have an influence on the process!



And all this needs to be documented and FDA be convinced of!

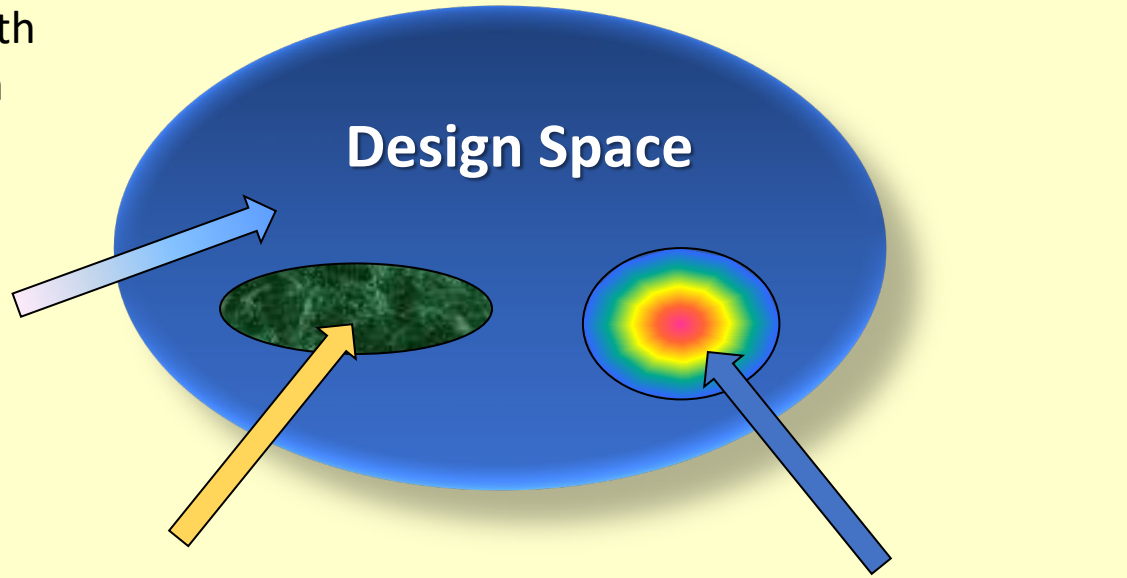
Advantages of doing a good job for FDA

- Successful convincing FDA of the production process may result in avoiding **preapproval inspections** (PAI).
- Further, a **design space** can be established to enable the company to freely act without regulatory oversight. This space will not stay static but is forever being refined. It is the area within which a company can operate with predictable outcomes to make acceptable products every time.
- Outside the design space regular change control is required.

Design space

Nondesign Space
(failure area; areas with regulatory submission required to operate)

Operation within design space using internal change control.

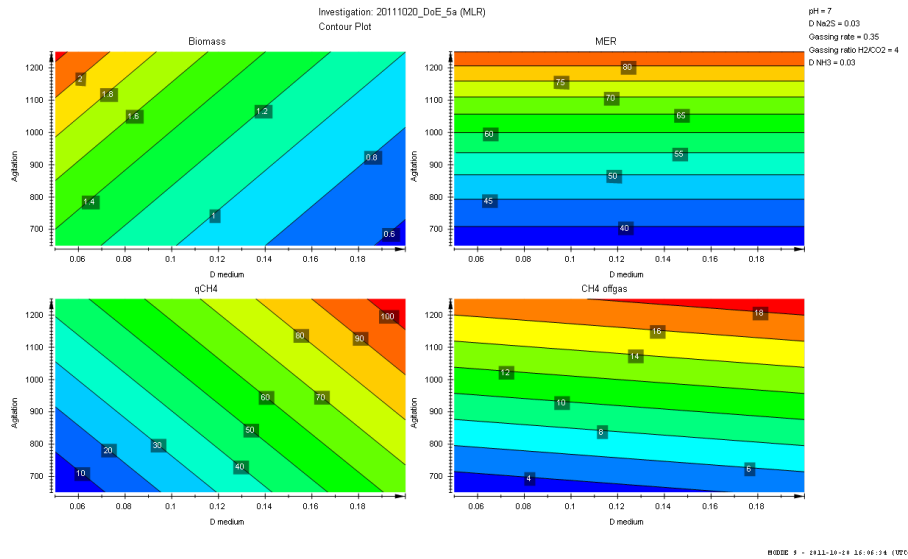


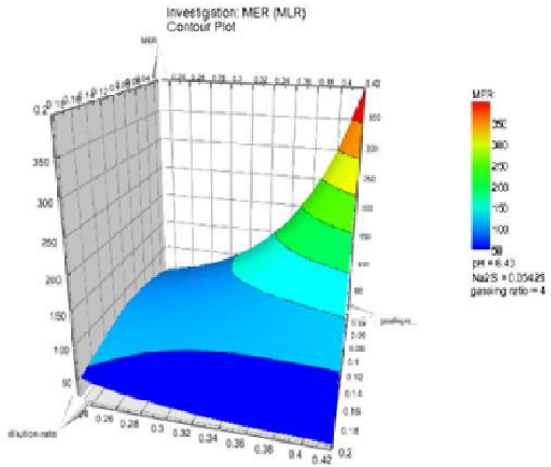
Alternative operation range
(requires no agency submission)

Normal operating range

Quality risk management

- Cost effective defining of the design space requires introduction of a **quality risk management** (QRM).
- Thousands of parameters can affect the design space. A suitable way is to use **directed efficient experimentation** (DoE).





Particular phylosophy of QbD

- Management must actively ensure that quality sytems are incorporated effectively into operations and foster **continuous improvement** for operations, products, and processes.
- **Change control** becomes a cornerstone. Each and every change contemplated should move a product closer to customer requirements.
- Nothing remains constant and everyone actively pursues continuous improvement, which becomes the driver for **increasing knowledge** after a product, process, and desing space are developed.

A few abbreviations

API:	**Active pharmaceutical ingredient**
COG:	Cost of goods
COGM:	Costs of goods manufactured
CPP:	**Critical process parameters**
CQA:	**Critical quality attributes**
DAQ:	Data acquisition
DCS:	Distributed control system
DoE:	**Design of experiment**
FMEA:	Failure mode and effects analysis
IT:	Information technology
MES:	Manufacturing execution system
MSA :	Measurement system analysis
MSPC:	Multiple setpoint control
MTBF:	Mean - time between failures
PA :	Process analytics
PAI:	Preapproval inspection
PAT:	**Process analytical technology**
PoC:	Proof of concept
PUC:	Process understanding and control
PV:	Process validation
QA/QC:	Quality assurance/Quality control
QbD:	**Quality by design**
RTA:	Real - time assurance
RTM:	Real - time (process) monitoring
SPC:	Setpoint control

What is PAT?

PAT [is] a system for designing, analyzing, and controlling manufacturing through timely measurements (i.e., during processing) of critical quality and performance attributes of raw and in-process materials and processes, with the goal of ensuring final product quality.

-

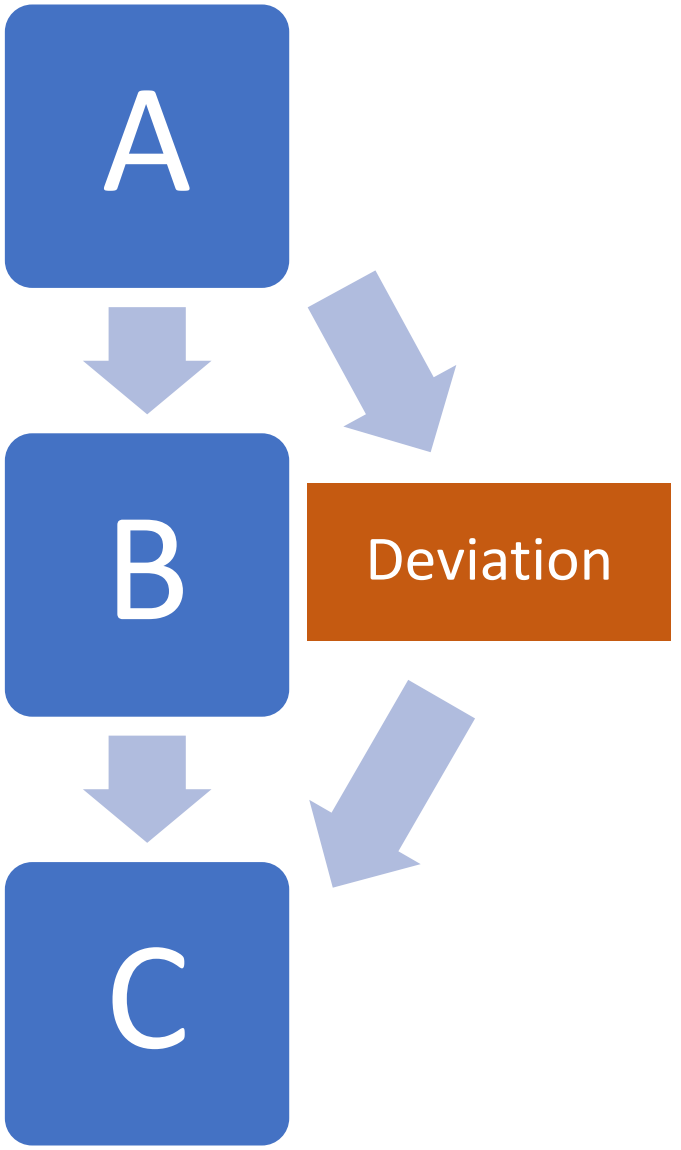
FDA, 2004

Typical motivations

- Quicker process development
  - **Sensors** help to remove the cycle experiment – off-line analytics – experiment
  - **Smart process** - easier establishment of an advanced control strategy
  - **Digital twin:** any digital solution which simulates processes needs information on what is currently going on.
- More economic processes
  - ✓Early detection of failures
  - ✓Working closer to optimal conditions
- Higher product quality through feed-back control

Improved root cause analysis

- Processes either run according to specifications (as defined in the relevant SOPs) or they deviate.
- Deviations are assessed by an expert, who tries to understand what was going wrong.
- Off-line analytics are often inappropriate for root cause investigations, since they typically provide only information at fixed times and fixed points in the process.
- PAT helps by at least providing continuous information (although the place of the information is still fixed)



Where does PAT make any sense?

- The analysis of **costs of goods manufactured** (COGM) and of the **failure mode and effects analysis** (FMEA) help to set priorities:

COGM	Real-time process analysis application
Receiving	Input material QC/QA (e.g.,) identification
Manufacturing	Process end points Process fault detection Quality attributes trending (SPC or MSPC) Process control (feedback or feed forward)
Product release	Determination of final product quality attributes
Plant operations	Real-time water quality Cleaning verification Waste stream monitoring and control
Environmental health and safety	Hazardous area monitoring Environmental monitoring & compliance

QA, quality attributes; SPC/MSPC, statistical process control/multivariate statistical process control; CPPs, critical process parameters.

Instrumentation in PAT

Process instrumentation types

- Process analyzers
  - Large instrumentations that carry out complex processes
  - Need an appropriate interface/sampling system
  - Price range: 50-350 kFr.
- Process sensors
  - Compact, lightweight, easier to operate
  - Most of supporting utilities are integrated
  - Everything is installed directly on the process equipment, e.g. mass flow meter
  - Price range <100 kFr.

Ruggedness of PAT equipment

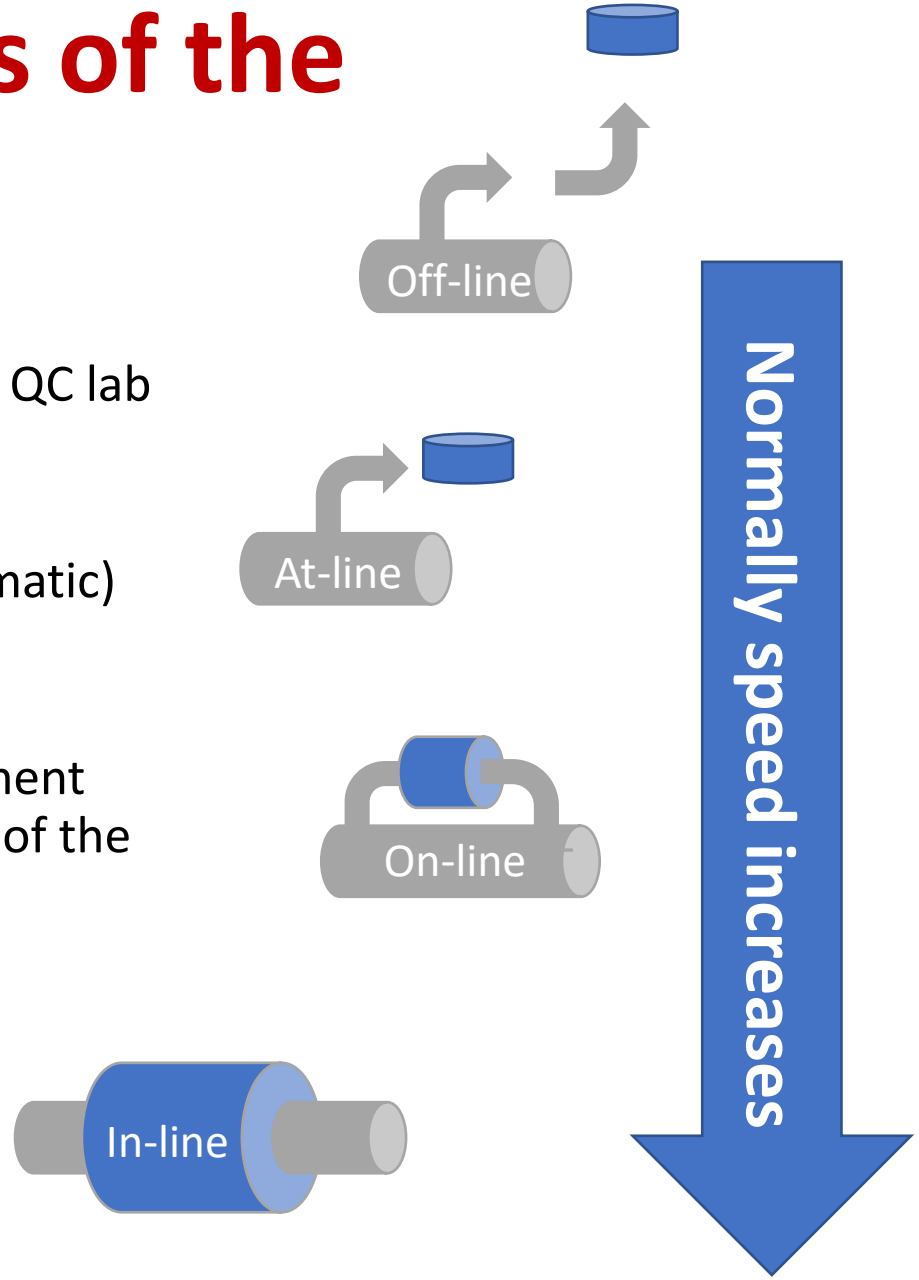
- PAT equipment in processes most be highly robust.
- One of the most important characteristics is the **mean time between failure** (MTBF); How often is the equipment expected to fail.
  - Typically, PAT equipment needs MTBF $\geq$ 10’000 h = 415 d ideally rather 50000 h = 5.7 y.
  - Moving parts are one of the biggest sources of failure. Ideally no valves, pumps etc.
- The equipment should run generally without operator intervention. Ideally no added activity of the staff in routine operation.

Lab

Process

Four common denominations of the measurement location

1) **Off-line** (>30 min)
   - Measured away from the shop floor (production area) in a QC lab
2) **At-line** (<30 min)
   - Close to the processing line
   - Isolation of sample → sampling required (manual or automatic)
3) **On-line** (<10 min)
   - Measured in a bypass of the main production line
   - No isolation but direct injection into measurement equipment
   - Due to direct connection, the bypass is considered as part of the process from a regulatory point of view
4) **In-line** (<20 sec)
   - In the process line (e.g. bioreactor, piping)
   - In situ (e.g., pH, pO_2, redox, etc.)

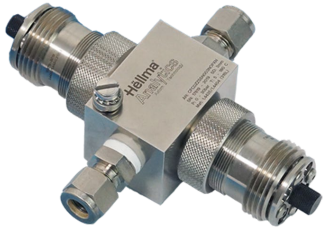


Probe vs. flow cell

- Probes are typically immersed into the process or measure through optical windows
  - Applicable to a wide variety of piping diameters and reactors
  - Port & adapter required by which the probe may be inserted
  - More common for production scale equipment
- Flow cells provide a dedicated piece of tubing for the measurements
  - Often used in laboratory scale equipment

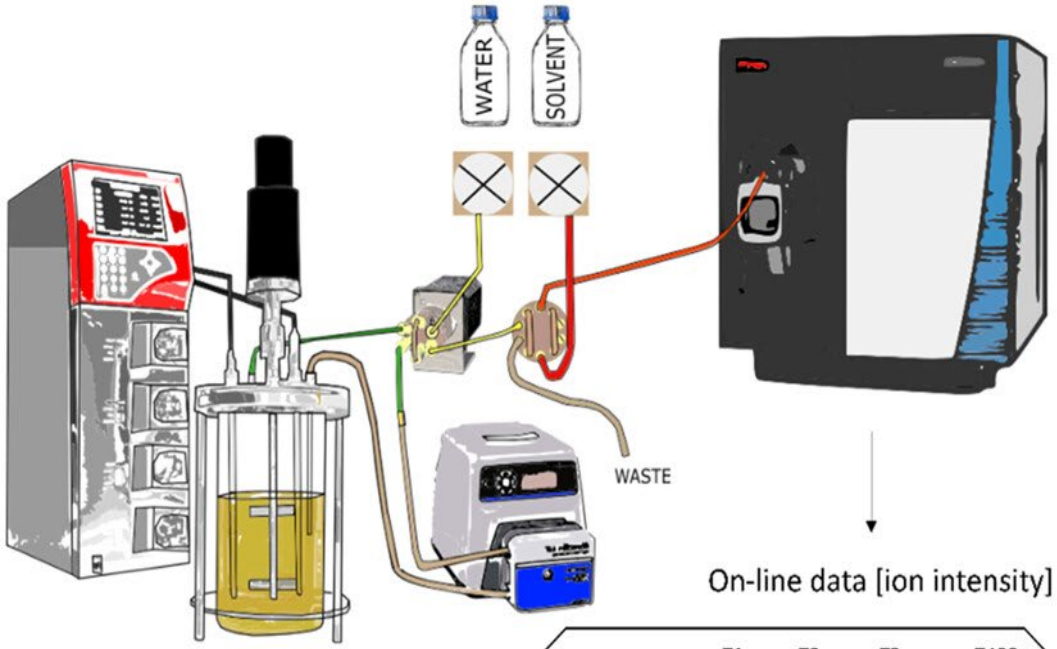
Figure 5.4 Collection of commercial Raman probes designed for different installations: (a) fifteen-foot immersion probe; (b) typical immersion probe, approximately one-foot long; and (c) probe body for a non-contact probe with fitting to accept different focal length optics. Adapted, with permission, Copyright © 2004 Kaiser Optical Systems, Inc.

UV flow cell



pH electrode and flow cell

Combination of off-line and online data



On-line data [ion intensity]

	T1	T2	T3	...	T400
Glucose	39657	40389	41107	...	5191
L-Alanine	9550	9076	8608	...	44401
L-Valine	3782	3511	3380	...	54568
S-Malate	0	0	0	...	18054
...					

Off-line data [ion intensity]

	T1	T2	T3	...	T15
Glucose	55243	47954	46724	...	23428
L-Alanine	0	0	0	...	267745
L-Valine	0	0	0	...	138721
S-Malate	0	0	0	...	124487
...					

1. Fermentation process – triplicate

Off-line samples taken every 1 -2 h
On-line mass spectrometry data acquired every 5 minutes

2. Off-line samples

~15 samples acquired in 33 h

2.1 Analysis by LC -MS

2.1.1 **Sample processing** : quenching, dilution
2.1.2 **LC-MS analysis** with calibration curve standards

2.2 Data preparation

2.2.1 Peak integration in Xcalibur ™
2.2.2 Missing value imputation (1/2 min value)
2.2.3 Build **linear calibration curves** with standards
2.2.4 **Quantify** off-line samples (off -line MS → g/L)

3. On-line samples

~400 samples acquired in 33 h

3.1 Analysis by MS

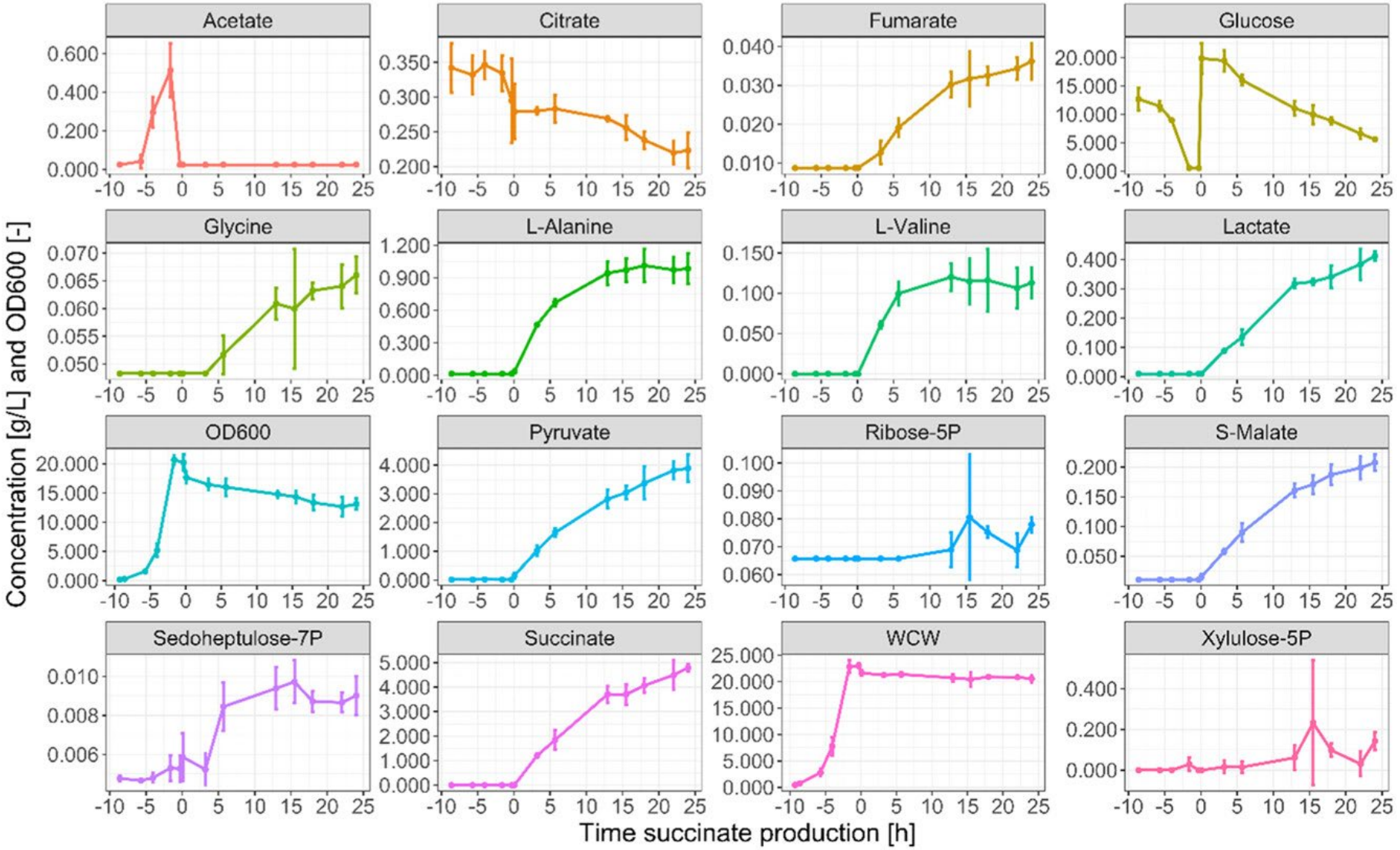
3.1.1 Peak integration in Xcalibur ™
3.1.2 Signals smoothed (LOESS)

3.2 Build on-line model

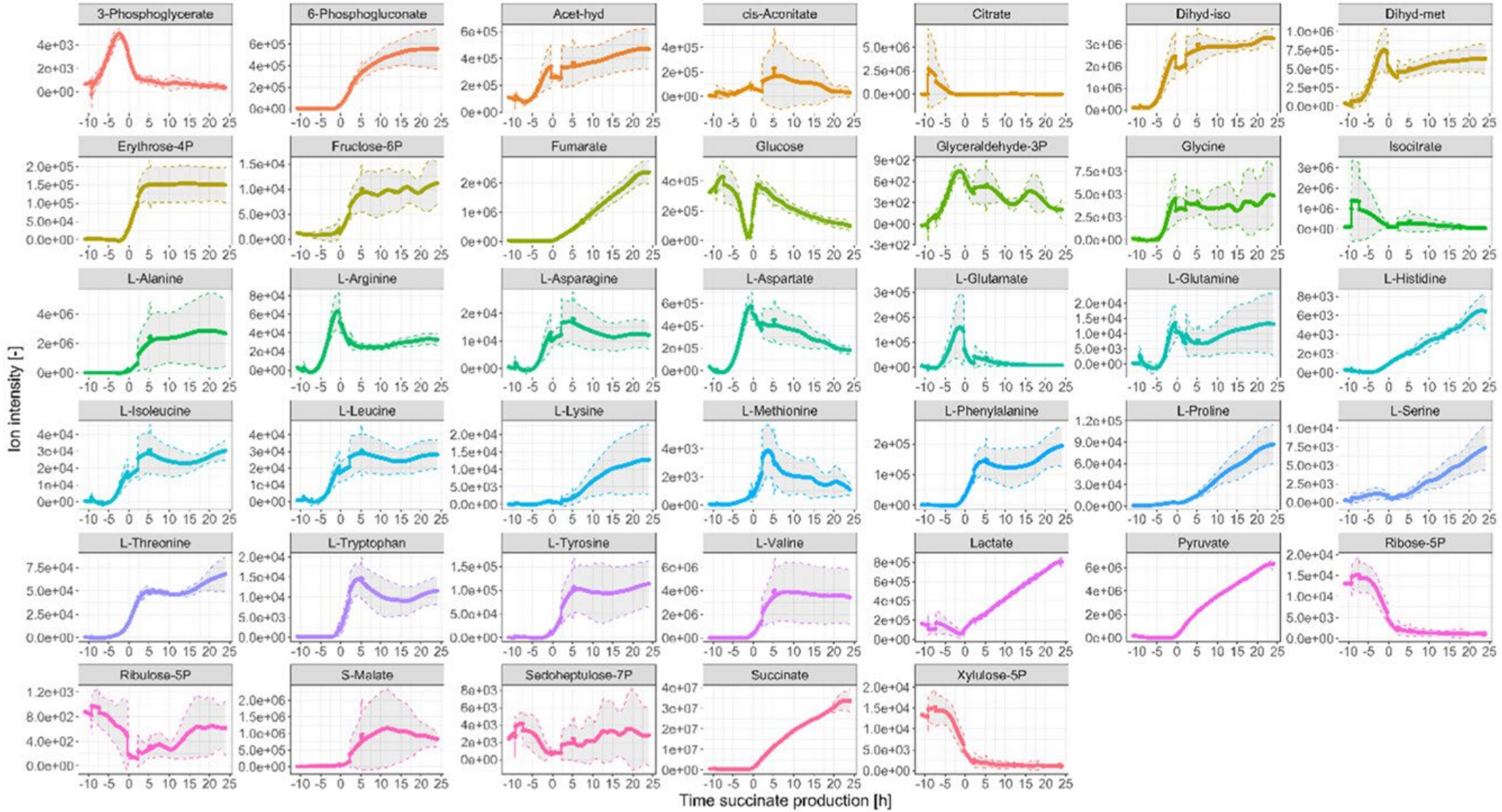
3.2.1 **Align off-line** and **on-line** time-course **data** (15 points/fermentation)
3.2.2 Build **linear model** (on-line MS → off-line g/L) using 10-fold cross-validation
3.2.3 **Model diagnostics**

https://onlinelibrary.wiley.com/doi/10.1002/bit.28599?af=R

Off-line analytics

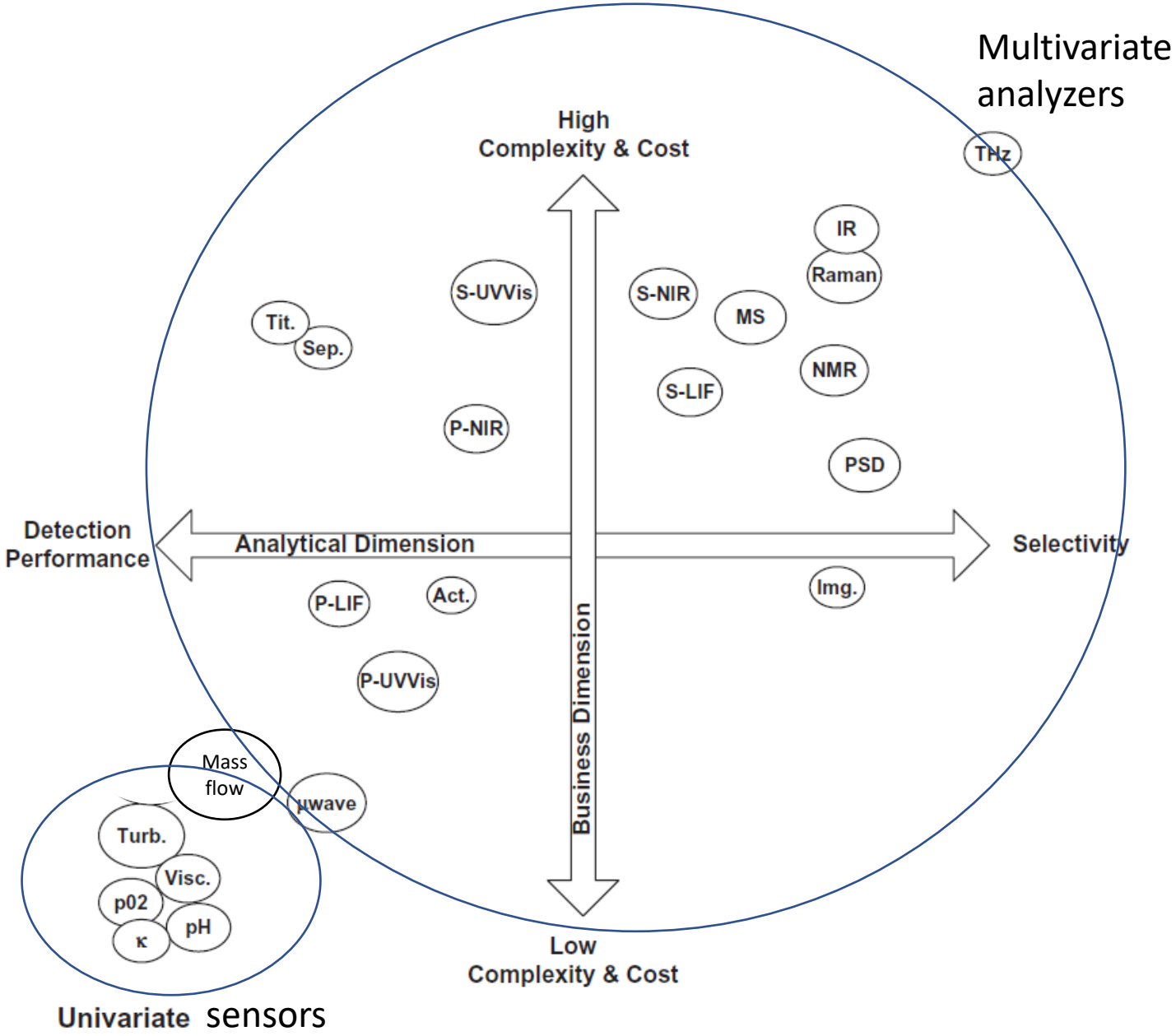


On-line analytics



Various instruments

Abbr	Instrument
Visc.	Viscometer
K	conductivity
Turb.	Turbidity
LIF	Laser induced fluorescence
Act	Accoustics
PSD	Particle size analyzers
Sep.	Separations (e.g. chromatography)
Tit.	Titration
NIR	Near Infrared
MS	Mass spectrometery
P-	Filter based (univariate or few WL)
S-	Spectral (multivariate)

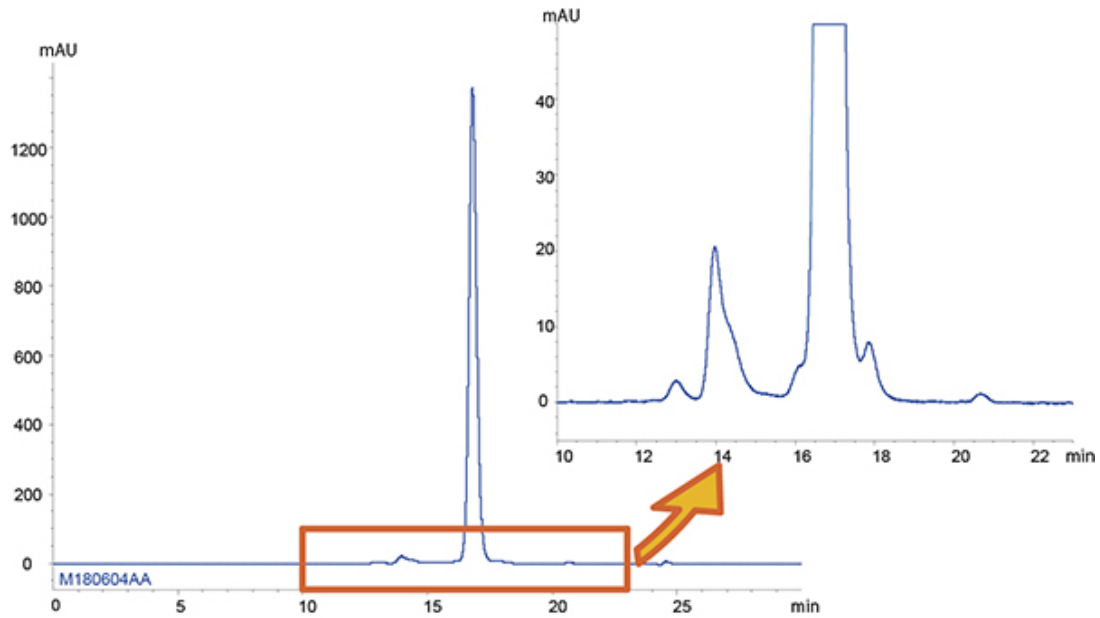


Univariate detectors

- Univariate sensors measure one (physical) attribute, this however very reliably and with high accuracy.
- “Simple” sensors which are widely used in processes
- May not provide information on relevant physical attributes (e.g. glucose content)
- Typical measurements in and around bioreactors
  - pH
  - pO_2
  - Temperature
  - Antifoam probe
  - Mass flow meters
  - Absorbance (optical density)

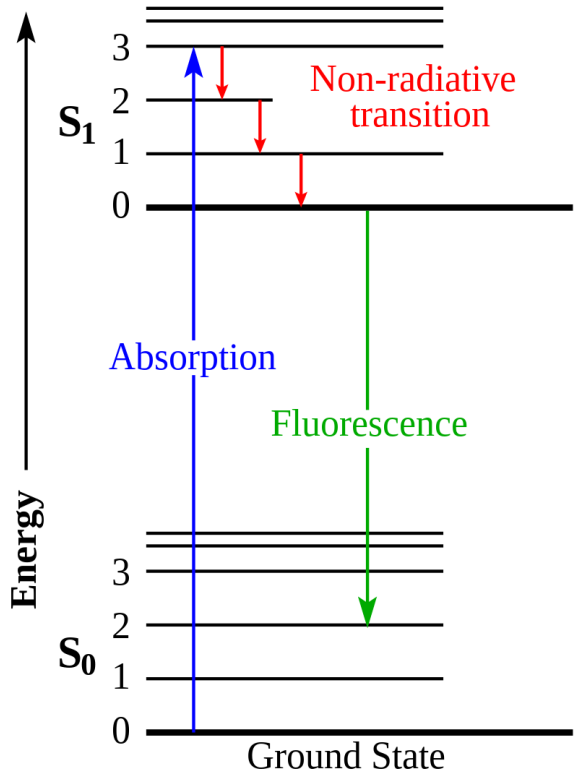
Multivariate sensors/process analyzer

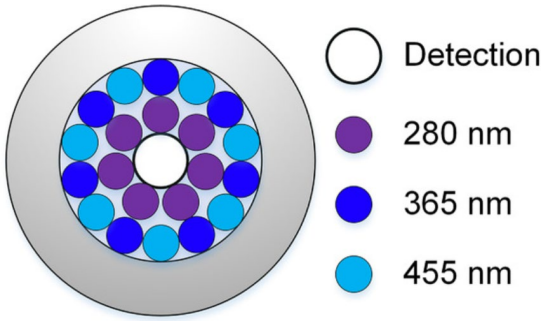
- Measurement consists of many variables
- The different variables provide (partly) independent information on the process.
- Each variable is affected by noise and is most often also correlated with the neighboring variables.
- Examples:
  - Spectra (UV/Vis, MS, NMR, IR, etc.)
  - Chromatograms (at-line chromatography)



Fluorescence

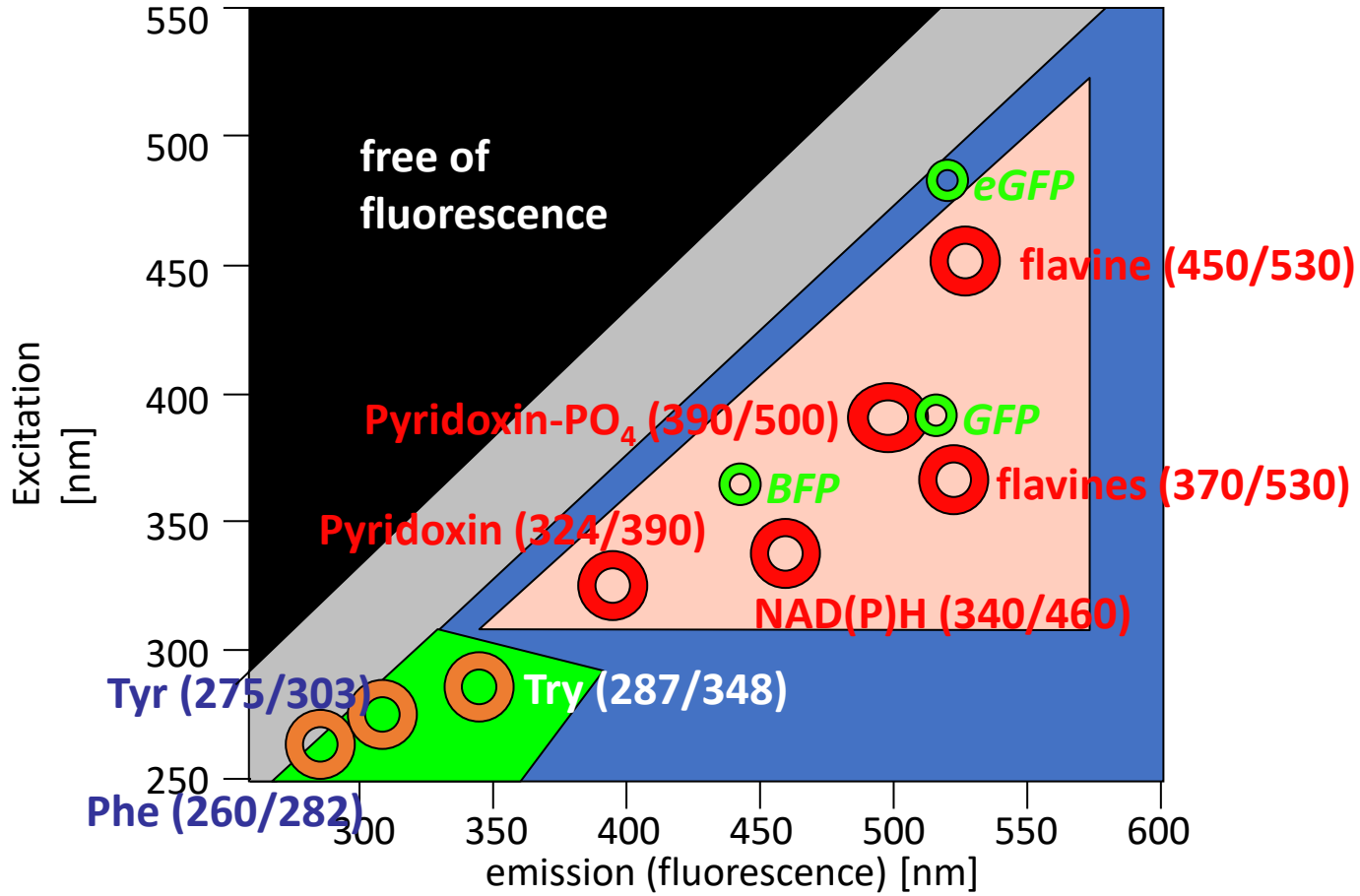
- Fluorescence is a process, where:
  1. A photon is absorbed by a molecule → light source required
  2. The electronic state of the molecule changes
  3. A photon is emitted at higher wavelengths (lower energy)
- The fluorescence detectors need to be more sensitive than for UV/VIS absorption spectroscopy since lower levels of light need to be detected
- Due to absorption within the sample, fluorescence behaves non-linear at elevated concentrations



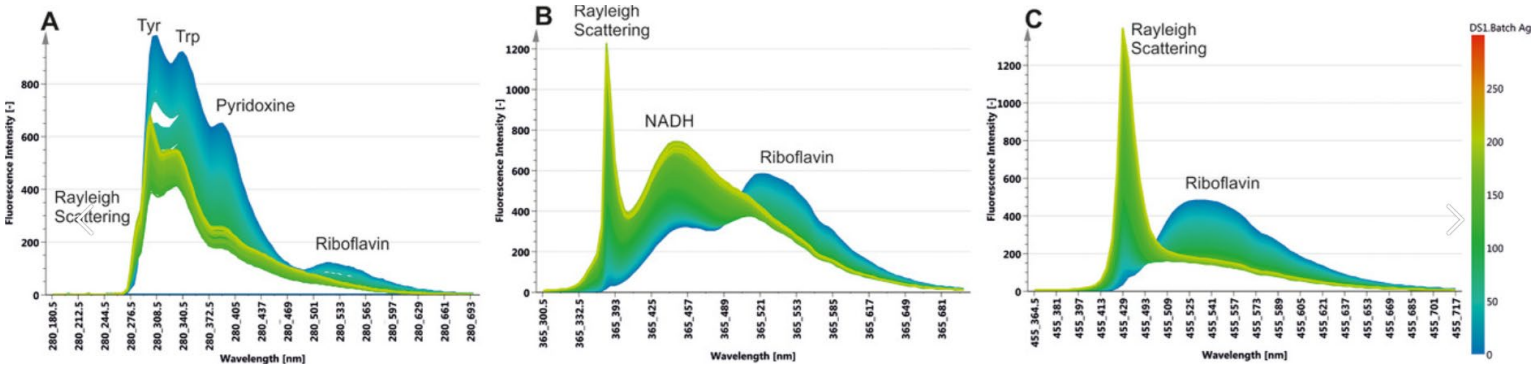


Fluorescence in bioreactors

- Rich in information on different biological components.
- Provides direct information on the oxidative state of cells.
- Deviations from linearity possible
- Sensitive towards local environment of fluorophores:
  - Detection of shifts during e.g., protein folding is possible



Hitzmann et al (1998) CAB7: 231-236



Claßen, Jens, et al. "Spectroscopic sensors for in-line bioprocess monitoring in research and pharmaceutical industrial application." *Analytical and bioanalytical chemistry* 409.3 (2017): 651-666.

Vibrational spectroscopy

- Measure the molecular vibrations of molecules, which is selective for chemical groups.
- The techniques allow to measure almost any molecule with covalent bonds.
- Three methods are commonly used
  - midIR absorbance
  - Near Infrared (NIR) absorbance
  - Raman spectroscopy

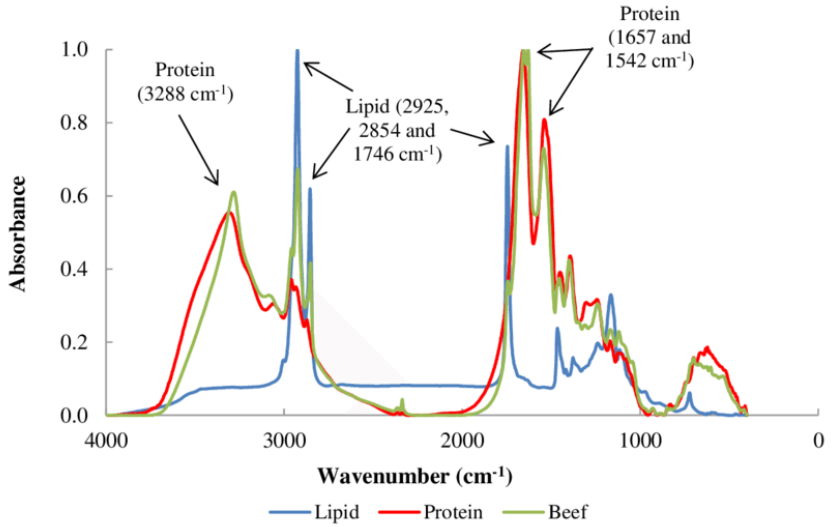
midIR and NIR

- Absorbance spectroscopy. therefore, the measurement is normally taken in reference to a standard intensity. This makes the technologies very linear up to absorbances of 2.5.

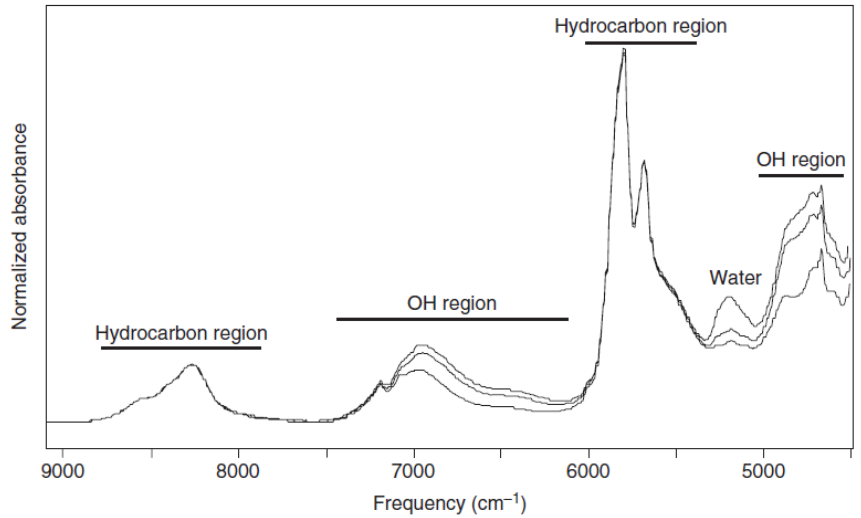
$$A = \log \frac{I_0}{I}$$

- midIR measures at longer wavelength than NIR. In NIR, the absorbance bands (peaks) are broader and less defined.
- Both technologies are strongly influenced by water which generates strong absorption bands.
- Both technologies are used in bioreactors to monitor various attributes.
  - E.g., lactate, glucose, viable cell density, etc.
  - Statistical models with limited transferability required

midIR

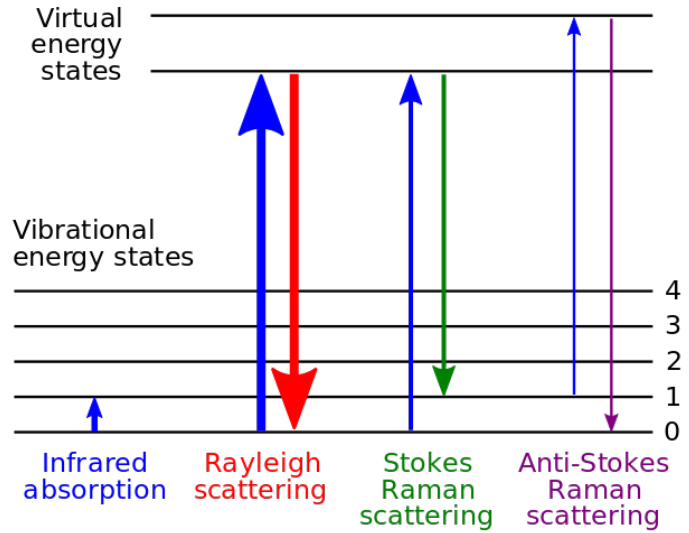


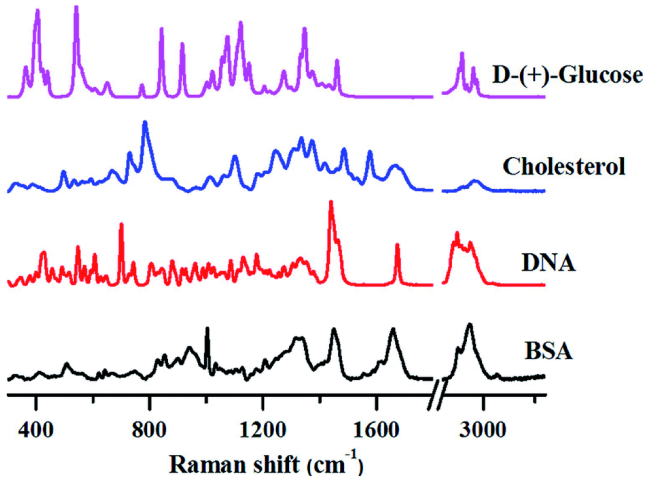
NIR



Raman spectroscopy

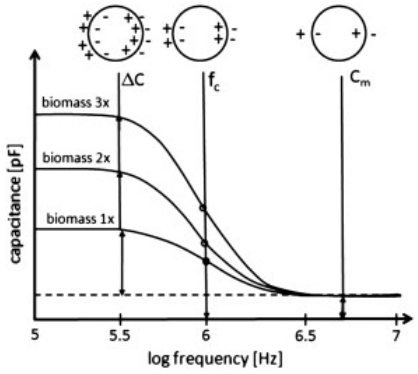
- Similar spectra to midIR
- Raman scattering is based on a two photon process:
  1. A photon (typically from a laser) comes close to the molecule
  2. It interacts, is scattered and transformed to another wavelength while the molecule changes into a different energy state.
  3. The shift in wavelength is called Raman shift and contains information on the molecular structure
- Insensitive to water and therefore well suited to applications in aqueous environments.
- Very sensitive to fluorescence at long wavelengths of the medium, typically >785 nm.
- Similar attributes measurable as IR
  - E.g. glucose, acetate, formate, lactate, and aromatic amino acids
  - Correlations to viable cell density observed
  - Statistical models with limited transferability required

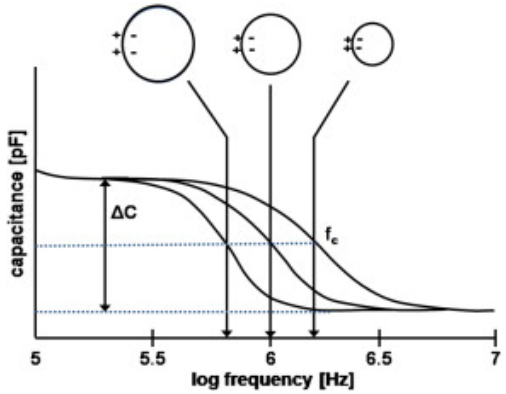




Dielectric spectroscopy

- Measures the impedance (capacity to store electrical fields) at different frequencies.
- Since living cells are small, particulate isolators, they influence the impedance
- Allows to directly follow important performance attributes of the process based on physical principles:
  - the viable cell density and
  - the size of cells.





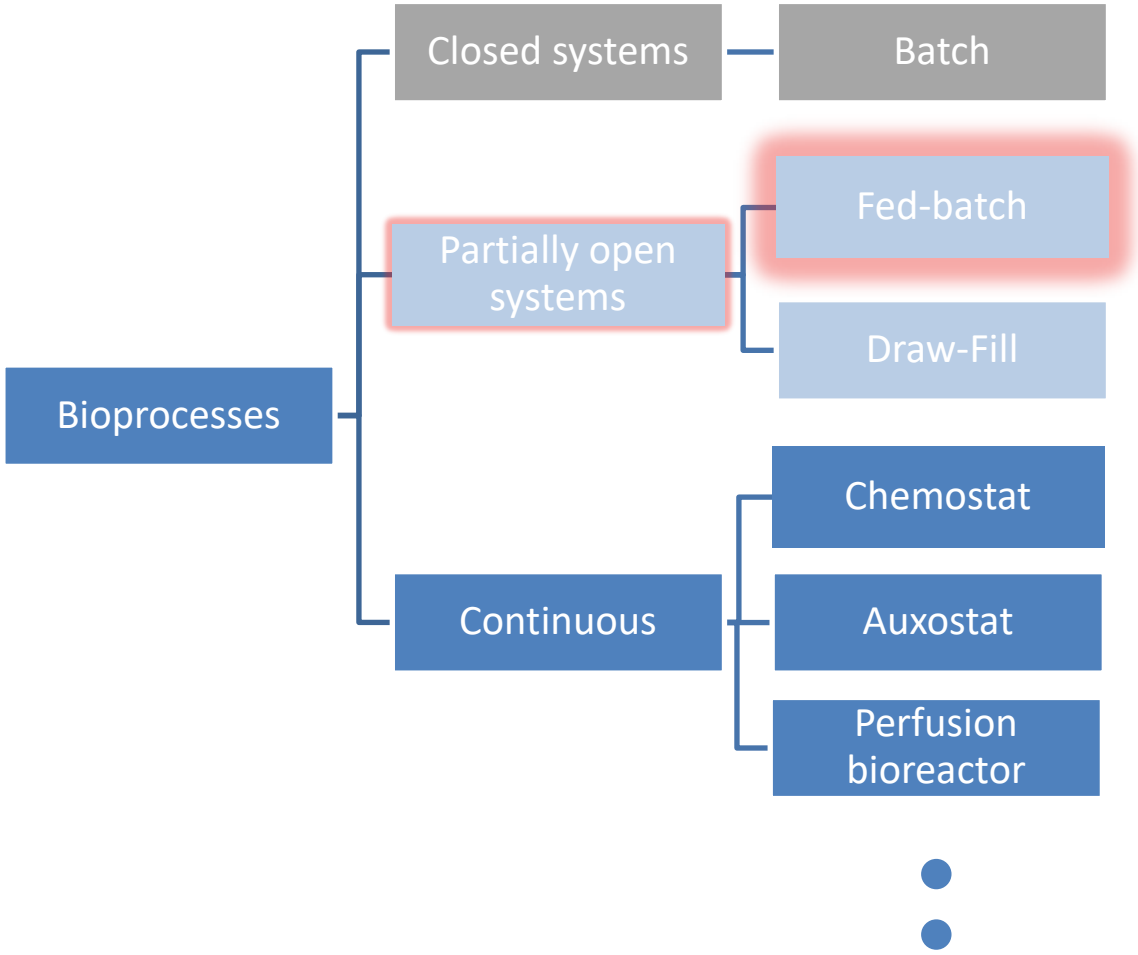
Justice, C., et al. "Process control in cell culture technology using dielectric spectroscopy." *Biotechnology advances* 29.4 (2011): 391-401.

Learning objectives

1. Define and use common vocabulary associated with PAT
2. Name business drivers for PAT and explain how PAT may improve process economics.
3. Name sensor technologies used for process analysis in bioreactors. Name advantages/problems associated different technologies.

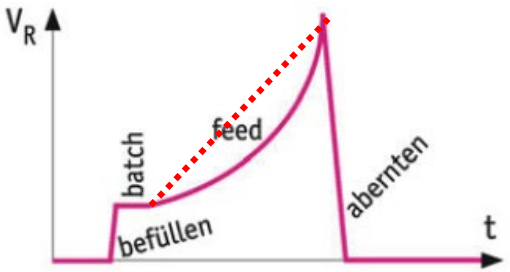
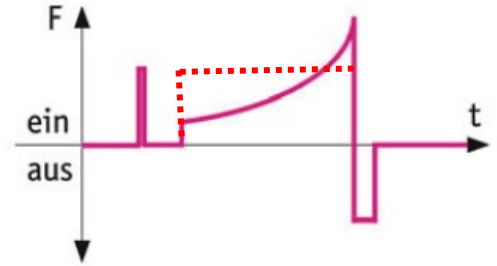
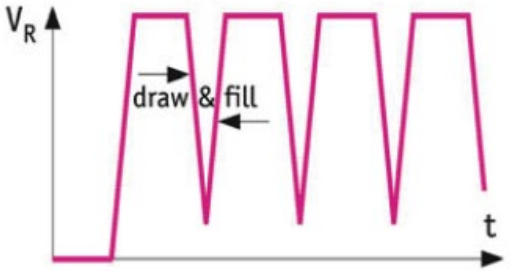
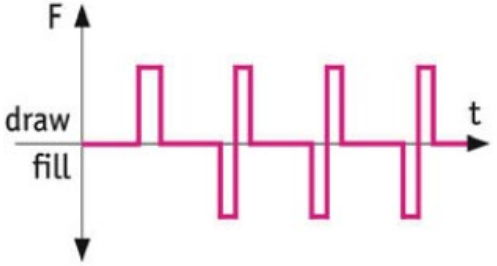
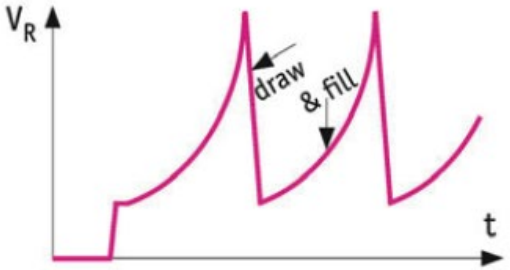
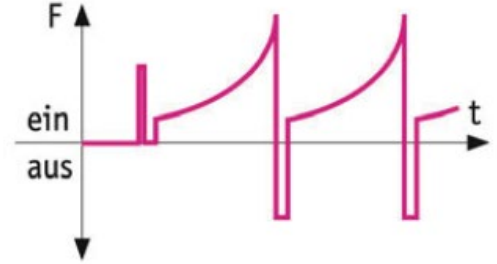
9. Fed-batch cultivation

A hierarchy of bioprocess modes



- Closed systems:
  - No substrate fed; no cell culture fluid withdrawn; volume remains (approximately) constant
- Partially open systems:
  - intermittent withdrawal/addition of significant amounts of liquids
- Continuous systems:
  - Continuous addition and withdrawal of liquid

Partially open systems

	Liquid volume	Global flow $F = F_{in} - F_{out}$
Fed-batch		
Repetitive batch		
Repetitive fed-batch		

This lecture

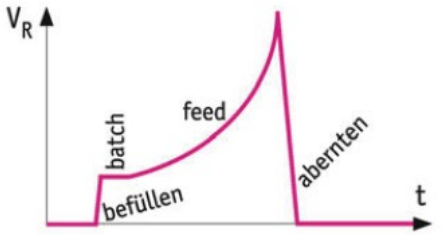
Constant feed

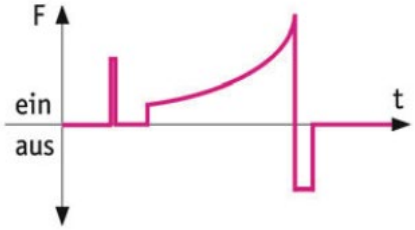
Exponential feed

Adapted from Chmiel, 2011

The four phases of a fed-batch culture

1. Initial fill ($t < t_{batch,start}$)
   - Before inoculating the batch, the bioreactor is filled with medium
2. Batch ($t_{batch,start} \leq t < 0$)
   - Initially, after inoculation, the cells are grown in a batch process to increase the cell density
   - During the late exponential or deceleration phase, the feed is started
3. Feed phase ($0 \leq t < t_{end}$)
   - The feed F supplies the limiting substrate(s) and dictates the growth rate of the cells
4. Harvest ($t_{end} \leq t$)
   - After the final volume is reached (max. 80% of reactor volume), the batch is harvested

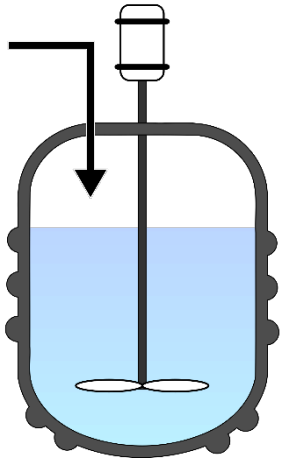




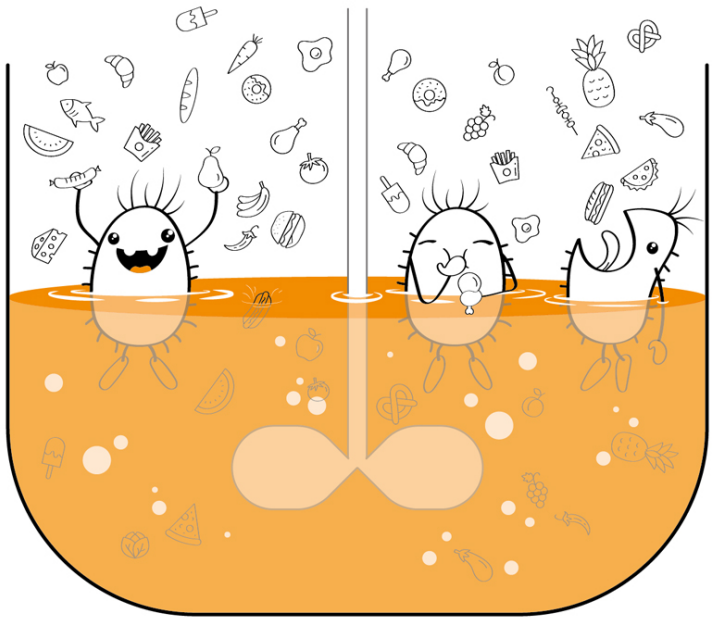
$$F_{in}(t)$$

$$F_{out}(t < t_{harvest}) = 0$$

$$\frac{dV}{dt} \neq 0$$



Question



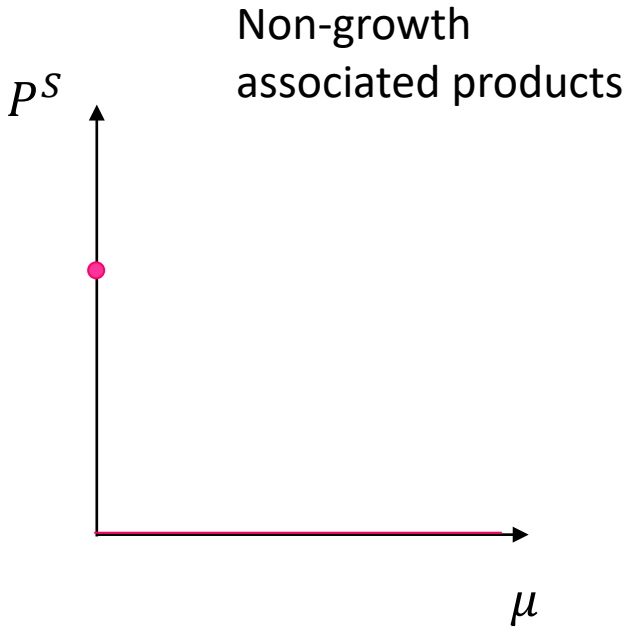
https://www.infors-ht.com/fr/blog/the-difference-between-batch-fed-batch-and-continuous-processes/

What are the key parameters that you need to know when you are asked to run a fed-batch experiment?

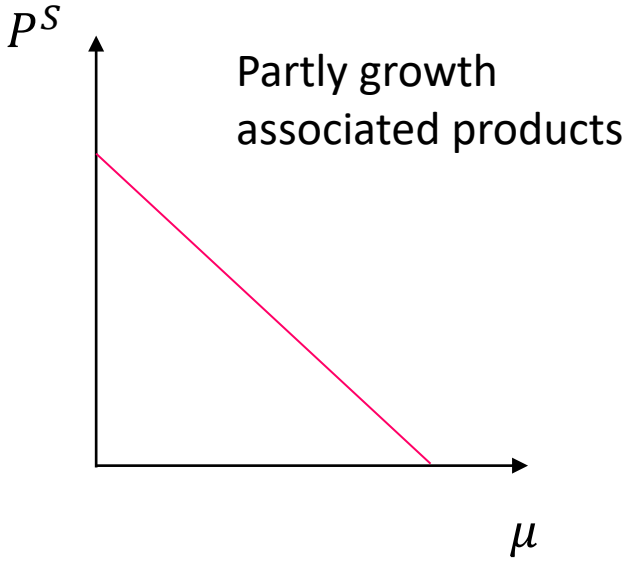
Why fed-batch cultures?

Many commercially important products are produced in highest yields at low nutrient or substrate concentrations.

In fed-batch reactors, feed is added, but effluent (and cells) are not removed. Thus fed-batch reactors can be used to maintain cells under low substrate or nutrient conditions **without wash-out** taking place.



As a large proportion of commercially important products are produced in a partly or non-growth associated manner (e.g., secondary metabolites), it is not surprising to find therefore that fed-batch reactors play such an important role in industry.



Fed-batch reactors are thus primarily used for producing products under low nutrient or substrate conditions.

General aspects of fed-batch

Advantages

- Increased productivity. Production of high cell densities by high substrate concentrations in feed and extension of working time compared to batch leads to higher volumetric productivities.
- Controlled conditions for the provision of the substrate in the reactor
- Control over metabolic pathway and catabolites. Different substrates may be fed in a time-specific approach
- Replacement of water lost due to evaporation
- No additional equipment required besides accurate feed pump

Disadvantages

- Organism needs to be characterized regarding its nutritional requirements and its physiology with respect to productivity.
- Requires more experience of the operator executing and scientist/process engineer developing & defining the process compared to a simple batch cultivation.
- Cyclic (fed-)batch cultivations need to ensure that all substrates are included in feed and no toxins accumulate

Metabolic aspects of (fed-)batch cultures

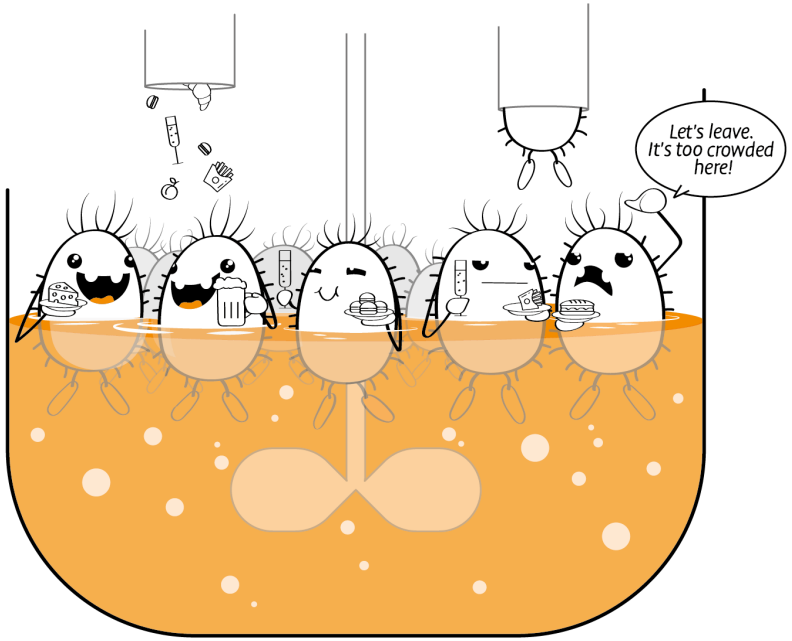
Processes inhibited by high substrate concentration

Process	*Inhibiting substrate*	*Effect*
S. cerevisiae	Glucose	Ethanol from over-flow metabolism
E. coli	Glucose	Acetate from over-flow metabolism
Animal cell cultures	Glucose Glutamine	Lactate and ammonia from over-flow metabol.
Antibiotics	Energy source	Catabolite repression
Proteases	Amino acids	Product repression
Amylases	Glucose	Product repression

What determines the maximum cell density in a fed-batch process

- Exhaustion of medium components (Mg2+, etc)
- Inhibitors from feed or cells
- **The substrate feed rate**
- **The maintenance demand**

Question

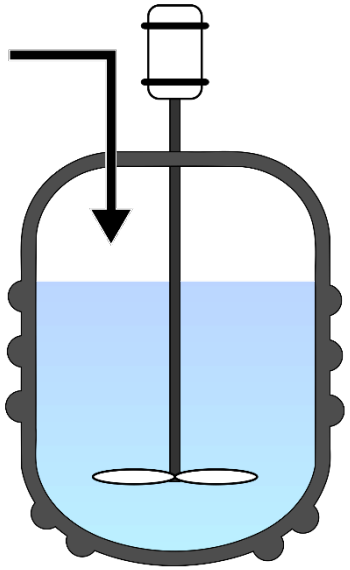


What are the PAT tools to achieve a HCD fed-batch culture?

What are the problems of a HCD fed-batch culture?

https://www.infors-ht.com/fr/blog/the-difference-between-batch-fed-batch-and-continuous-processes/

Reactor volume in fed-batches



$$F_{in}(t) = \frac{dV}{dt}\Big|_{t<t_{harvest}}$$

$$F_{out}(t < t_{harvest}) = 0$$

$$\frac{dV}{dt} \neq 0$$

- The total volume of a fed-batch bioreactor is a function of time. We can write:

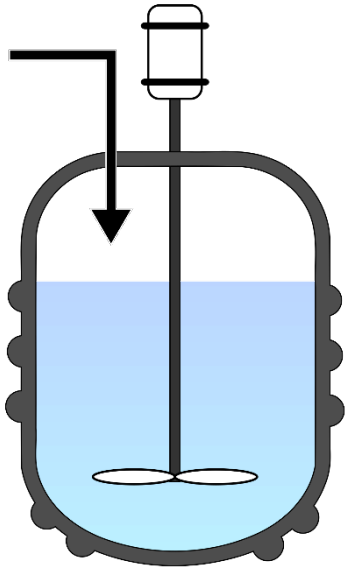
$$\frac{dV}{dt} = F(t)$$

- Integrating:

$$V(t) = V_0 + \int_0^t F(t)\,dt$$

- The exact reactor volume over time thus depends on the feed rate and feed profile.
  - Constant feed → linear dependence of reactor volume on time
  - Exponential feed → exponential dependence of reactor volume on time

Differential mass balance: biomass 1



$$F_{in}(t) = \frac{dV}{dt}\Big|_{t<t_{harvest}}$$

$$F_{out}(t < t_{harvest}) = 0$$

$$\frac{dV}{dt} \neq 0$$

- We can construct a differential biomass balance according to first assumption of Monod's model

$$\frac{dX}{dt} = \frac{d(xV)}{dt} = \mu X$$

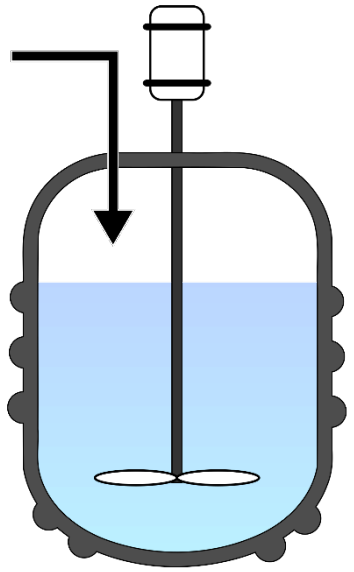
- Contrary to chemostat and batch processes, the volume in the reactor is not constant, and we cannot simply pull V out of the derivative. Instead, we apply the product rule.

$$\frac{d(xV)}{dt} = V\frac{dx}{dt} + x\frac{dV}{dt}$$

- By using the definition of the feed flow

$$\frac{d(xV)}{dt} = V(t)\frac{dx}{dt} + xF_{in}$$

Differential mass balance: biomass 2



$$F_{in}(t) = \frac{dV}{dt}\Big|_{t<t_{harvest}}$$

$$F_{out}(t < t_{harvest}) = 0$$

$$\frac{dV}{dt} \neq 0$$

- Summarizing the previous equations

$$V(t)\frac{dx}{dt} + xF_{in} = \mu X$$

- We define once again $D(t) = \frac{F_{in}(t)}{V(t)}$ and divide the above equation by $V(t)$

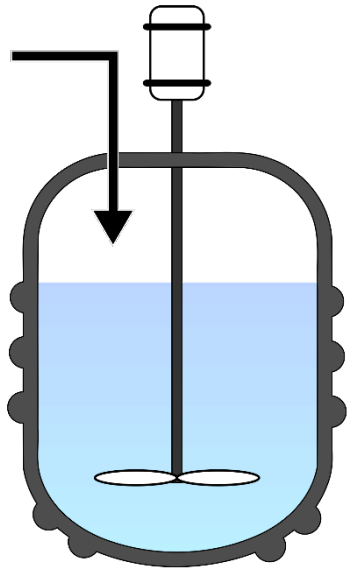
$$\frac{dx}{dt} + xD = \mu x$$

$$\Leftrightarrow$$

$$\frac{dx}{dt} = (\mu - D)x$$

In a quasi-steady state, the cells grow as fast as the dilution rate

Differential mass balance: substrate



$$F_{in}(t) = \frac{dV}{dt}\Big|_{t<t_{harvest}}$$

$$F_{out}(t < t_{harvest}) = 0$$

$$\frac{dV}{dt} \neq 0$$

- We define the substrate mass balance with **no** maintenance requirements and **no** substrate consumption for product generation as follows:

$$\frac{dS}{dt} = s_{in}F_{in} - \frac{\mu X}{Y_{\frac{X}{S}}}$$

- Converting the derivative to concentrations by applying the product rule (see previous slides)

$$\frac{d(sV)}{dt} = V(t)\frac{ds}{dt} + sF_{in}$$

- Combining the two equations, dividing by $V(t)$

$$\frac{ds}{dt} = (s_{in} - s)D - \frac{\mu x}{Y_{\frac{X}{S}}}$$

Fed-Batch Culture

- When the substrate is totally consumed, $S \approx 0$, and $X = X_m$
  - At this point, $dX/dt = 0$, and the system is at quasi-steady state (nutrient consumption rate is nearly equal to nutrient feed rate), therefore:

$$\mu_{net} = D \qquad (10.7)$$

- If maintenance energy can be neglected,

$$\mu_{net} = \mu_m \frac{S}{K_s + S} \quad \text{and} \quad S \cong \frac{K_s D}{\mu_m - D} \qquad (10.8)$$

Fed-Batch Culture

- The balance on the rate-limiting substrate without maintenance energy is:

$$\frac{dS^t}{dt} = FS_0 - \frac{\mu_{net} X^t}{Y^M_{X/S}} \qquad (10.9)$$

  - Where S^t is the total amount of the rate-limiting substrate in the culture, and S_0 is the concentration of S in the feedstream
  - At quasi-steady state, $X^t = VX_m$, and essentially all substrate is consumed, therefore:

$$\frac{dX^t}{dt} = X_m\left(\frac{dV}{dt}\right) = X_m F = FY^M_{X/S} S_0 \qquad (10.10$$

Fed-Batch Culture

- Integration of eq. 10.10 from $t=0$ to t, with the initial biomass concentration in the reactor being X_0^t yields:

$$X^t = X_0^t + FY_{X/S}^M S_0 t \qquad (10.11)$$

  - That is, the total amount of cells in the culture increases linearly with time
  - Dilution rate and μ_{net} decrease with time
  - Since $\mu_{net}=D$ at quasi-steady state, the growth rate is controlled by the dilution factor

Fed-Batch Culture

- For product formation in a fed-batch reactor, at quasi-steady state ($S<<S_0$): $P \cong Y_{P/S} S_0$ (10.12)
- Or the potential product output is:

$$\frac{dP^t}{dt} = q_p X^t \quad (10.13)$$

- When the specific rate of product formation (q_p) is constant:

$$FP \approx Y_{P/S} S_0 F \quad (10.14)$$

  - Where P^t is the total amount of product in culture

Fed-Batch Culture

- Substituting $X^t=(V_0+Ft)X_m$ into eq. 10.14 yields:

$$P^t = P_0^t + q_p X_m \left(V_0 + \frac{Ft}{2} \right) t \qquad (10.15)$$

- Integration of eq. 10.15 yields:

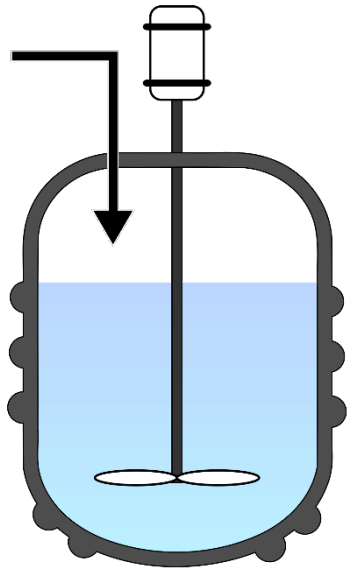
$$\frac{dP^t}{dt} = q_p X_m \left(V_0 + Ft \right) \qquad (10.16)$$

- Eq. 10.16 can be written in terms of product:

$$P = P_0 \frac{V_0}{V} + q_p X_m \left(\frac{V_0}{V} + \frac{Dt}{2} \right) t \qquad (10.17)$$

Note: P, P_0 and X_m are concentrations [g L^{-1}]

Time-dependent feed rates 1



$$F_{in}(t) = \frac{dV}{dt}\Big|_{t<t_{harvest}}$$

$$F_{out}(t < t_{harvest}) = 0$$

- To achieve a quasi steady-state ($s, x = \text{const}$), D is held constant over time. A target quasi steady-state growth rate μ is selected for the process

$$D = \frac{F(t)}{V(t)} = \frac{1}{V}\frac{dV}{dt} = \mu$$

- Integrating

$$V = V_0 \exp \mu t$$

- And calculating the feed rate

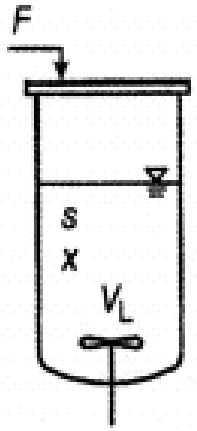
$$F(t) = V_0 \mu \exp \mu t$$

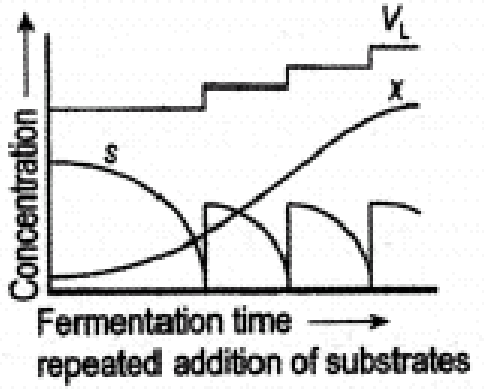
Note: sometimes a similar equation is derived on the presumption that $\frac{ds}{dt}$ is held constant. However, that approach requires more assumptions and leads qualitatively to the same result. μ can be replaced by D under quasi steady-state conditions.

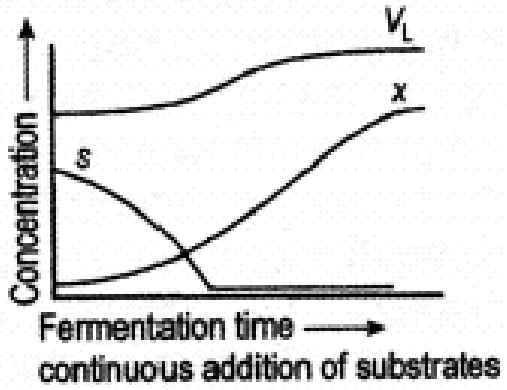
Time-dependent feed rates 2

- Exponential feed profiles are not the only approach used in industry. In practice, different feed profiles are applied. Most frequently:
  1. $F = const$: First growth with a high μ but then steady decrease!
  2. $s, x = const$: Exponential feed profiles

$$F_t = \frac{\mu * V_0 * x_0 * e^{\mu * t}}{Y_{X/S} * (s_i - s)}$$

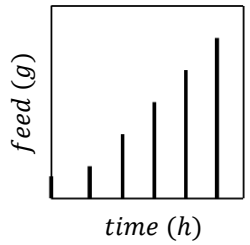
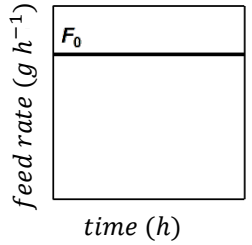
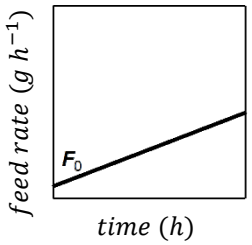
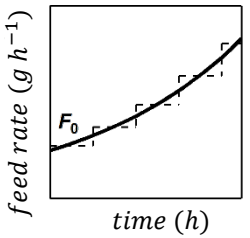
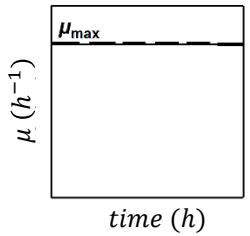
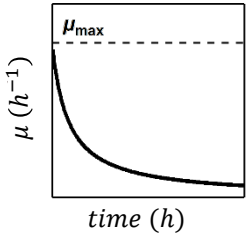
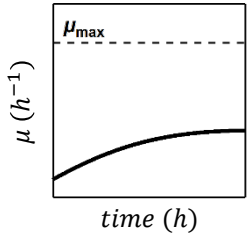
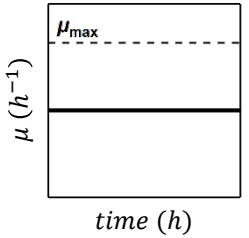






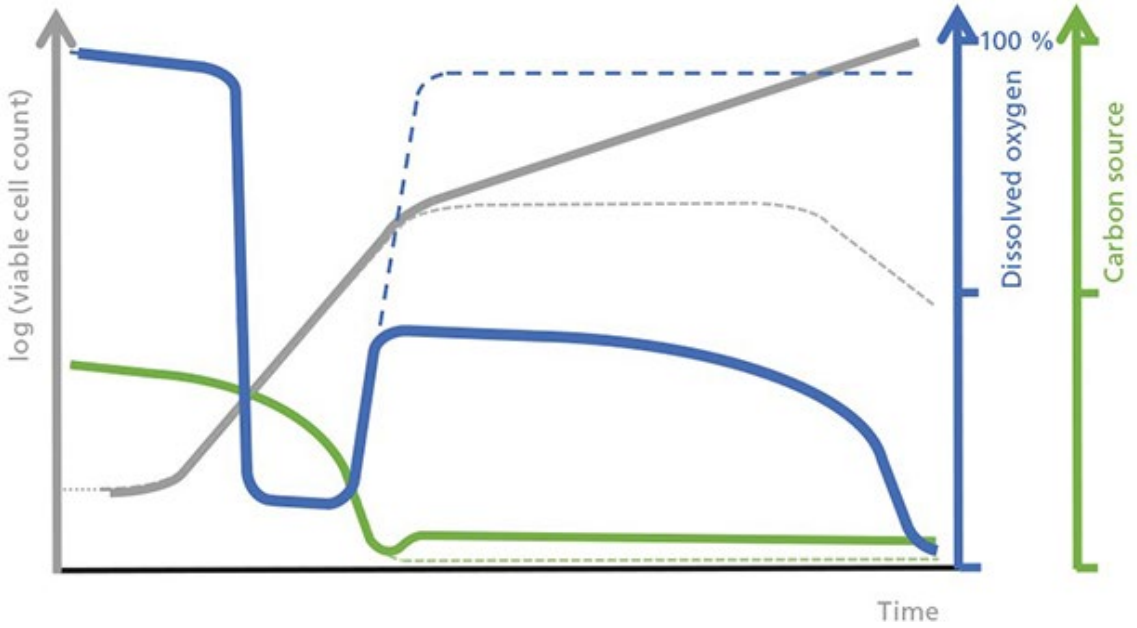
- If the feed follows a pre-defined profile (no feedback), it is also called **open-loop** control. In this case, the cells stabilize the quasi-steady state. This is feasible as long as
  - D changes slowly
  - the cells behave robustly regarding small variations in substrate concentration

Different fed-batch feeds and their influence on μ

Pulsed feed	Fed-batch feed profiles		
pulsed	constant feed	linear increase	exponential increase
$F(t) = \sum_{i=1}^{n} s_i \cdot V_i$	$F(t) = F_0$	$F(t) = a \cdot tF_0$	$F(t) = F_0 \cdot e^{\mu \cdot t}$
			
			

Question

What feed profile would you use to achieve a HCD culture?



Further aspects of fed-batch cultures

Processes inhibited by high substrate concentration

Process	*Inhibiting substrate*	*Effect*
S. cerevisiae	Glucose	Ethanol from over-flow metabolism
E. coli	Glucose	Acetate from over-flow metabolism
Animal cell cultures	Glucose Glutamine	Lactate and ammonia from over-flow metabol.
Antibiotics	Energy source	Catabolite repression
Proteases	Amino acids	Product repression
Amylases	Glucose	Product repression

What determines the maximum cell density in a fed-batch process

- Exhaustion of medium components (Mg^{2+}, etc)
- Inhibitors from feed or cells
- **The substrate feed rate**
- **The maintenance demand**

Example: Inhibition of growth by acetate

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1990, p. 1004–1011 Vol. 56, No. 4
0099-2240/90/041004-08$02.00/0

Comparison of Growth, Acetate Production, and Acetate Inhibition of *Escherichia coli* Strains in Batch and Fed-Batch Fermentations

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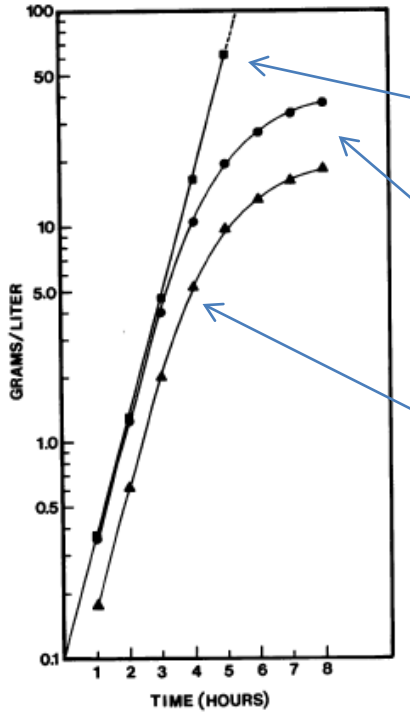
Received 18 October 1989/Accepted 11 January 1990



Growth of biomass not inhibited by acetate

Growth of biomass inhibited by acetate

Concentration of acetate during inhibition

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Journal of Biotechnology 39 (1995) 59–65

journal of biotechnology

Simple fed-batch technique for high cell density cultivation of *Escherichia coli*

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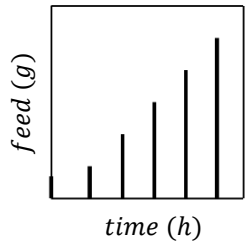
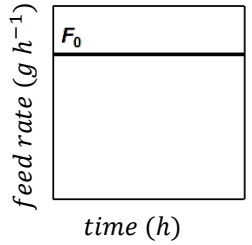
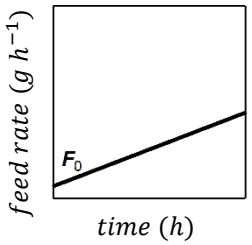
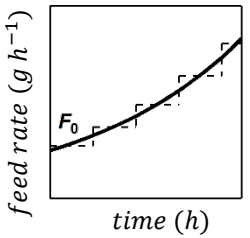
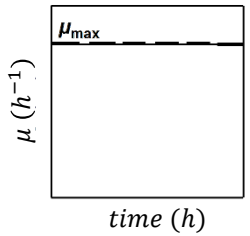
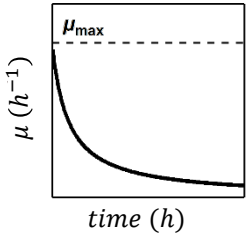
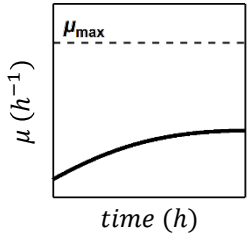
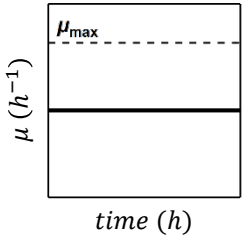
Abstract

A simple fed-batch process for high cell density cultivation of *Escherichia coli* TG1 was developed. A pre-determined feeding strategy was chosen to maintain carbon-limited growth using a defined medium. Feeding was carried out to increase the cell mass concentration exponentially in the bioreactor controlling biomass accumulation at growth rates which do not cause the formation of acetic acid ($\mu < \mu_{crit}$). Cell concentrations of 128 and 148 g per l dry cell weight (g l^{-1} DCW) were obtained using glucose or glycerol as carbon source, respectively.

Keywords: Escherichia coli; Fed-batch; Cultivation, high cell density

Open loop control of bioprocess with $D < \mu_{crit} = 0.17\ h^{-1}$

Different fed-batch feeds and their influence on μ

Pulsed feed	Fed-batch feed profiles		
pulsed	constant feed	linear increase	exponential increase
$F(t) = \sum_{i=1}^{n} s_i \cdot V_i$	$F(t) = F_0$	$F(t) = a \cdot tF_0$	$F(t) = F_0 \cdot e^{\mu \cdot t}$
			
			

Production kinetics

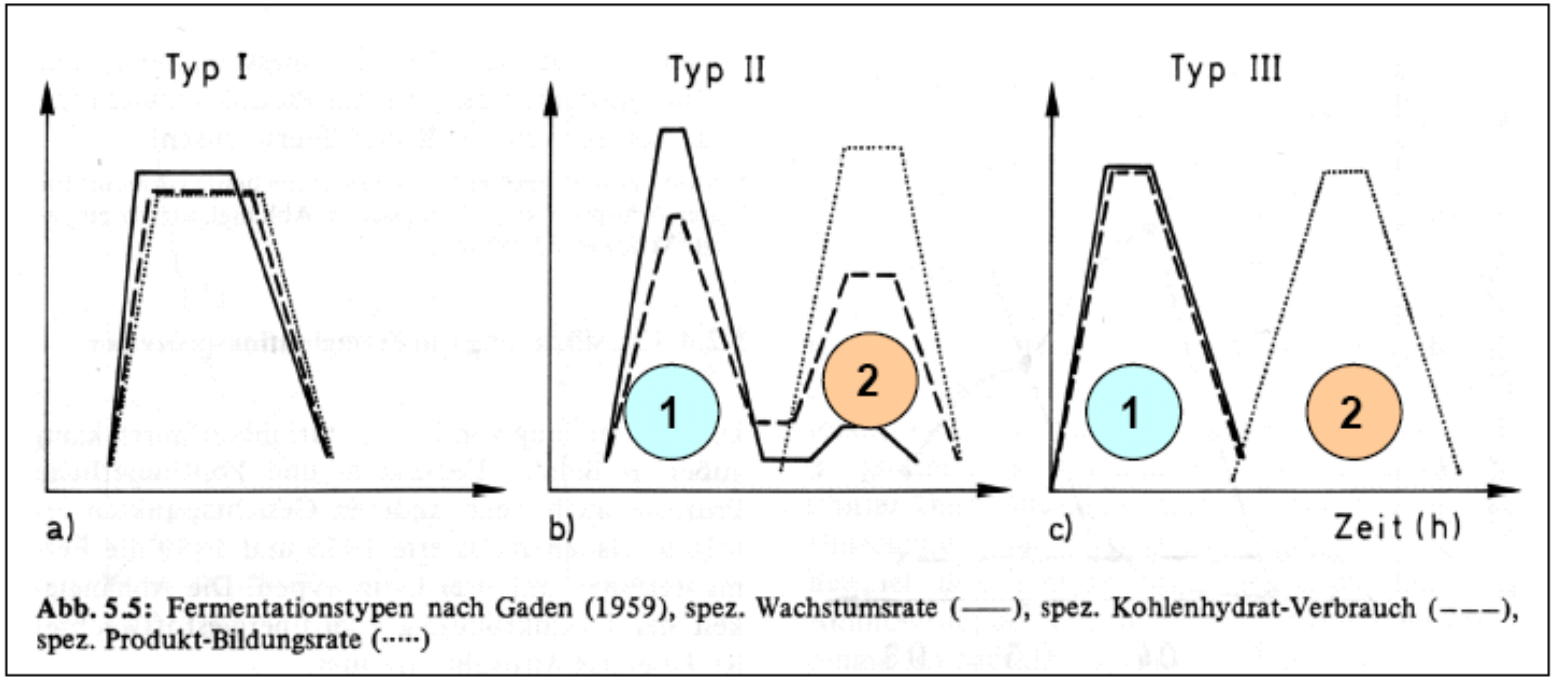


Abb. 5.5: Fermentationstypen nach Gaden (1959), spez. Wachstumsrate (——), spez. Kohlenhydrat-Verbrauch (– – –), spez. Produkt-Bildungsrate (·····)

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

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

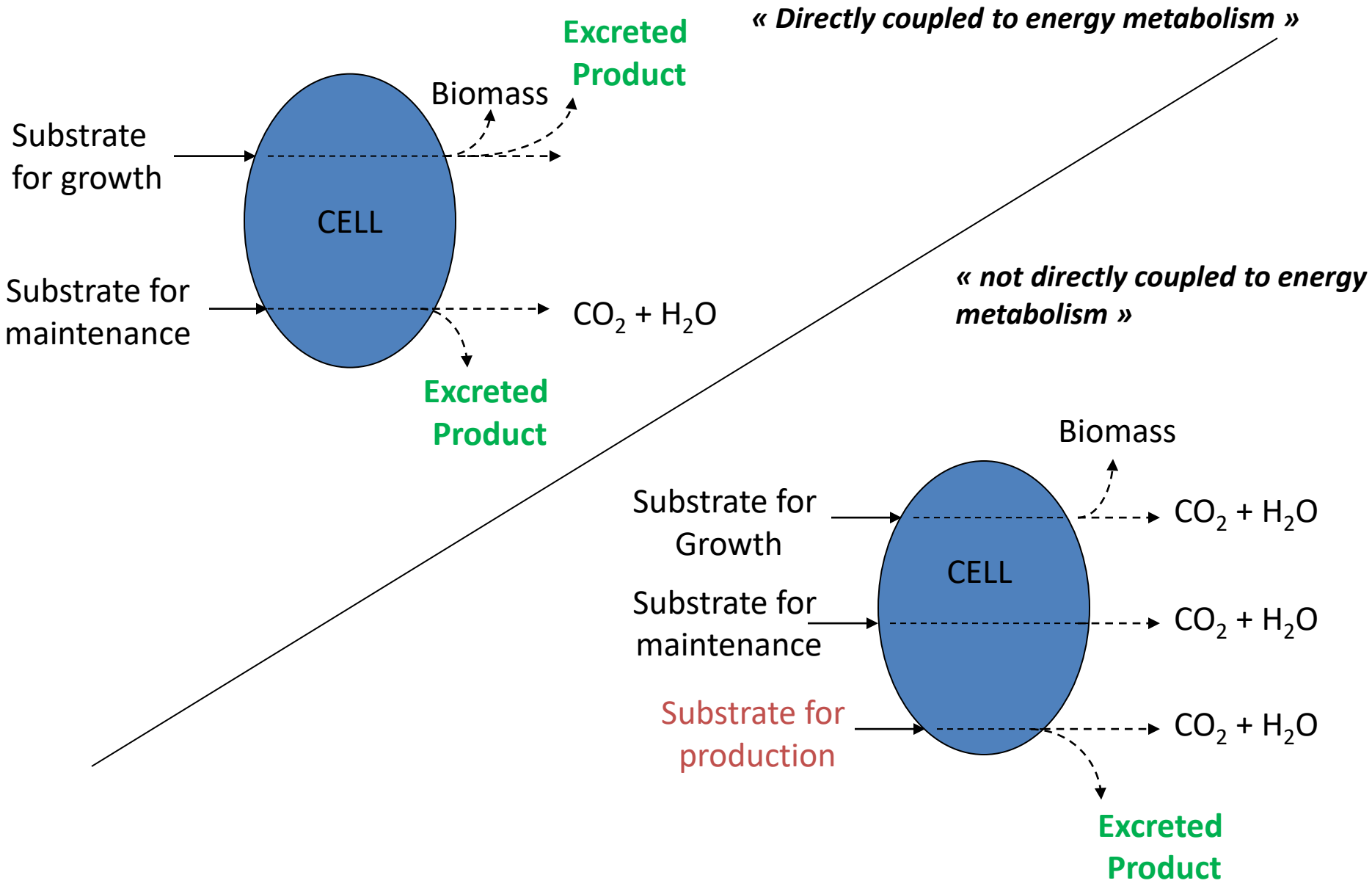
where q_P^{max} and k are constants. The formation of cyclodextrin from starch by *Bacillus macerans* and spore production by *Bacillus subtilis* are similar.

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.

Substrate uptake with product formation



Closed-loop control

- PAT is used to measure the content of limiting substrate. This is generally the case for mammalian cultivations.
  - Frequently, off-line or at-line measurements are performed 1-2 times a day (exact frequency depends on cultured cells).

- The feed rate is adjusted to maintain a constant concentration of the limiting substrate by a feedback control, e.g., through:
  - PID (proportional–integral–derivative) controller
  - Model-based controller
  - Manual adjustment

High cell-density culture of non-recombinant *E. coli* strains

Host strain	Medium[a]	Temperature (°C)	Culture method/ feeding mode	Cultivation time (h)	Final cell mass [g(DCW) l^{-1}]
B54125	Defined	36.5	Exponential	10	86
TG1	Defined	28	Exponential	24	128
TG1	Defined (glycerol)	28	Exponential	44	148
W3350	Defined	37	Specific growth-rate control	21	53
TG1	Defined	28	Specific growth-rate control	35	110
B (OSU333)	Defined	32	Glucose concentration control	9	65
W3110	Defined (glycerol)	37	Constant feeding with dialysis	23	174
W	Defined (sucrose)	37	pH-stat	36	105.4
ATCC10536	Semi-defined	37	DO-stat	12	110.2
B	Semi-defined	36	DO-stat	11	125
ATCC8739	Defined	30	DO-stat	14	111.4
ATCC8739	Defined	37	DO-stat	12	105

[a] Except where indicated, glucose was used as the carbon source.

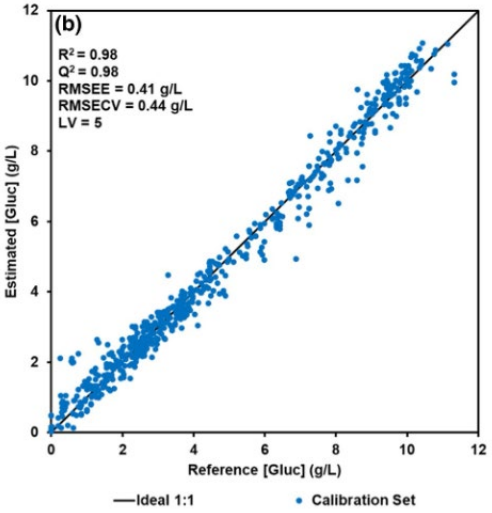
Successful application of fed-batches using recombinant *E. coli* strains

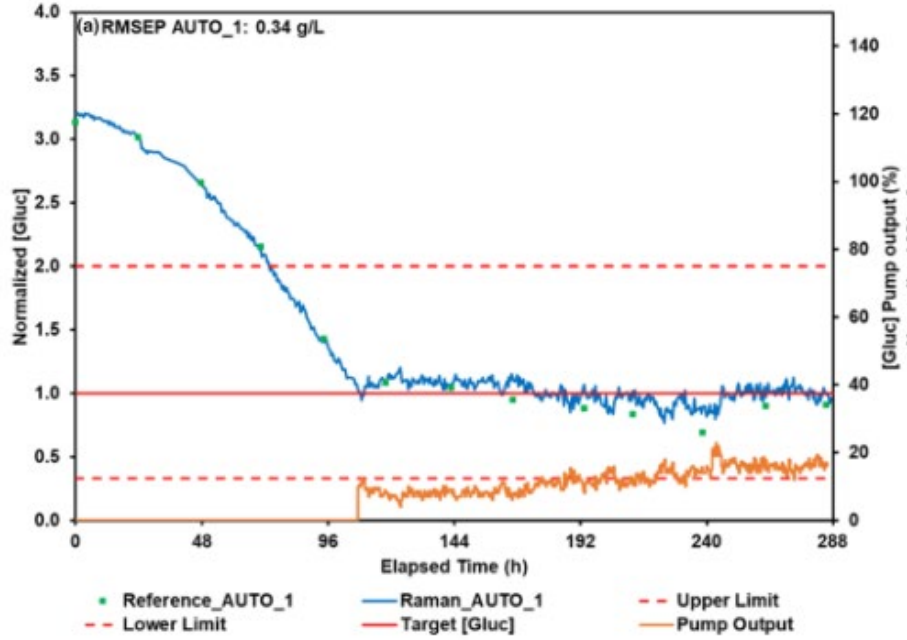
Final cell mass [g(DCW) l^{-1}]	Product	Product concentration
OD_{525} = 120	Human growth hormone	1.08 g l^{-1}
20	Human insuling-like growth factor-1	600 mg l^{-1}
OD_{525} = 11	Human growth hormone	1.75 g l^{-1}
68	Human α consensus interferon	5.6 g l^{-1}
92	Trypsin	56 mg l^{-1}
55	Human interleukin 1β	2.15 g l^{-1}
26	Human leukocyte interferon	1×10^9 IU l^{-1}
77	ProteinA–β-galactosidase fusion	19.2 g l^{-1}
60	Human interferon α1	5.5×10^8 IU g(DCW)$^{-1}$
50	Mini-antibody	1.04 g l^{-1}
58	Human interferon α1	1.26×10^9 IU l^{-1}
95.5	Aprotinin–β-galactosidase fusion	2.85×10^6 U l^{-1}
40	Human parathyroid hormone	338 mg l^{-1}
OD_{600} = 100	Bovine somatotropin	2.9 g l^{-1}
63	β-isopropylmalate dehydrogenase	16.00 U g(protein)$^{-1}$
101.4	PHB[g]	81.2 g l^{-1}
175.4	PHB	65.5 g l^{-1}
124.6	PHB	34.3 g l^{-1}
OD_{660} = 134.4	Human Proapo A-I	6.0 g l^{-1}
59.5	Human interleukin 2	1.2 g l^{-1}
125	*Bacillus thuringiensis* toxin	6.6 g l^{-1}
36	Phenylalanine	46 g l^{-1}
OD_{680} = 90	Human interleukin 2	–
OD_{680} = 75	Human interleukin 2	3.3 g l^{-1}
102	*E. coli* tryptophan synthase	1.6×10^5 U g(protein)$^{-1}$
21	Human epidermal growth factor	60 mg l^{-1}
84	β-galactosidase	4600 U OD_{600}^{-1}
145	Penicillin acylase	6.0 U mg^{-1}

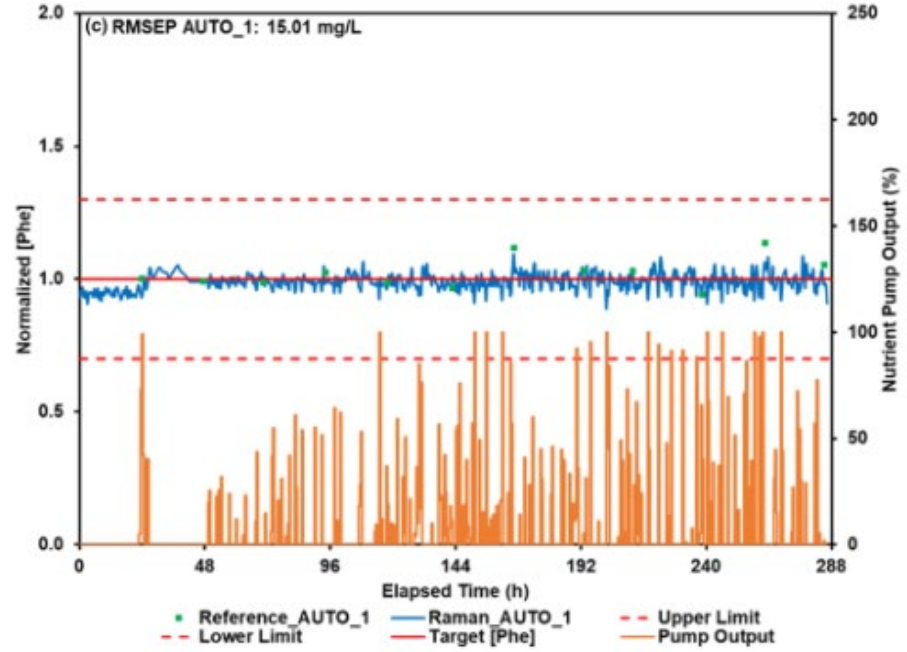
Example: closed-loop control of CHO cultivation with Raman spectroscopy

- Glucose and phenylalanine monitored in real-time by in-line Raman spectroscopy
- Feed-rates of two different feeds is adjusted to maintain a stable concentration of both substrates
- Productivity could be increased by ca. 20% compared to off-line measurements.

Question: The glucose feed rate remains almost stable over time. What does this indicate regarding the growth rate?

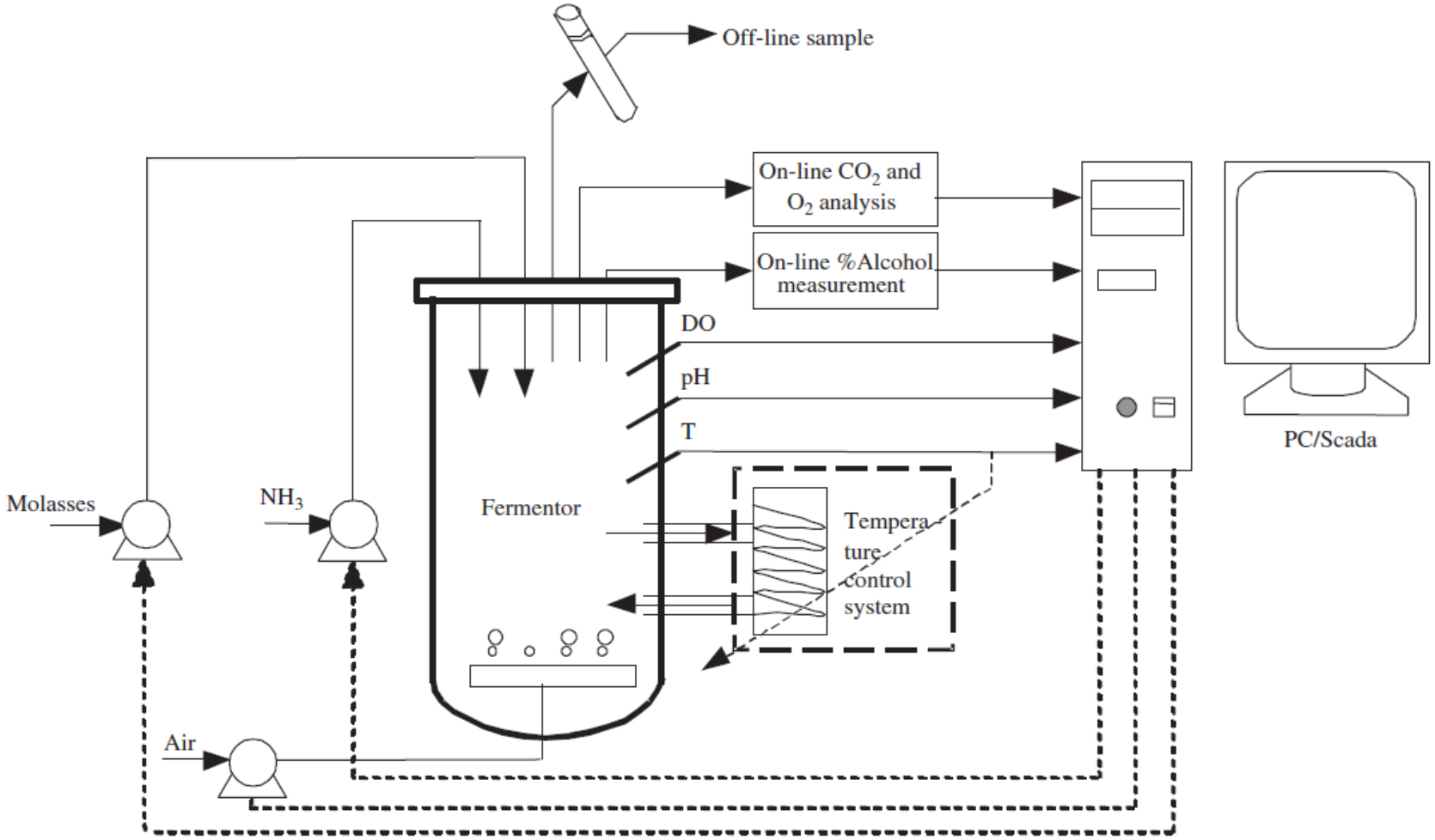






Webster, T. A., Hadley, B. C., Dickson, M., Busa, J. K., Jaques, C., & Mason, C. (2021). Feedback control of two supplemental feeds during fed-batch culture on a platform process using inline Raman models for glucose and phenylalanine concentration. *Bioprocess and Biosystems Engineering*, *44*(1), 127-140.

Example: Closed loop control of *Saccharomyces cerevisiae* fed-batch



Control Engineering Practice 14 (2006) 959–974

Total masses in fed-batches

- In fed batches run for **biomass** or a **primary product**, the substrate provisioning will typically be large. Thus:
  - The maintenance is small compared to the substrate used for cell growth
  - Mainly growth-related (primary) metabolites are produced
- The yield coefficients remain **constant** throughout the process. This includes the batch and the fed-batch phase.
- **All substrate** entering the bioreactor is consumed.
- Calculating the total substrate amount consumed per batch:

$$S_{tot} = S_{batch} + S_{feed} = S_{batch} + \int_0^{t_{end}} F s_{in} dt$$

S_{batch}: Substrate amount in batch phase [kg or g]

Total biomass and product mass in fed-batches

- Based on the biomass yield coefficient $Y_{\frac{X}{S}}$, we convert the total provided substrate into **biomass**.

$$X_{tot} = X_{inoc} + Y_{X/S} S_{tot}$$

- The same approach may be used for a **primary product**

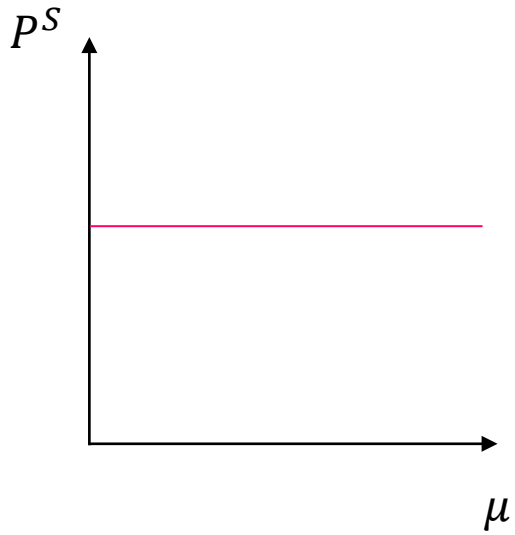
$$P_{tot} = P_{inoc} + \mathrm{Y}_{\frac{\mathrm{P}}{\mathrm{S}}} \mathrm{S}_{\mathrm{tot}}$$

X_{inoc}: biomass in the inoculum [kg or g]
P_{inoc}: product amount in the inoculum [kg or g]

Total product mass in fed-batches

- Some products are produced independently of the growth rate but proportional to the amount of biomass available with a reaction rate q.
- Assuming that all substrate is immediately converted to biomass:

$$\frac{dP}{dt} = r_p = qX = q_p Y_{\frac{X}{S}} S_{tot}(t) = q_p \left(X_0 + Y_{\frac{X}{S}} \int_0^t F s_{in} dt \right)$$



Integrating from 0 to t results in

$$P(t) = P_0 + qp \left(X_0 t + \int_0^t \int_0^t F s_{in} Y_{\frac{X}{S}} \, dt \, dt \right)$$

$S_{tot}(t)$: total added substrate amount up to time t [g]
X_0: biomass at feed start [g]
P_0: product concentration at feed start [g]
q_p: reaction rate [g $g^{-1}h^{-1}$]

Question

Why is it necessary to set up the differential equations for fed-batch based on total amounts (biomass, products, etc.)?

Are fed-batch or repetitive fed-batch cultivations always better than batch cultivations?

Summary of fed-batch technology

Positive	Negative
• High cell density	• Closed-loop control requires expensive PAT infrastructure
• Control of growth / metabolism	• Exponential feed needs high levels of oxygen and nutrients
• Always low nutrient concentrations	
• High product titers	• With constant feed rates change in metabolism by successively changing dilution rate
• Increased space-time-yield	• Technical limitations are often reached (e.g., limited by OTR)
• Control of product quantity and quality (post-translation modification)	

- In biotechnology, (fed-)batch cultures dominate the field of bioprocesses (ease of implement, prior knowlegde, regulatory requirements).
- Fed-batch cultivations are very suitable for reaching high cell-densities from substrates that usually lead to substrate inhibition phenomenons. However, for very high cell densities, the maintenance energy and aeration needs to be considered (see also TP fed-batch & lecture on gas transfer).
- When working with differential equations, always start from the mass balance since the volume is changing!
- Depending on the type of product formation (e.g., directly coupled to growth) and the production strain particular fermentation techniques need to be applied.
- Screening technology enables the selection of the optimal production strain. Direct and indirect biomass measurements simplify the follow up of biomass evolution.

Task 12

In a fed-batch culture operating with intermittent addition of glucose solution, values of the following parameters are given at time t = 2 h, when the system is at quasi-steady state.

$V = 1000$ ml $\qquad$ $dV/dt = 200$ ml h^{-1}

$s_0 = 100$ g glucose L^{-1} $\qquad$ $\mu_{max} = 0.3\ h^{-1}$

$K_s = 0.1$ g gluscose L^{-1} $\qquad$ $Y_{XS} = 0.5$ g cells / g glucose

$X_0 = 30$ g (total = x_o^t)

a) Find V_0 (the inital volume of the culture).

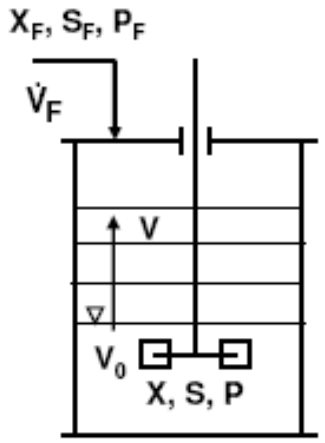
b) Determine the concentration of growth-limiting substrate in the vessel at quasi-steady state.

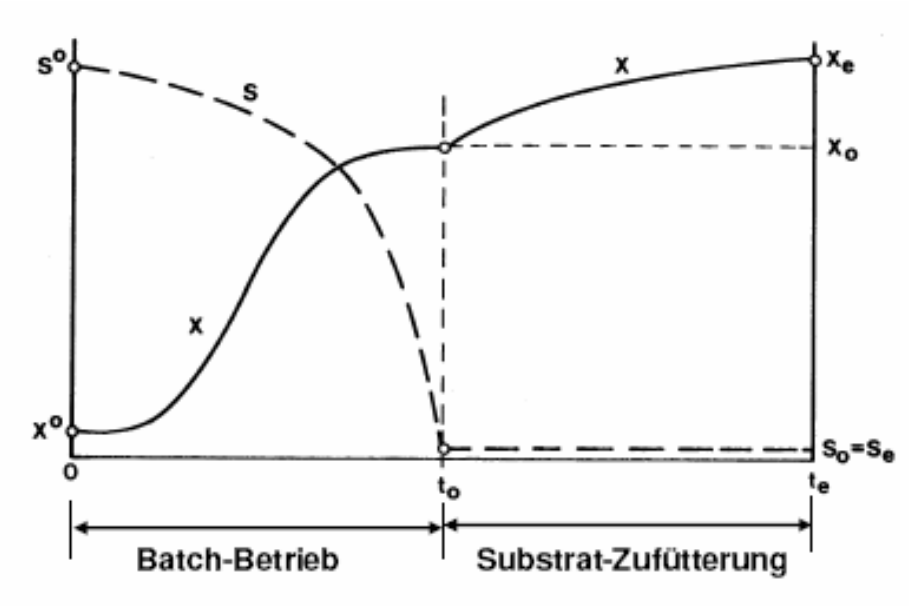
c) Determine the concentration and total amount of biomass in the vessel at t = 2 h (at quasi-steady state).

d) If $q_p = 0.2$ g product /g cells h, $p_0 = 0$, determine the concentration of product in the vessel at t = 2h.

Task 13

The concentration of the substrate should be kept constant at a very low concentration in a fed-batch culture. Please explain how the feed rate of the pump should look like in order to maintain a very low residual substrate concentration (there are several solutions).





Task 14

a) Analyse the dynamics of the fed-batch culture with respect to x, s and μ, when the feed rate is kept constant.

b) In a fed-batch culture, the following problem requires a solution:
How can you compute the feed rate of substrate in order to keep the value of μ constant (μ can be respresented by a function of substrate concentration).