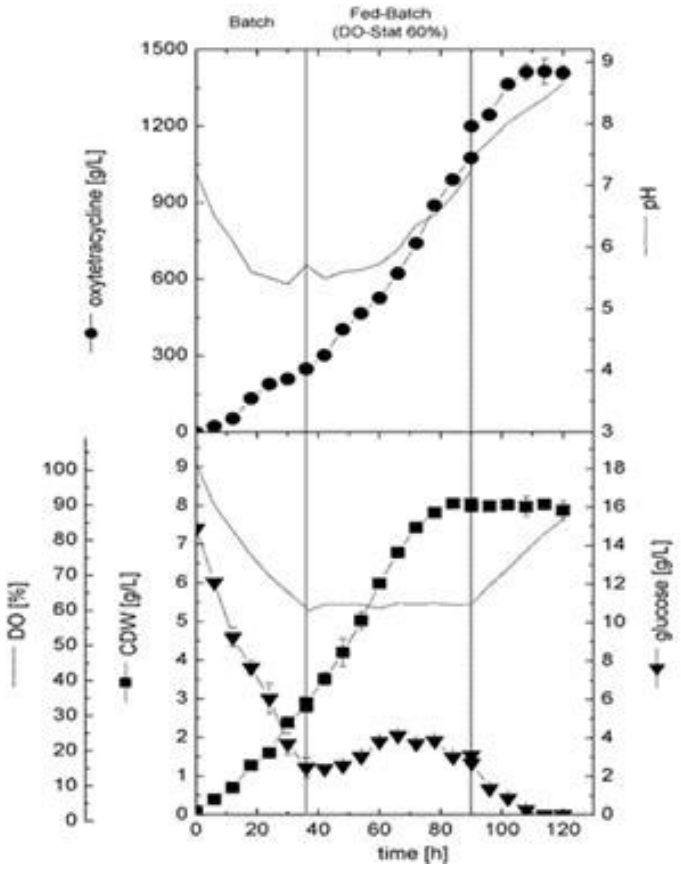
Improvement of Productivity & Fed-Batch Cultivation

Course ChE-311
"Biochemical Engineering"

manfred.zinn@epfl.ch

Agenda

- Improvements of batch performance
- Bioprocess analytics
- Calculations concerning fed-batch cultivation
- Fed-batch applications



https://doi.org/10.1590/S1516-89132015050184

Strategies to improve the productivity of a bioprocess

Generally, a bioprocess can be improved at three levels:

i. Strain development

ii. Medium optimization

iii. Improvement of the bioprocess unit operations (*e.g.,* preculture, feeding strategy)

i) Improvement of productivity by cell engineering

Improvement of production cells

There is a possibility to increase the productivity by genetic engineering.

The objectives are:

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

Techniques:

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

Typical host cells

Host Organism	Type	Advantages	Application Examples
Escherichia coli (E. coli)	Gram-negative bacterium	Fast growth, easy manipulation, cost-effective	Insulin, growth hormones, enzymes
Bacillus subtilis	Gram-positive bacterium	Secretes proteins, lacks endotoxins	Proteases, amylases, industrial enzymes
Corynebacterium glutamicum	Gram-positive bacterium	High tolerance to metabolites, good for amino acid production	Amino acids, recombinant proteins
Saccharomyces cerevisiae	Yeast (fungus)	Performs PTMs, GRAS, well-characterized	Bioethanol, vaccines, enzymes
Pichia pastoris (*Komagataella phaffii*)	Yeast (fungus)	High-density fermentation, strong promoters	Biopharmaceuticals, enzymes
Yarrowia lipolytica	Yeast (fungus)	High lipid metabolism, protein secretion	Lipases, specialty chemicals, proteins
Aspergillus niger / *Trichoderma reesei*	Filamentous fungi	Excellent secretion, robust enzyme producers	Cellulases, amylases, citric acid
Streptomyces spp.	Actinobacteria	Naturally produces antibiotics, handles complex proteins	Antibiotics, secondary metabolites
Sf9 / Sf21 (Insect cells)	Insect cells	Eukaryotic PTMs, high protein yield	Vaccines, VLPs, complex proteins
CHO / HEK293 (Mammalian cells)	Mammalian cells	Authentic human-like PTMs	Monoclonal antibodies, therapeutics

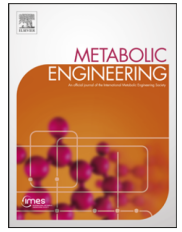
Increase of product yield: Simulation of pathways

Metabolic Engineering 35 (2016) 148–159

Contents lists available at ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/ymben



Identification of metabolic engineering targets for the enhancement of 1,4-butanediol production in recombinant *E. coli* using large-scale kinetic models ☆

Stefano Andreozzi [a,b,1], Anirikh Chakrabarti [a,b,1], Keng Cher Soh [a,b], Anthony Burgard [c], Tae Hoon Yang [c], Stephen Van Dien [c], Ljubisa Miskovic [a,b], Vassily Hatzimanikatis [a,b,*]

[a] *Laboratory of Computational Systems Biotechnology (LCSB), Swiss Federal Institute of Technology (EPFL), CH-1015 Lausanne, Switzerland*
[b] *Swiss Institute of Bioinformatics, CH-1015 Lausanne, Switzerland*
[c] *Genomatica, Inc., San Diego, CA, USA*

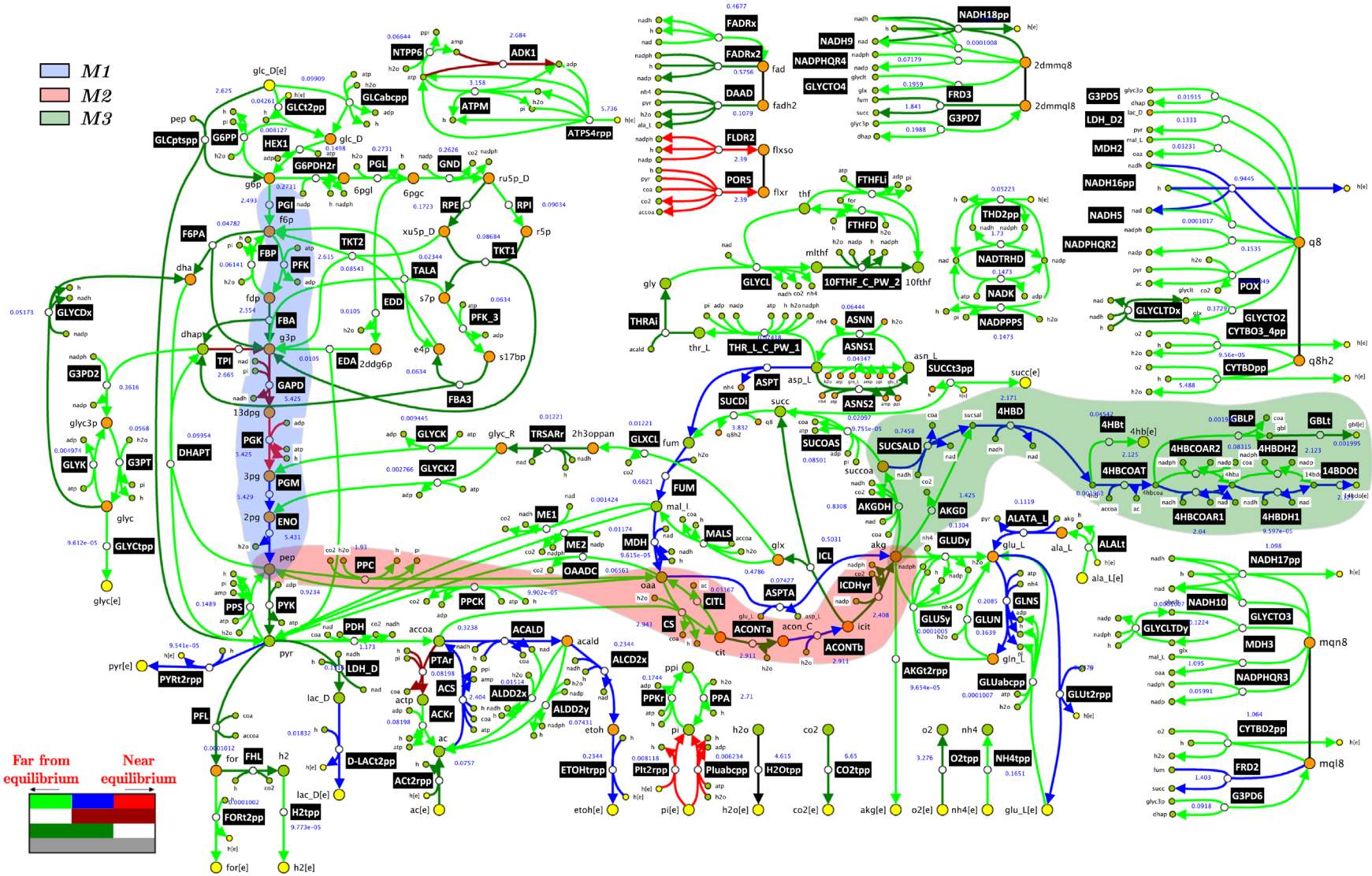
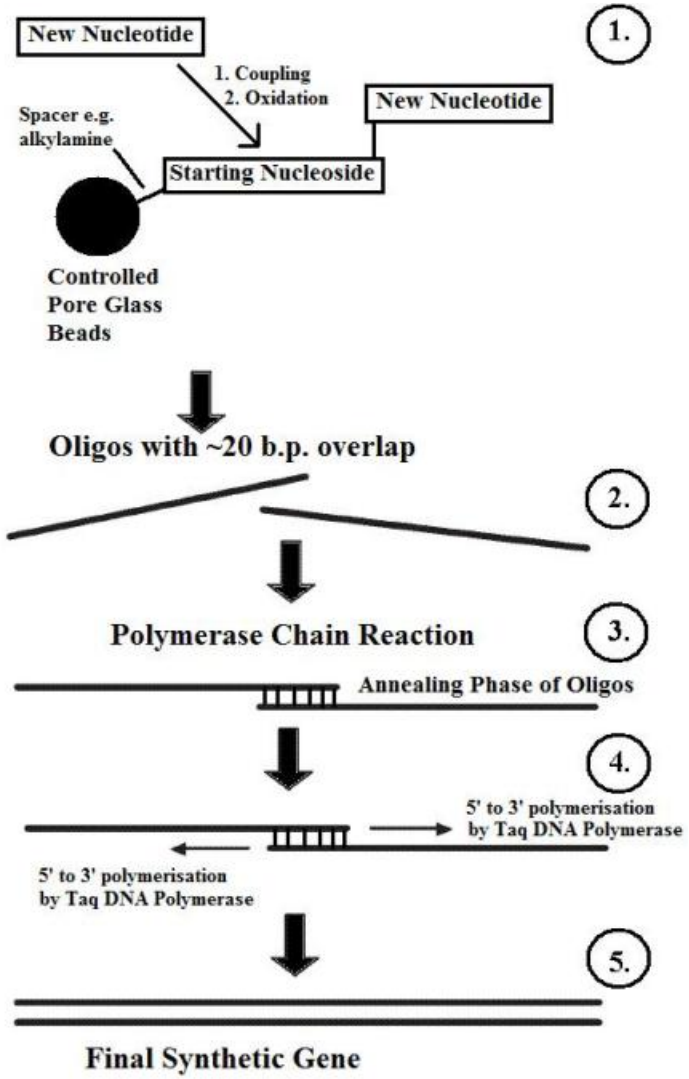


Fig. 1. BDO producing *E. coli* metabolic network. Color-coding of the reactions denotes the distance from the thermodynamic equilibrium of their enzymes. The control over BDO production is centered around: Module 1 (*M*1)-focusing around PFK, FBA, GAPD, PGK and PGI; Module 2 (*M*2)-focusing around PPC, CS, ACONTa; Module 3 (*M*3)-focusing around AKGD, 4HBCOAT and 4HBDH2.

Buy your missing gene: Gene synthesis service



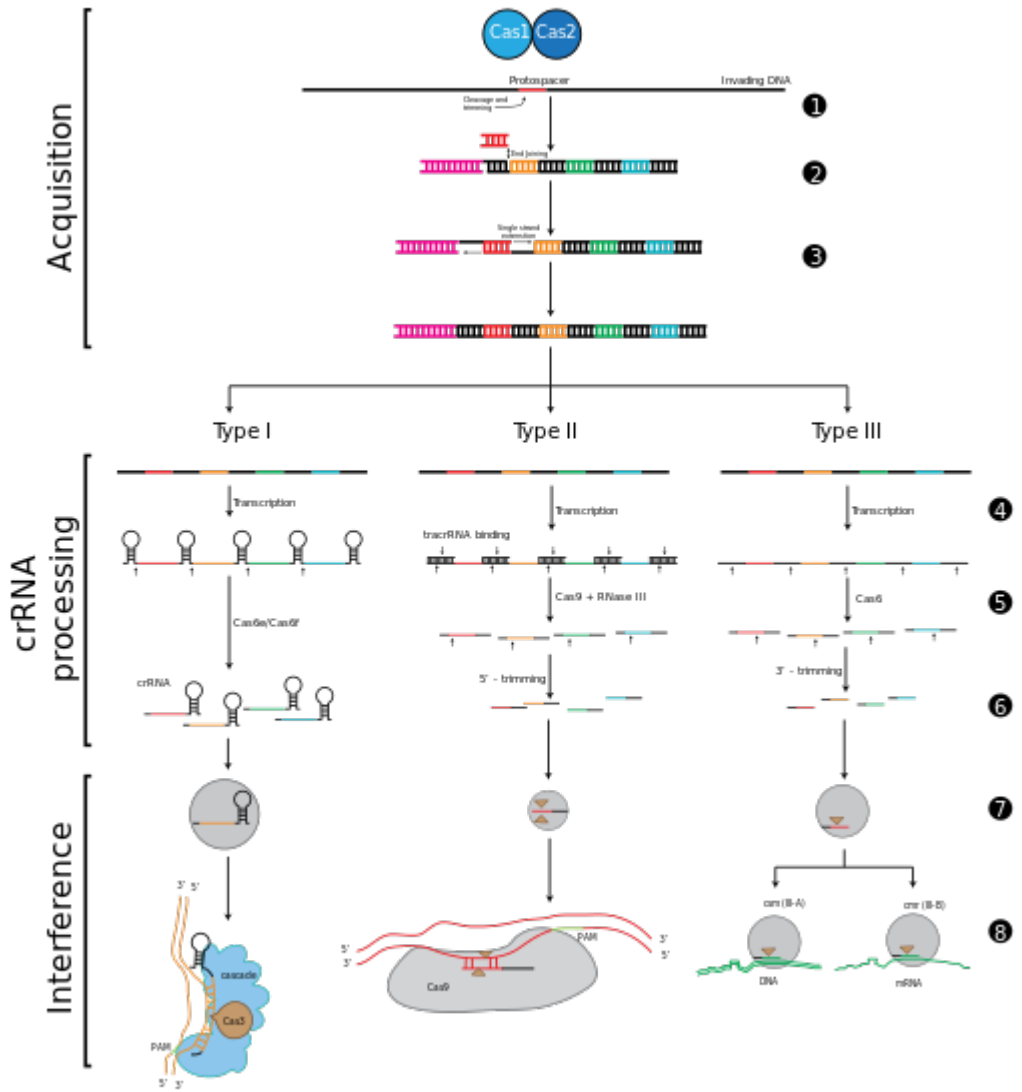
http://www.genscript.com/gene_synthesis.html

https://www.brain-biotech.de/en/

Recent methods to enhance strain performance using mutagenesis

- Directed mutagenesis (assisted by evolution)
- Site directed mutagenesis (genetic engineering became very efficient because of known genome sequences)
- PCR mutagenesis (error prone PCR of gene sequence)
- Transposon mutagenesis
- Signature tagged mutagenesis (used in research of pathogens)
- Insertional mutagenesis (change of reading frame by insertion of one or more bases)
- CRISPR/CAS9 Method to put the gene on the chromosome: => Maintenance of a stable production yield!

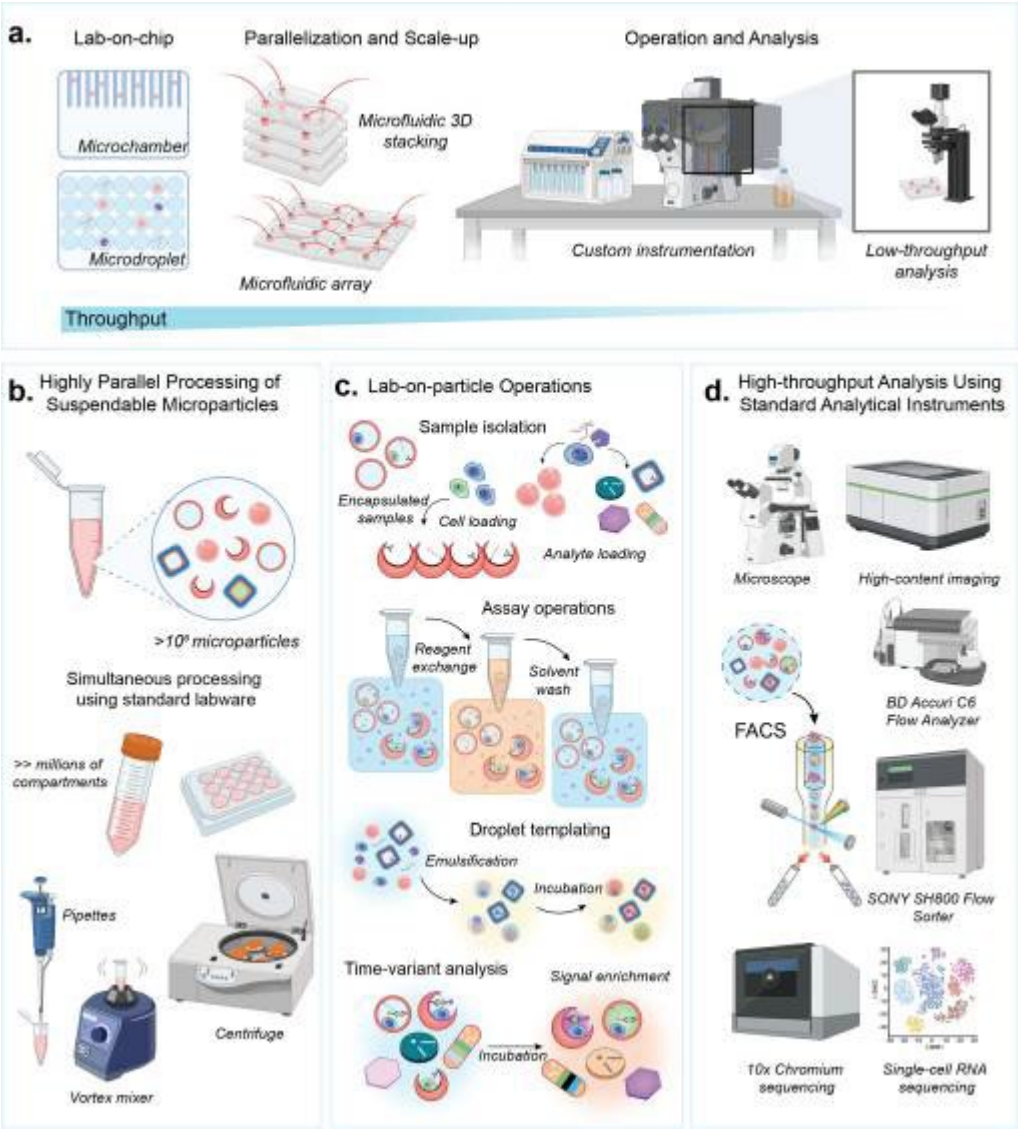
Clustered regularly interspaced short palindromic repeats (CRISPR)



The stages of CRISPR immunity for each of the three major types of adaptive immunity. (1) Acquisition begins by recognition of invading DNA by Cas1 and Cas2 and cleavage of a protospacer. (2) The protospacer is ligated to the direct repeat adjacent to the leader sequence and (3) single strand extension repairs the CRISPR and duplicates the direct repeat. The crRNA processing and interference stages occur differently in each of the three major CRISPR systems. (4) The primary CRISPR transcript is cleaved by cas genes to produce crRNAs. (5) In type I systems Cas6e/Cas6f cleave at the junction of ssRNA and dsRNA formed by hairpin loops in the direct repeat. Type II systems use a trans-activating (tracr) RNA to form dsRNA, which is cleaved by Cas9 and RNaseIII. Type III systems use a Cas6 homolog that does not require hairpin loops in the direct repeat for cleavage. (6) In type II and type III systems secondary trimming is performed at either the 5' or 3' end to produce mature crRNAs. (7) Mature crRNAs associate with Cas proteins to form interference complexes. (8) In type I and type II systems, interactions between the protein and PAM sequence are required for degradation of invading DNA. Type III systems do not require a PAM for successful degradation and in type III-A systems basepairing occurs between the crRNA and mRNA rather than the DNA, targeted by type III-B systems.

https://en.wikipedia.org/wiki/CRISPR

From lab on a chip to lab on a particle.

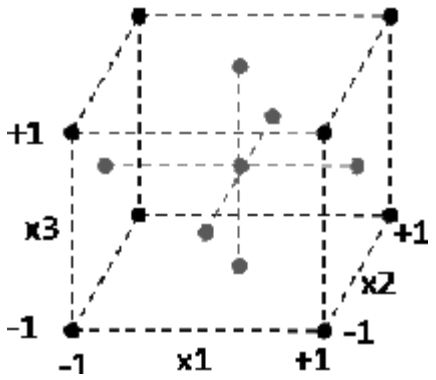


(a) **Lab on a chip technology** uses microchambers or droplets to confine reactions, enabling the analysis of target cells or molecules with high precision. Parallelization and scale-up rely on the 2D surfaces of chips and custom instrumentation, which often lead to reduced analysis throughput. **(b)** **Lab on a particle technology** enables millions of microparticle-based compartments to be scaled in 3D in standard tubes, where fluidic operations are performed using standard laboratory equipment. **(c)** Operations on particles include cell loading and encapsulation, analyte binding, reagent exchange and washing, and templating of water-in-oil emulsions. Signal enrichment can occur on particles through reactions that are either confined or locally bound. Microparticles are barcoded by shape, size, pattern, color, or other means to enable time-variant analysis as reactions or cell behavior progresses over time. **(d)** Microparticles are analyzed using standard analytical instruments compatible with cells, such as microscopes, flow cytometers, fluorescence activated cell sorters and single-cell sequencing instruments.

ii) Improvement of the productivity by medium optimization

Composition of the growth medium

The medium composition plays a crucial role for an optimal production. Besides using literature data to design a medium and thus considering growth yields, the statistical approach (*design of experiments (DOE)*) helps to determine the most significant parameters for cultivation in a very efficient way.



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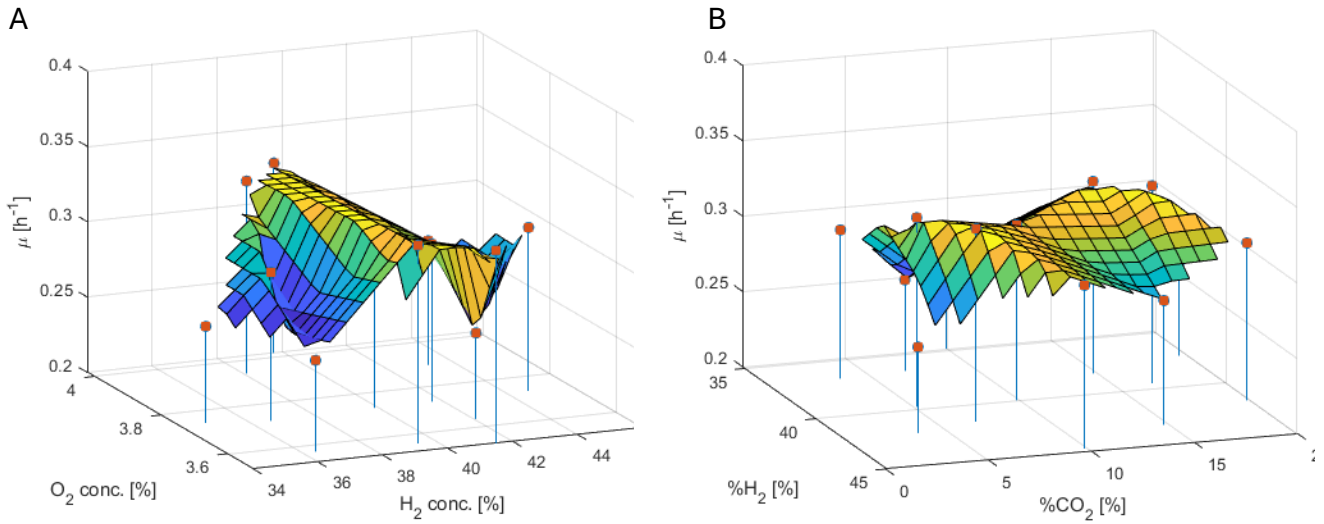


Figure 1: Effect of the gas composition on the specific growth rate (μ) during cultivations in a 1 L bioreactor without CO for A) a variation of O_2 and H_2 concentration and B) for a variation of H_2 and CO_2 concentrations.

Florian Miserez (Growth of Idionella sp., unpublished data)

See also chapter «Medium»

iii) Improvement by Bioprocess Engineering

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*)

Why do we need microbioreactors?

≠

Optimization prior to scale-up = reduction of time and costs

Why do we need microbioreactors?

"To restate the obvious, protein expression is an inexact science at present…

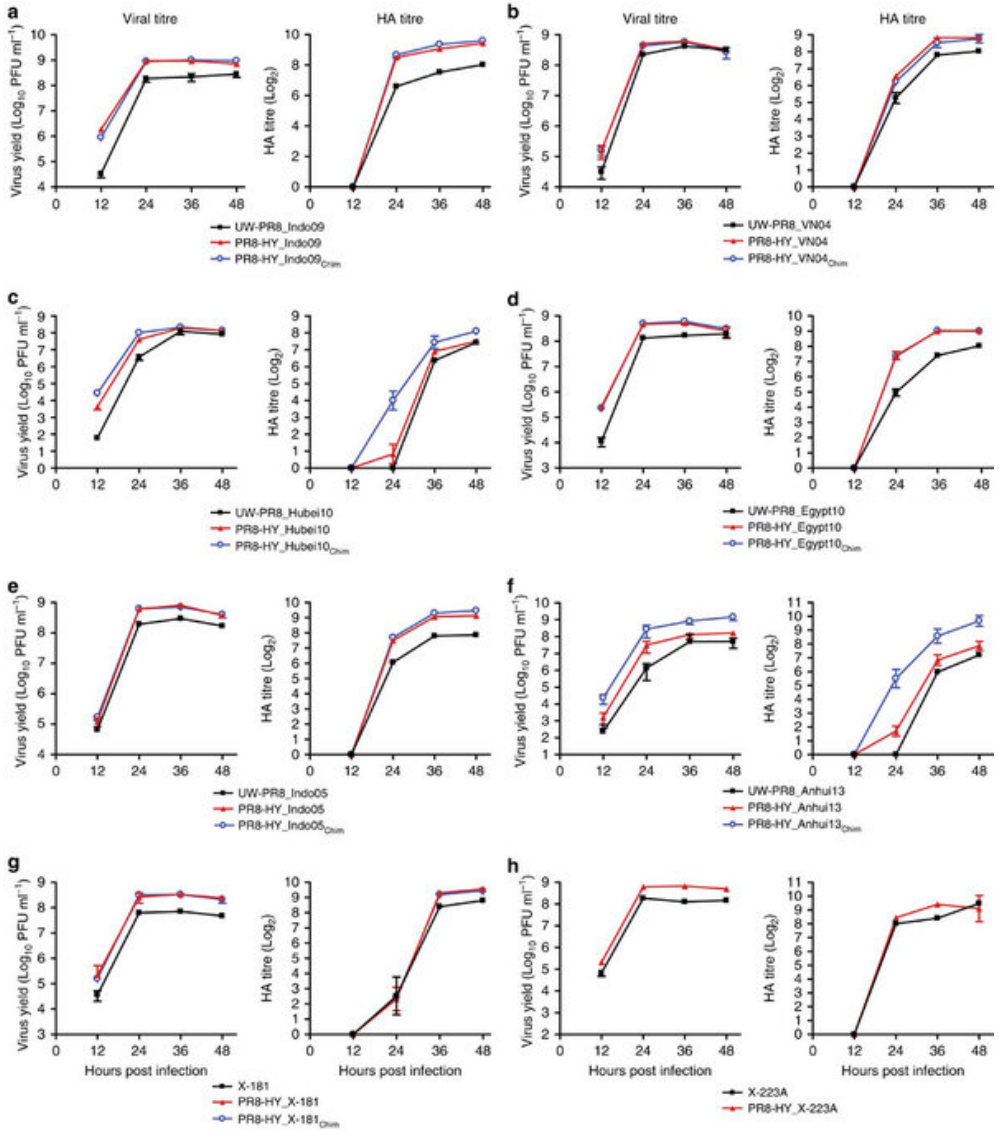
The solution: Implementation of microbioreactors

…The procedures employed are relatively quick and uncomplicated, and the rewards for success are great."

Contributed by Paul F. Schendel
Current Protocols in Molecular Biology (1998) 16.1.1-16.1.3

Medium throughput screening of as many parameters as possible…
…as early as possible!

Evaluation of growth: Classical methods



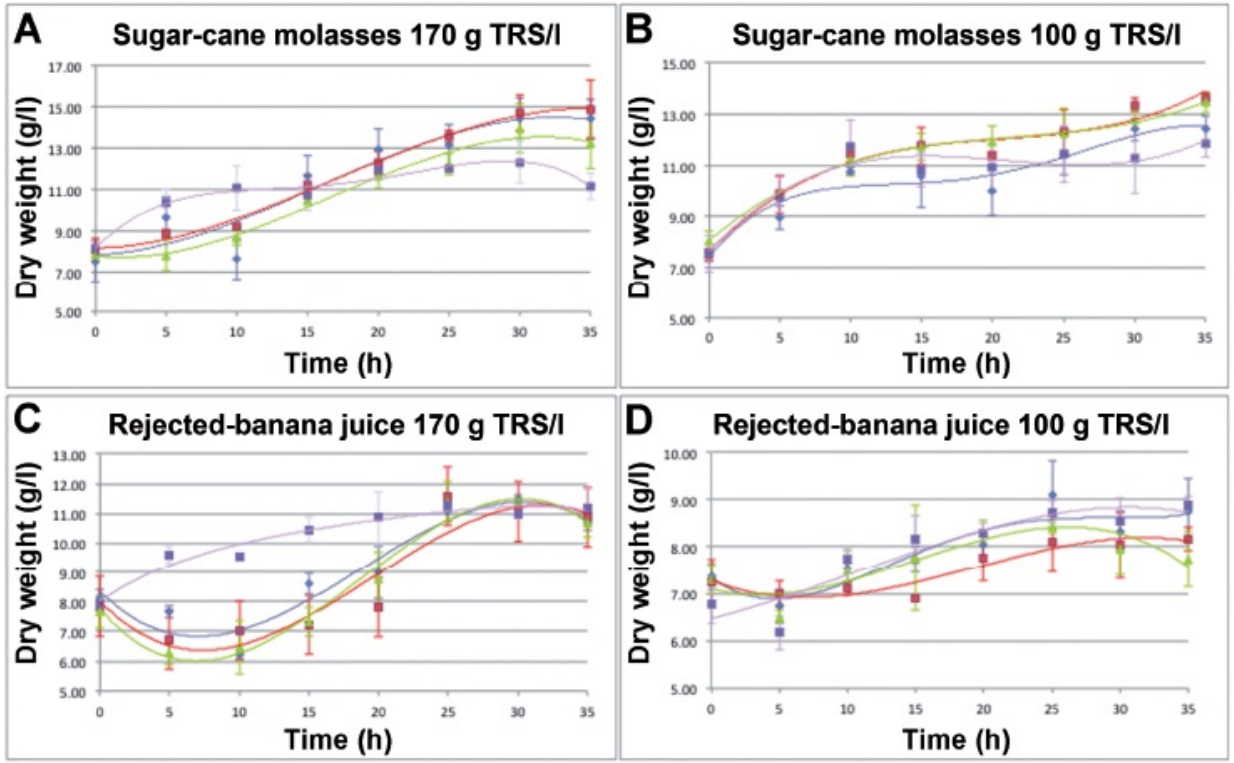
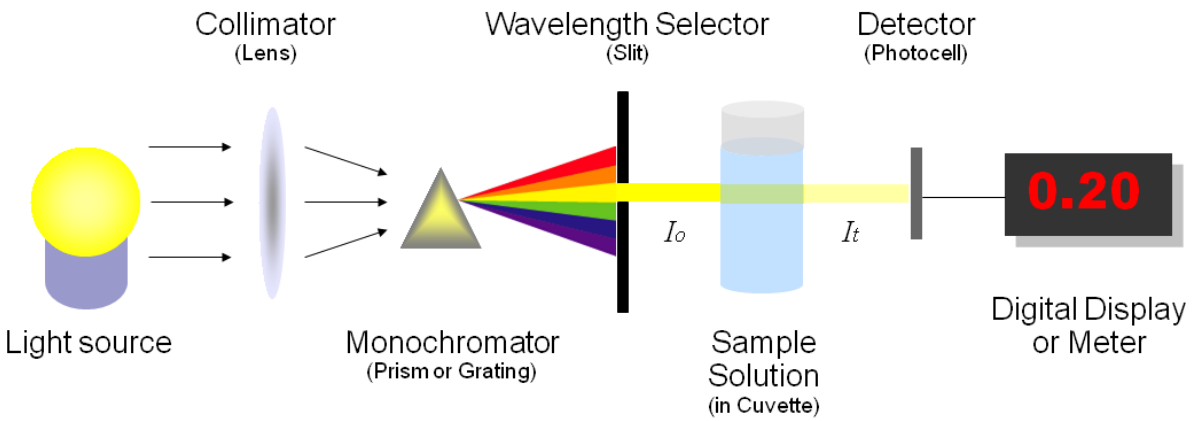


Figure 1. Kinetics of cellular biomass production of *Saccharomyces cerevisiae* (Fungi: Ascomycota) strains: **CBS8066** (♦), **recombinant GG570-CIBI** (▪), **recombinant GG570-CIBII** (▲) and **Ethanol Red®** (X) under anaerobic conditions and in sugar-cane molasses with *170* (**A**) or *100 g/l TRS* (**B**) or rejected-banana juice with *170* (**C**) or *100 g/l TRS* (**D**)

Only distinct data points!

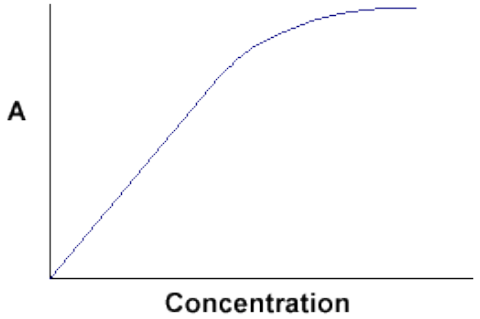
Direct measurement of microbial growth

Optical density



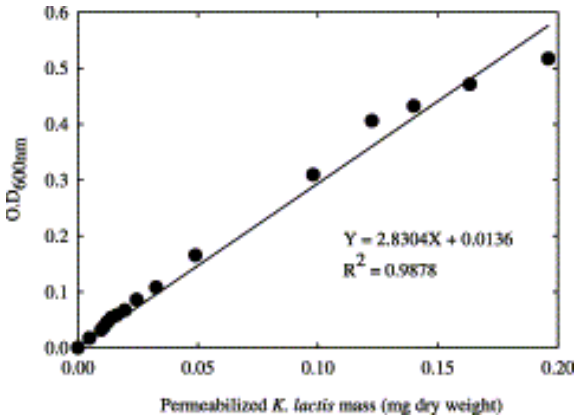
Beer-Lambert law

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



Dilution with blank solution (e.g. dH_2O)

$$OD_{cells} = OD_{measured} \cdot f - OD_{medium}$$ (f = dilution factor)



Correlation with CDW

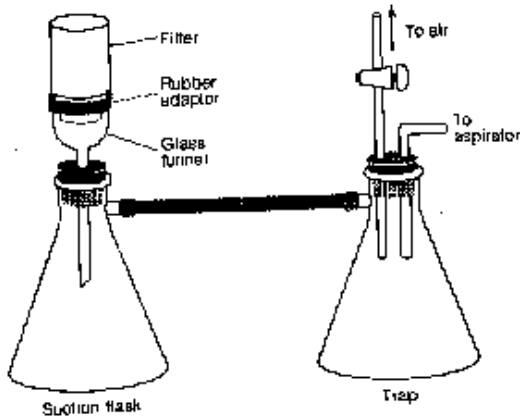
Direct measurement of microbial growth

Cell dry weight:

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

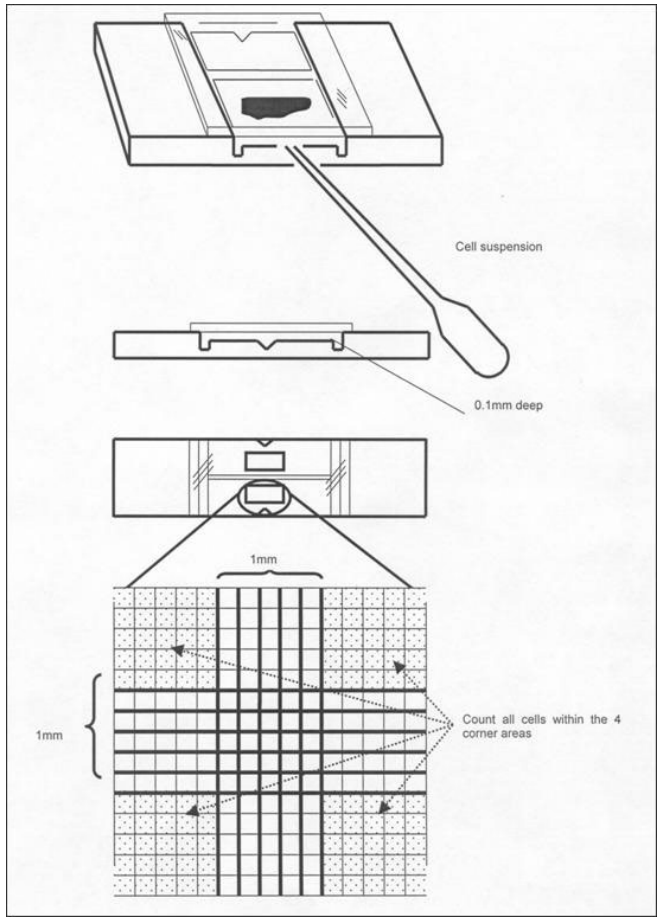
- Use triplicates.
- Wash loaded filters with ca. 10 mL of 10mM $MgSO_4$ or 0.9% NaCl solution.
- Dry loaded filters at 90°C for 20 hours and cool down in a desiccator.

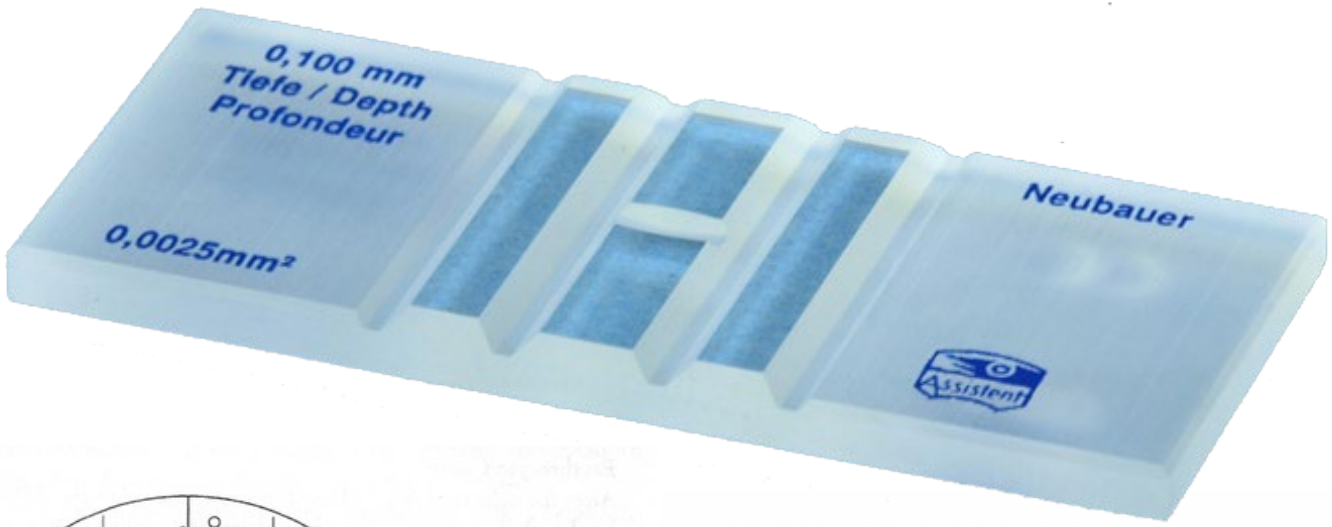
- Alternatively also preweighed and dried Eppendorf or glas tubes can be used.

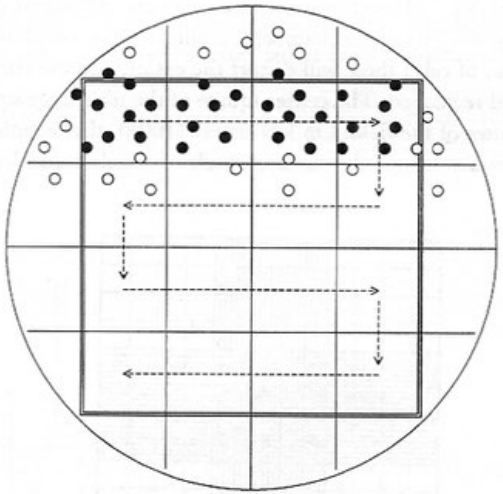


Direct measurement of microbial growth

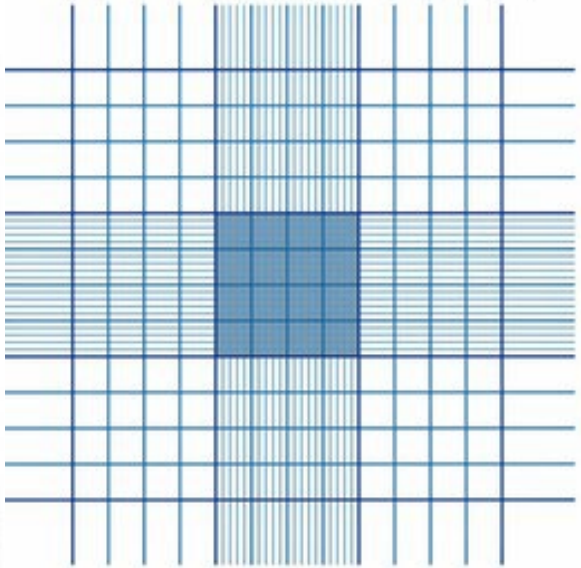
Cell number determination using microscope







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.



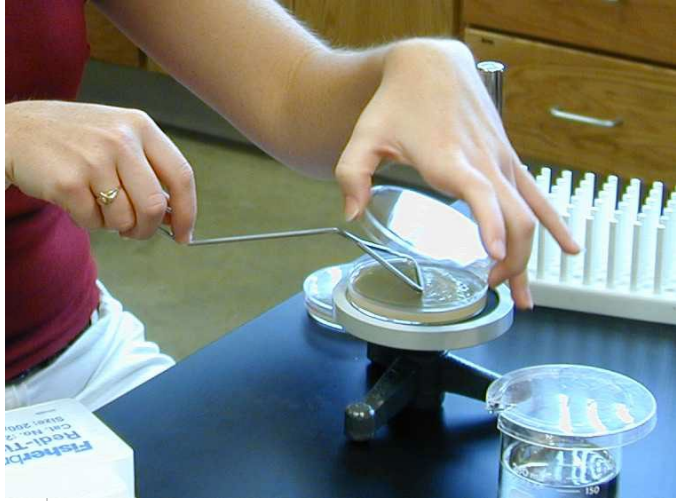
The area of a small square is $0{,}0025\,mm^2$.

A large square contains $25 \cdot 16 = 400$ small squares.

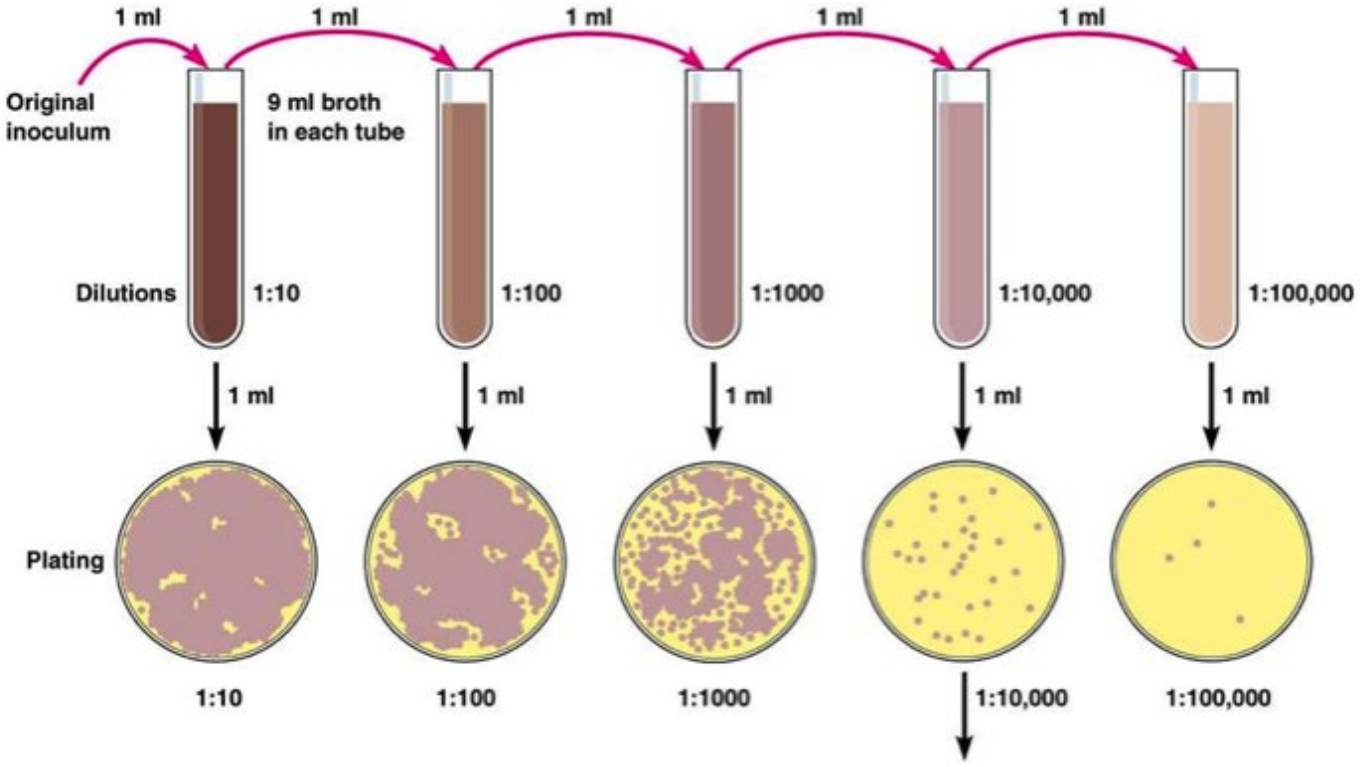
area of a large square:
$400 \cdot 0{,}0025\,mm^2 = 1\,mm^2$.

volume over a large square:
(area · depth = volume)
$1\,mm^2 \cdot 0{,}1\,mm = 0{,}1\,mm^3$
$= 0{,}1\,\mu l$

Direct measurement of microbial growth



Cell number determination plate counts

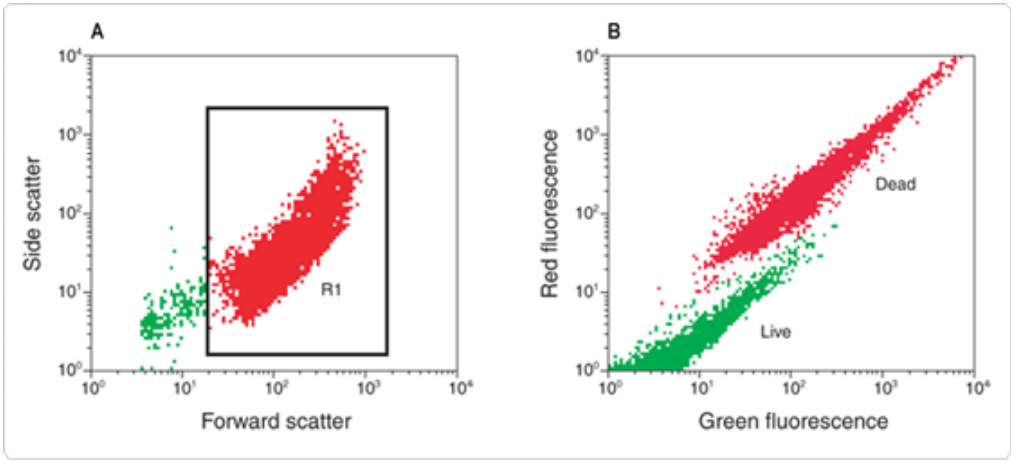


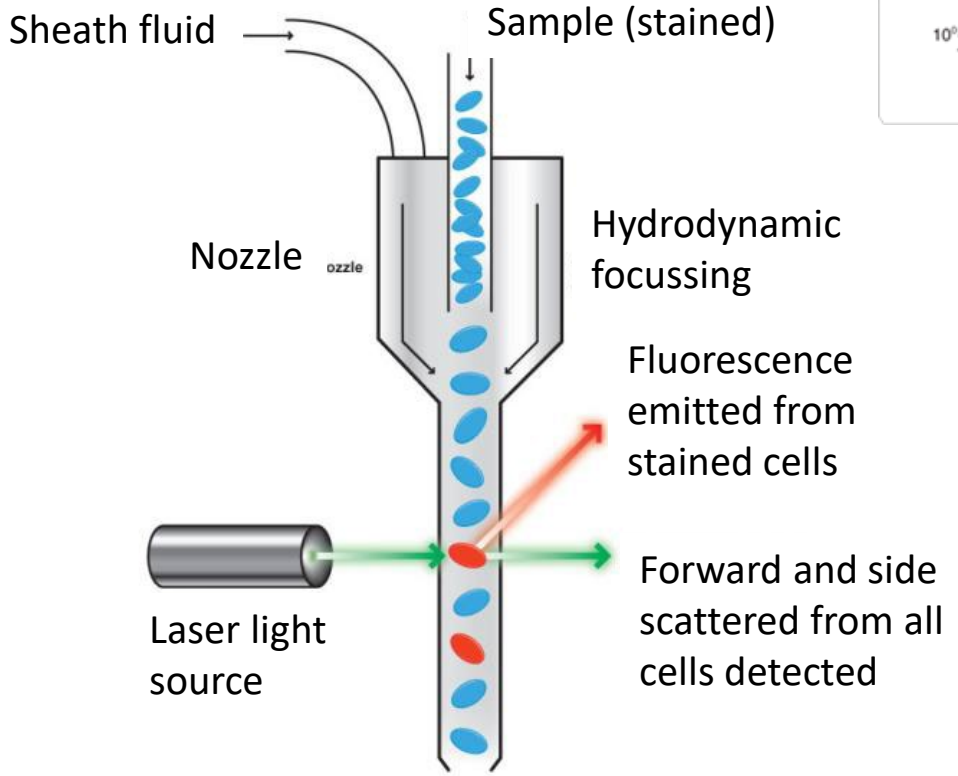
Calculation: Number of colonies on plate × reciprocal of dilution of sample = number of bacteria/ml
(For example, if 32 colonies are on a plate of $^{1}/_{10,000}$ dilution, then the count is 32 × 10,000 = 320,000 bacteria/ml in sample.)

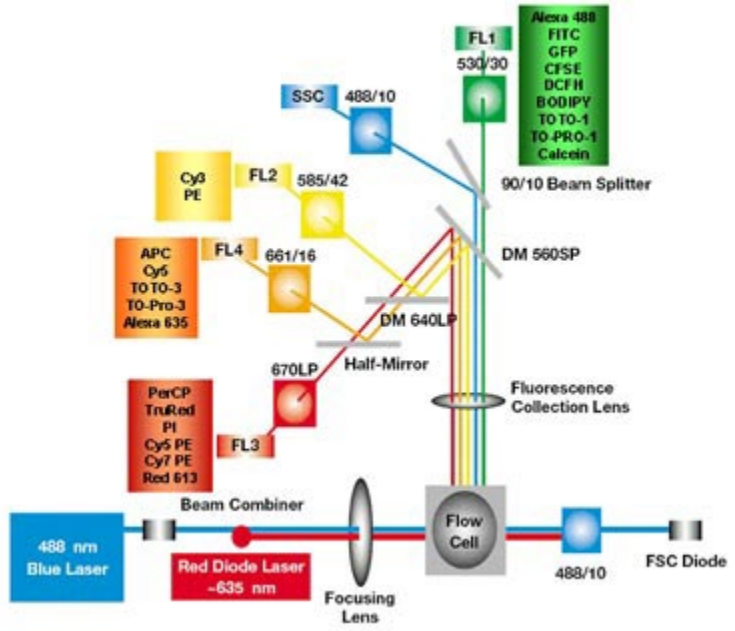
Note:

- 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

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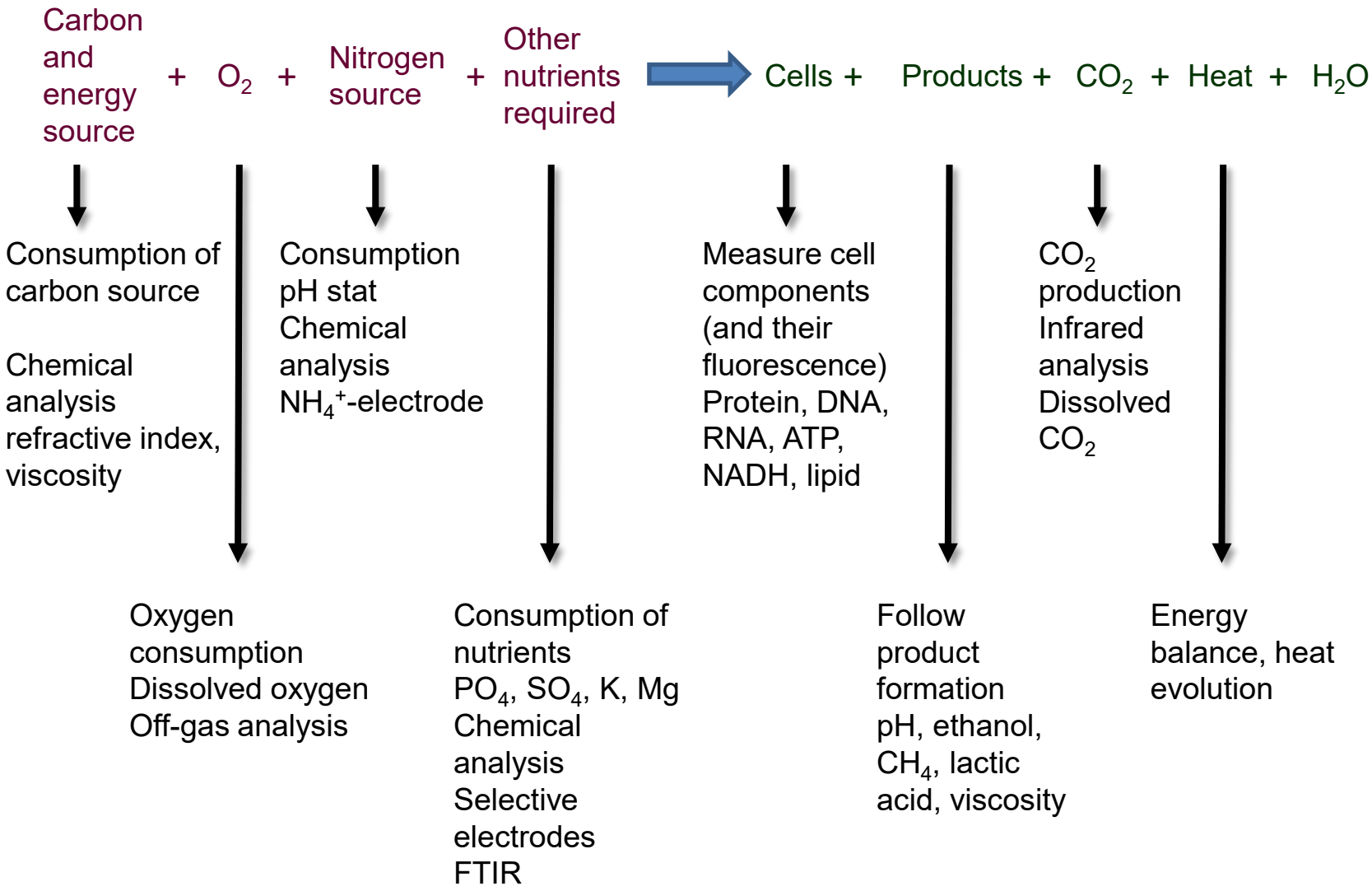
Indirect measurement of growth



APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 2000, p. 2641–2646 Vol. 66, No. 6
0099-2240/00/$04.00+0

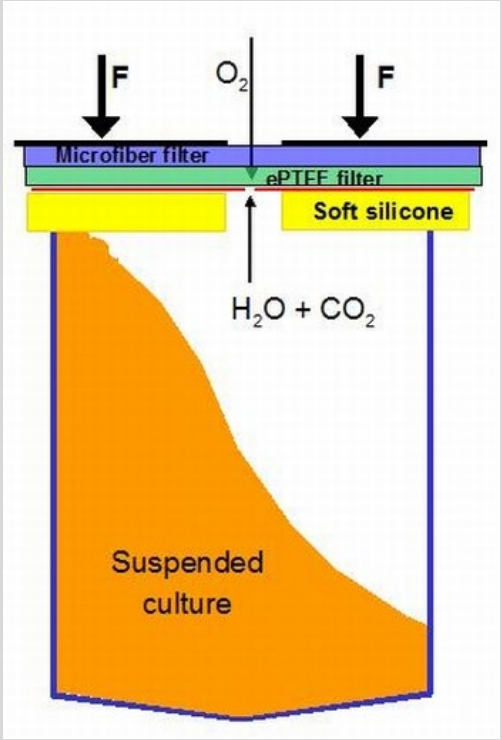
Methods for Intense Aeration, Growth, Storage, and Replication of Bacterial Strains in Microtiter Plates

WOUTER A. DUETZ,[1]* LORENZ RÜEDI,[1] ROBERT HERMANN,[2] KEVIN O'CONNOR,[1] JOCHEN BÜCHS,[2] AND BERNARD WITHOLT[1]

Institute of Biotechnology, ETH Hönggerberg, CH 8093, Zürich, Switzerland,[1] and Chair of Biochemical Engineering, Aachen University of Technology, D-52074 Aachen, Germany[2]

Received 15 November 1999/Accepted 30 March 2000

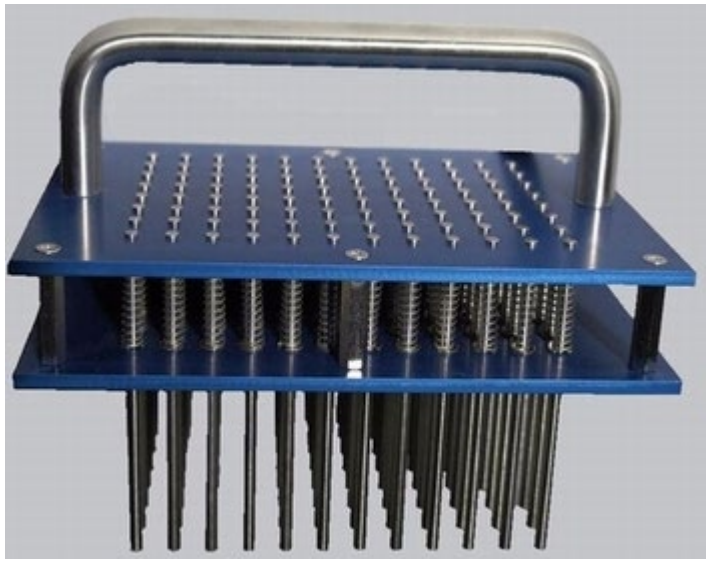
Miniaturized growth systems for heterogeneous culture collections are not only attractive in reducing demands for incubation space and medium but also in making the parallel handling of large numbers of strains more practicable. We report here on the optimization of oxygen transfer rates in deep-well 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.

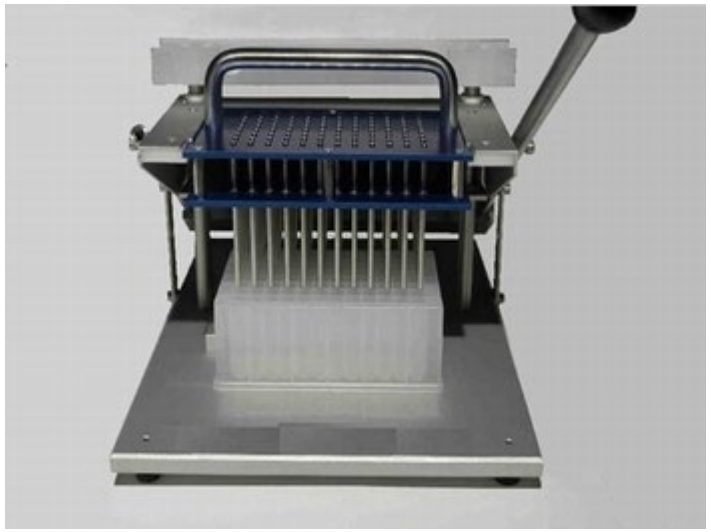


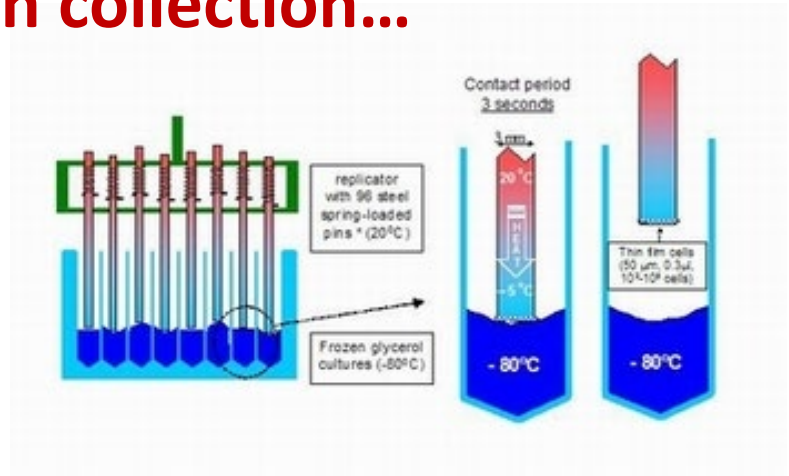
Establishment of a screening system using stock cultures

https://www.youtube.com/watch?v=r-9si98zdR8

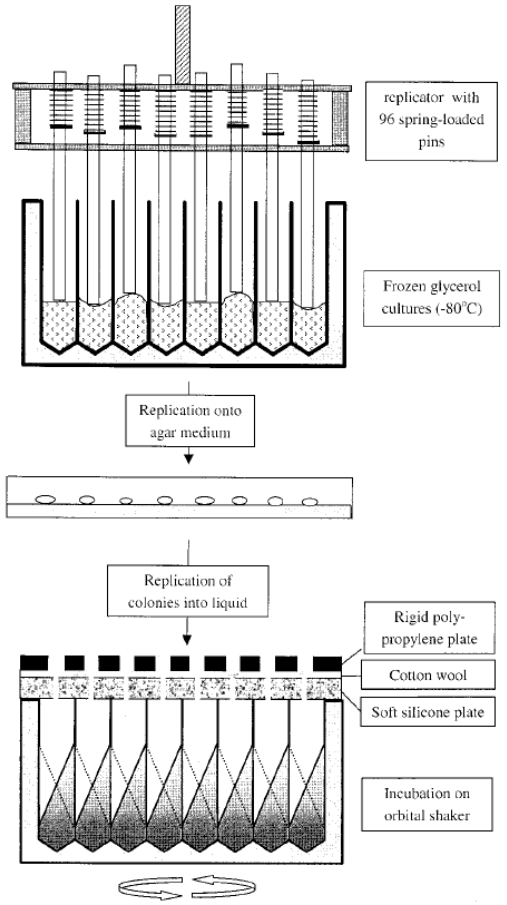
Establish your own strain collection…





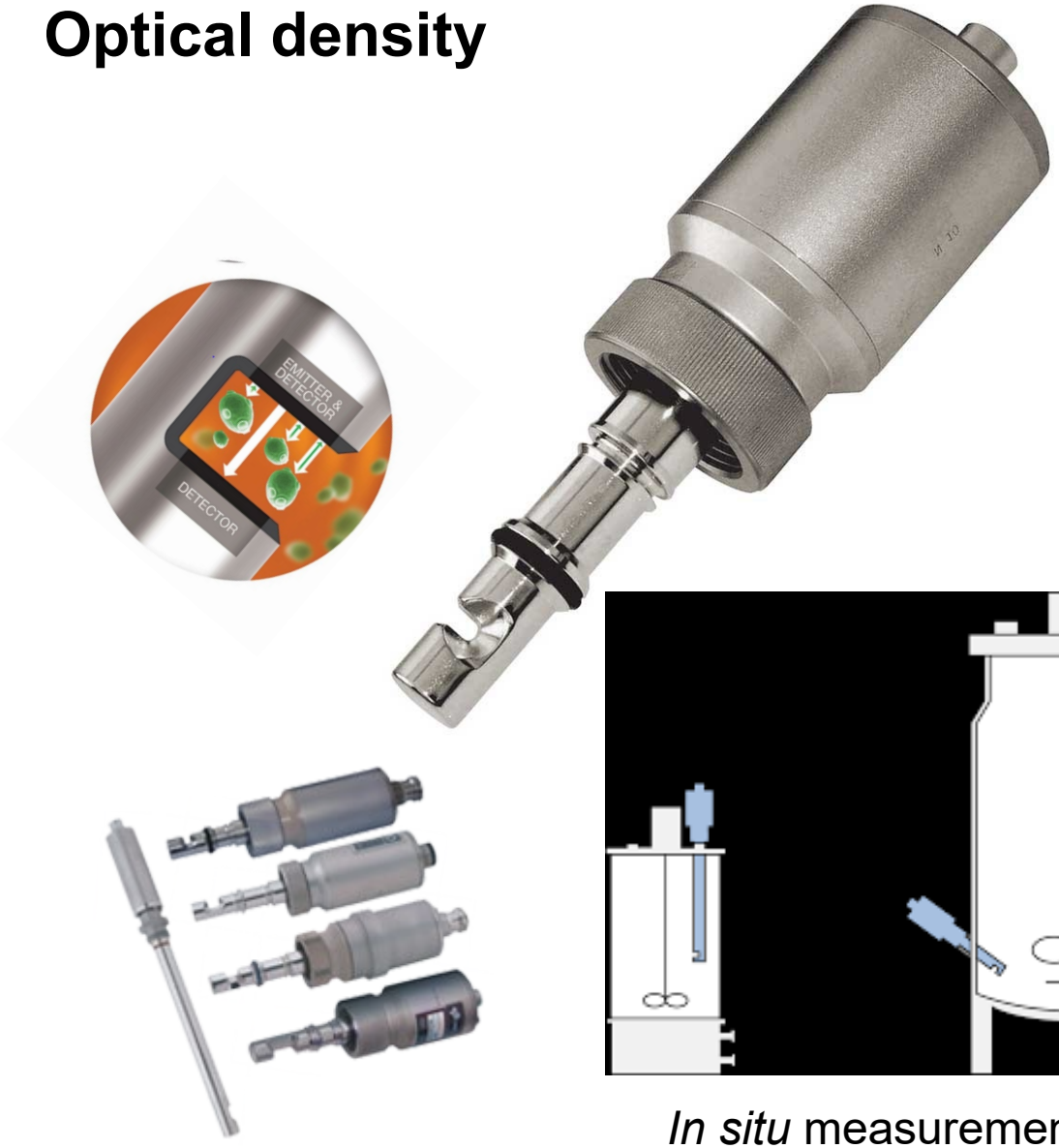


The different strains are stored in 96-well plates at -80°C.
Culture plates are inoculated by pins pressed into frozen stocks.



Direct measurement of microbial growth

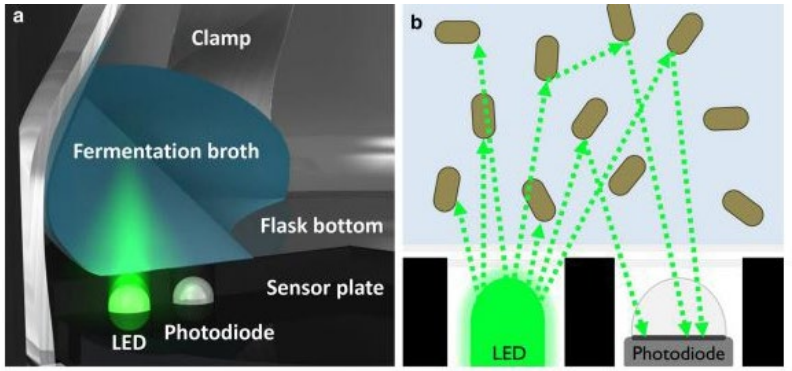
Optical density



In situ measurement

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

Back scattering

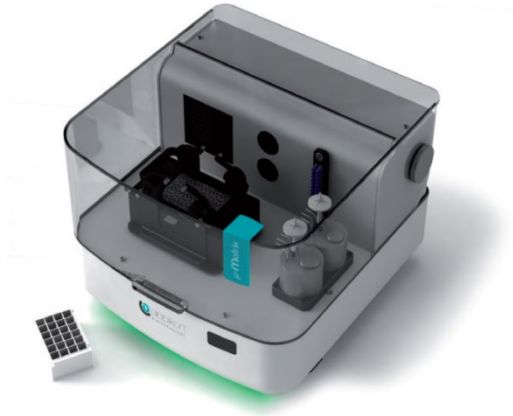


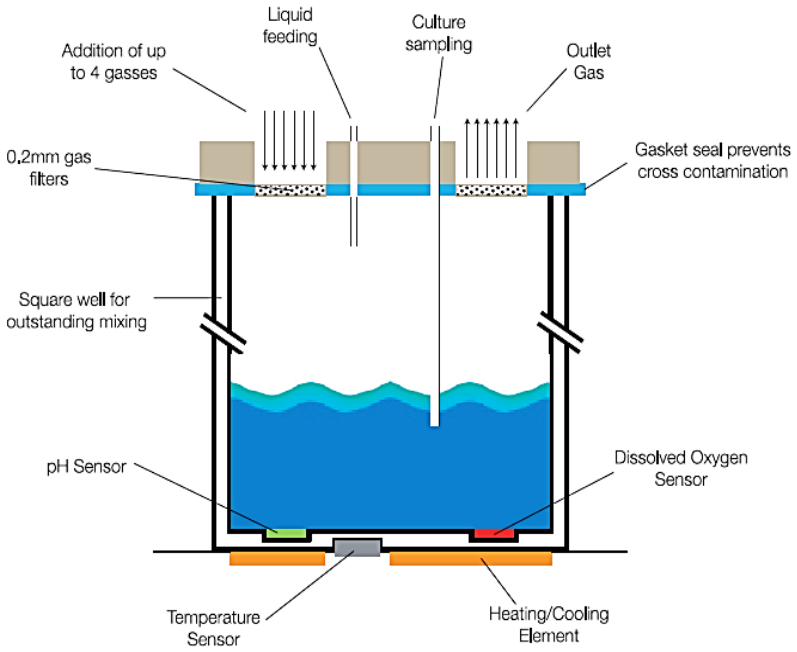
Parameter \ Measuring principle	Optical Density	Backscattering
Known technique	Yes	No
Biomass estimation	Good	Good
Sample size	Small	Not required [1]
Results with low cell densities	Good	Poor
Results with high cell densities	Poor / With dilution	Good
Small changes in biomass observable	Good [2]	Good [2]
Invasive	Variable [3]	No [1]
Automation	No	Yes
Can be used with planktonic cells	Yes	Yes
Can be used with filamentous growing organisms	No	Yes
Measuring range (OD, app.)	0 – 1	0.5 - 70
Dilution required	Yes if > 0.8	No
Comparable to other parameters	Requires calibration	Requires calibration

[1] System is often automated
[2] When within measurement range
[3] Samples must be taken

https://www.infors-ht.com/de/blog/measuring-biomass-in-shake-flasks-offline-od-vs-online-backscatter-light/

Micro-Matrix (Applikon Biosystems)





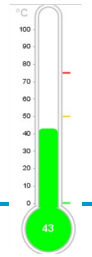
Schematic diagram illustrating the functioning of the micro-Matrix control loops.

no of wells	24
volume	2-5 mL working volume
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.reseabiotec.ch/our-products/micro-matrix/

BioLector XT Microbioreactor Features with Microfluidics Module

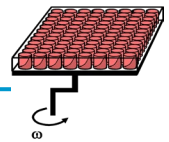
Temperature Control
10 – 50 °C

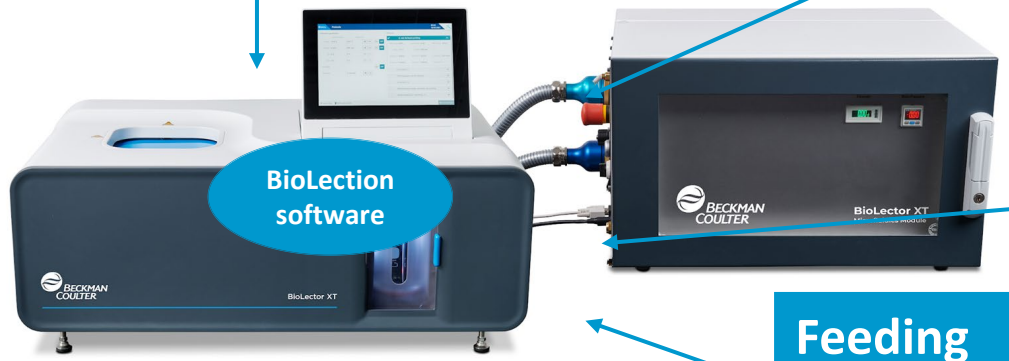


Humidified
All gasses are humidified

DO Measurement
Real-time online measurement

Shaker
For mixing and oxygen transfer





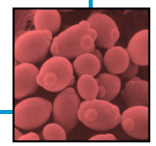
pH Measurement
Real-time online measurement

pH Control
Continuous pH control using acid or base

Feeding
Controlled, fully flexible feeding

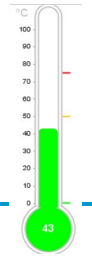
Optical Filter Modules
Up to six filter combinations for different parameters (fluorescence)

Biomass Measurement
Scattered light, *in situ*, no dilution
Up to OD_{600} = 250



BioLector XT Microbioreactor Features with Light Array Module (LAM)

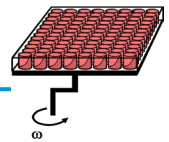
Temperature Control
10 – 50 °C

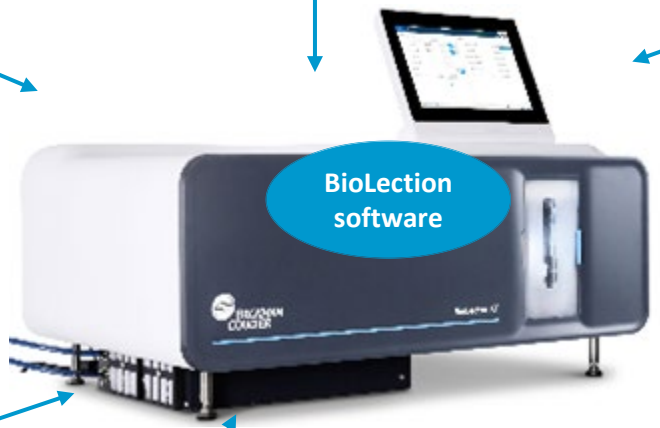


Humidified
All gases are humidified

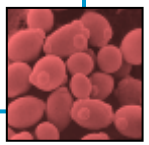
pH Measurement
Real-time offline measurement

Shaker
For mixing and oxygen transfer





Biomass Measurement
Scattered light, *in situ*, no dilution
Up to OD_{600} = 250

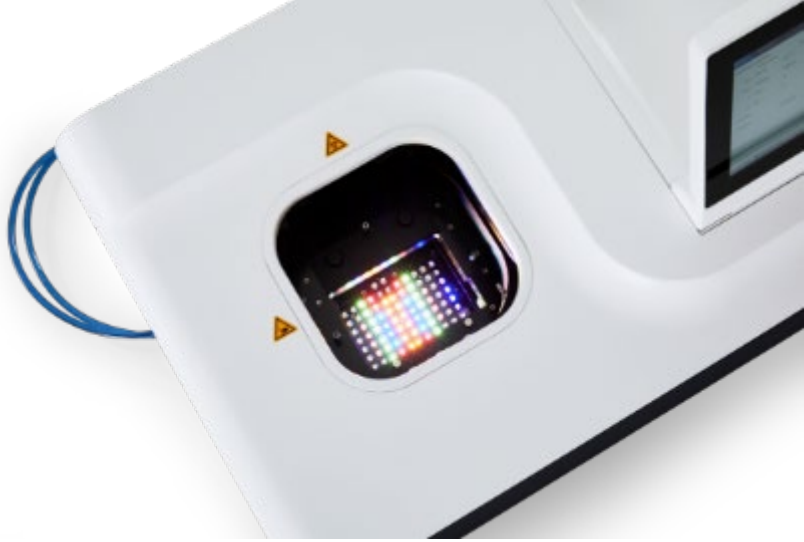


Light Irradiation
Allows phototrophic cultivation of algae and cyanobacteria

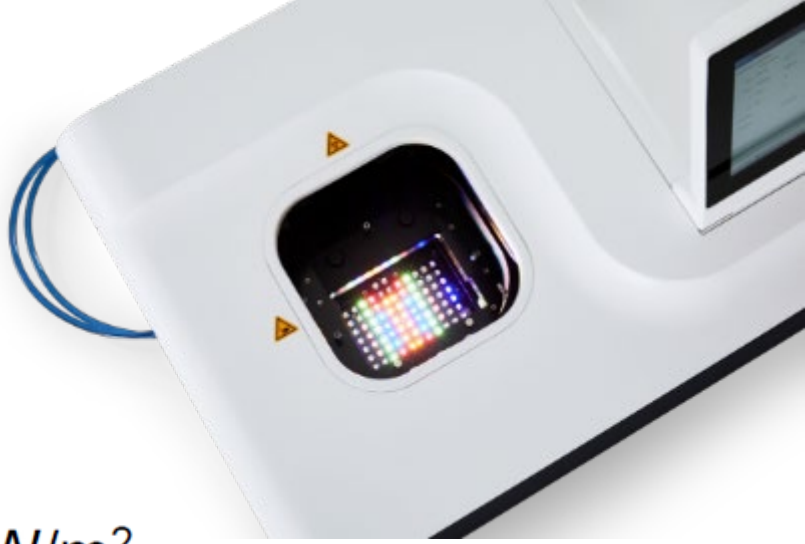
Optical Filter Modules
Up to six filter combinations for different parameters (fluorescence, Chlorophyll)

BioLector XT LAM (Light Array Module)

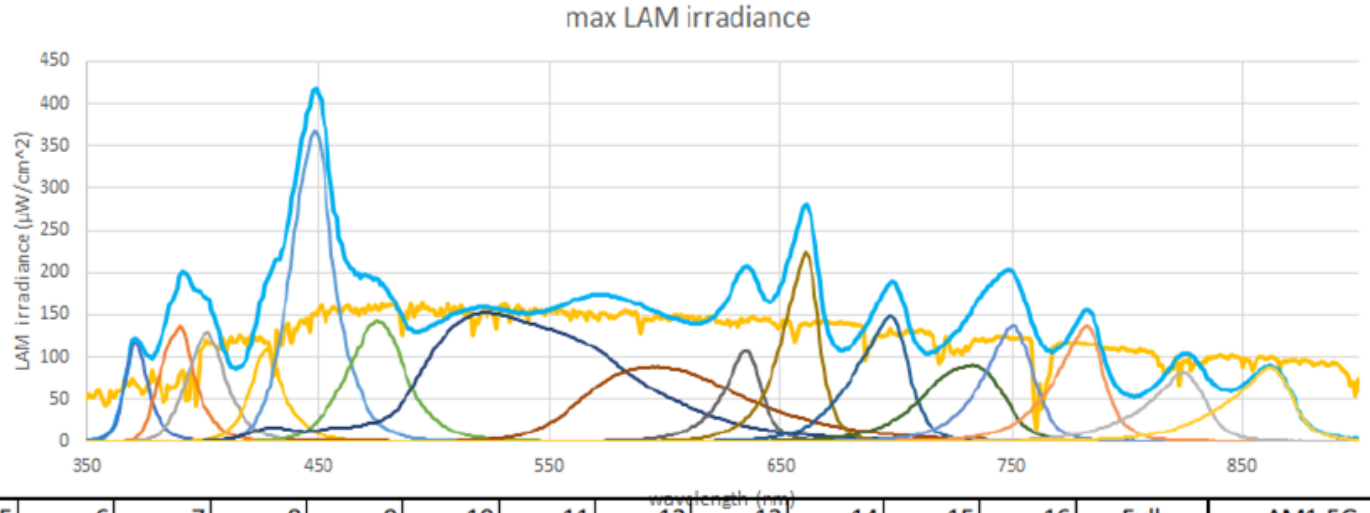
- All gassing modules work together with the LAM (CO_2)
- Chiller unit necessary to cool down the LED array during operation



BioLector XT LAM



- 100 LEDs, 16 LED-types
- Peak emission wavelengths between 365 and 850 nm
  - Photosynthetically active range 400 – 700 nm
- Maximum photon flux density ~3500 µmol/m²/s or ~700 W/m²
  - Sunlight: ~2500 µmol/m²/s
- Spectral flexibility
- Pulsed or non-pulsed
- Illumination profile, e.g. day-night rhythm



LED Channel		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Full	AM1.5G
Peak Wavelength		365nm	385nm	405nm	420nm	450nm	470nm	520nm	590nm	620nm	660nm	690nm	730nm	750nm	780nm	820nm	850nm	Spectrum	(350nm-900nm)
irradiance @ 100% LED Power	W/m2	16.24	26.49	29.05	21.91	91.29	47.79	161.53	81.10	24.64	49.05	45.58	40.98	40.45	40.19	28.79	33.45	778.52	669.77
PFD @ 100% LED Power	µmol/m2/s	50.52	86.43	97.78	78.60	342.12	190.20	734.07	414.90	129.88	268.99	263.07	248.62	252.03	260.71	196.29	238.10	3852.31	3457.00

ambr (TAP Biosystems)

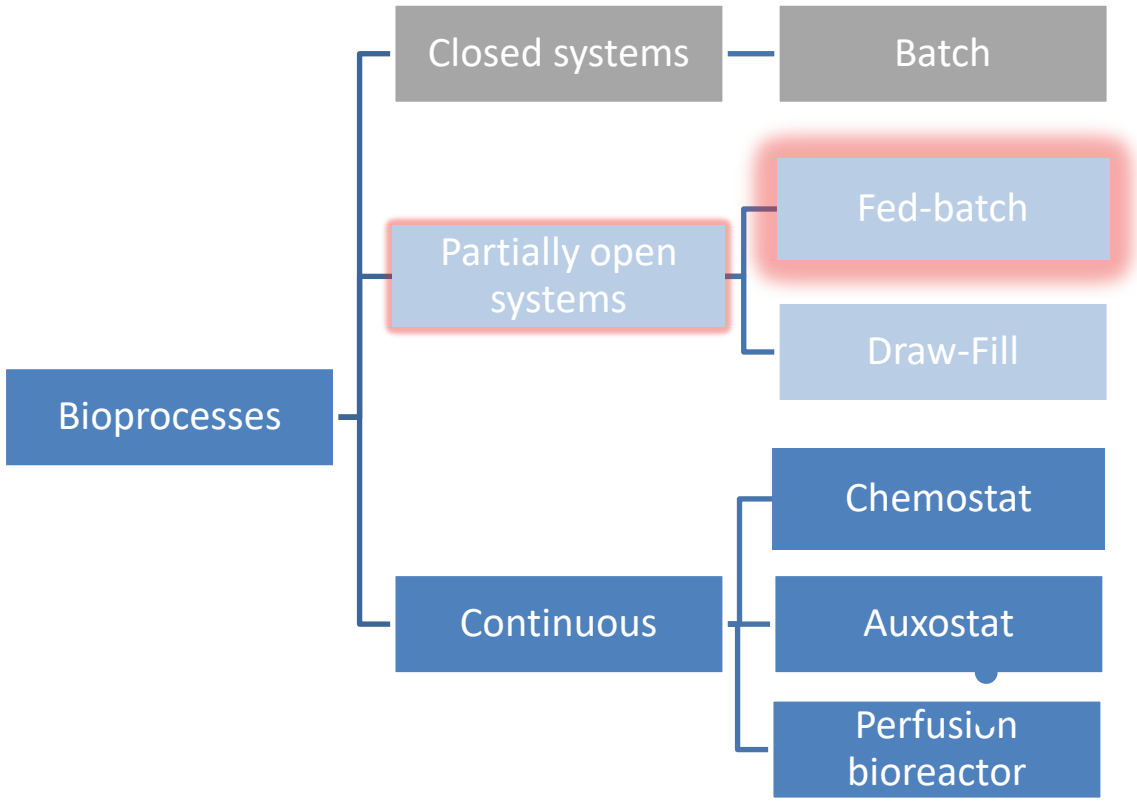
No. of wells	24/48
Volume	10-15 ml
Mixing	stirring
Lid	no
Automated handling	yes
Temperature	no
pH	online measure/control
DO	online measure/control
Gas addition	top
Feed	yes
cell types	Bacteria?, yeast?, **animal cells**

https://www.sartorius.com/en/products/fermentation-bioreactors/ambr-multi-parallel-bioreactors/ambr-15-cell-culture#id-1157764

Fed-batch cultures: the best way of reaching high cell-densities

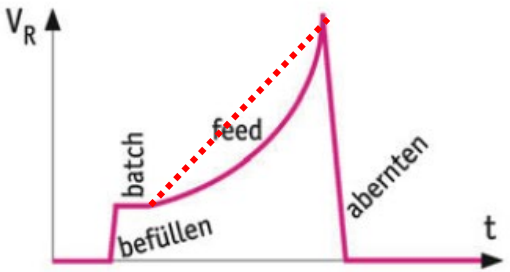
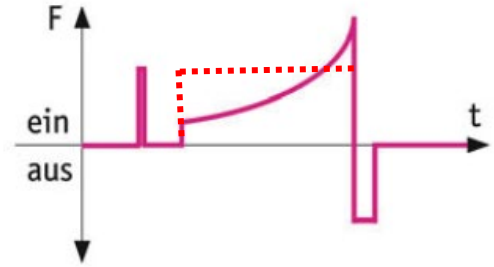
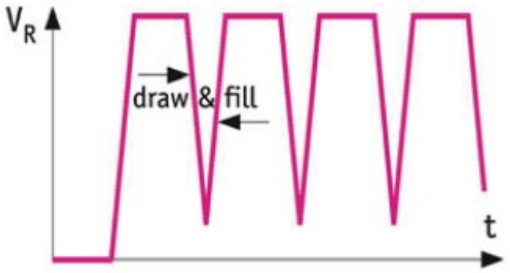
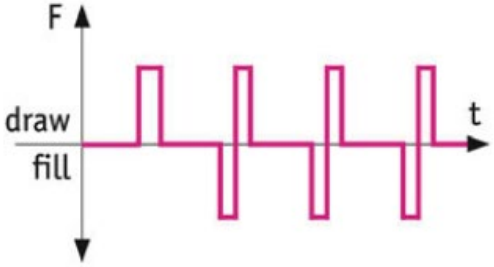
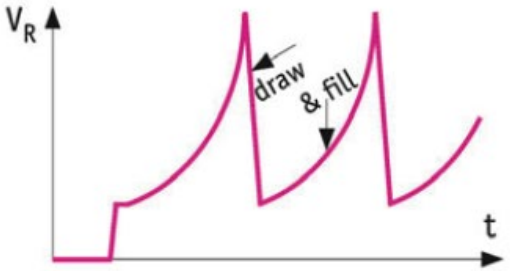
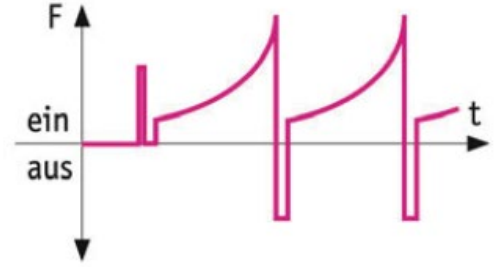
http://www.novasep.com/Technologies/

Different 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		

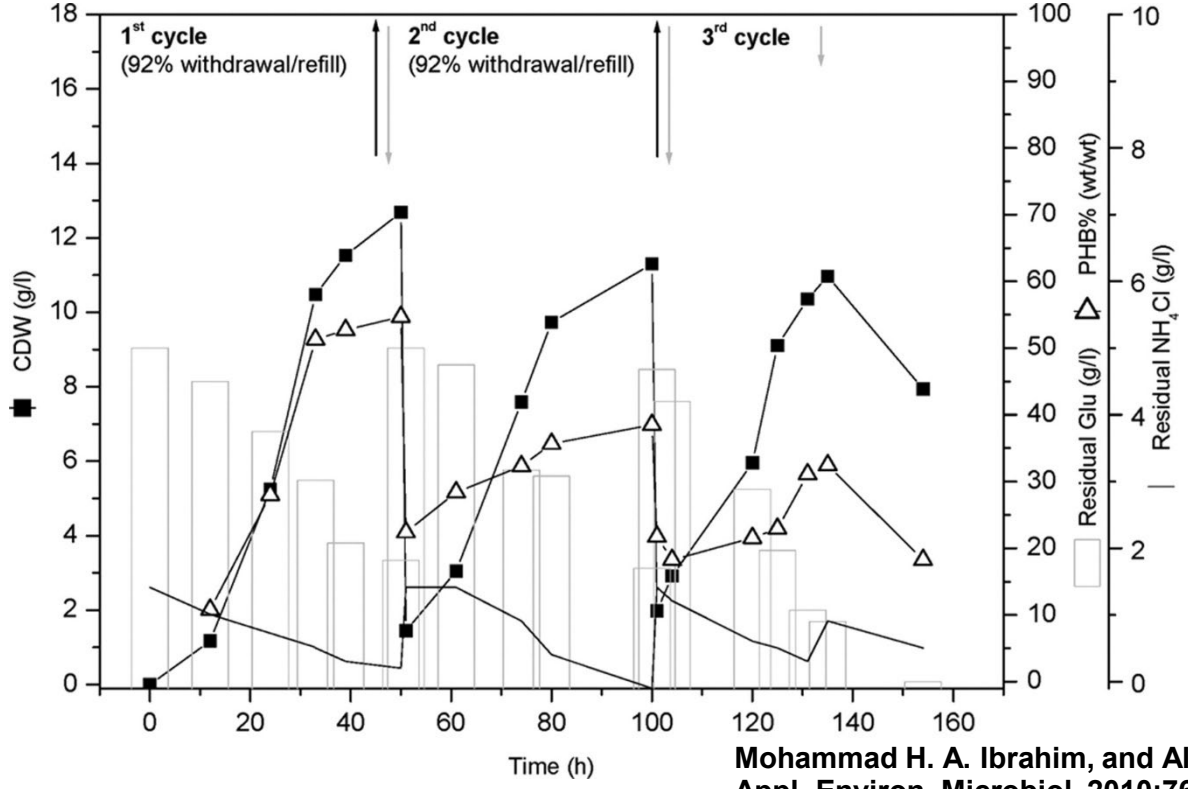
This lecture

Constant feed

Exponential feed

Adapted from Chmiel, 2011

Cyclic batch fermentation (CBF) for cultivation of *Chelatococcus* sp.

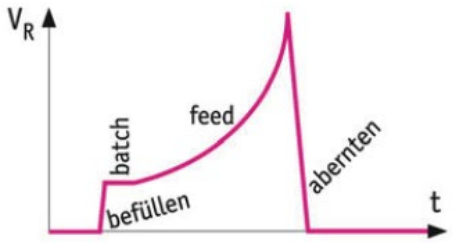


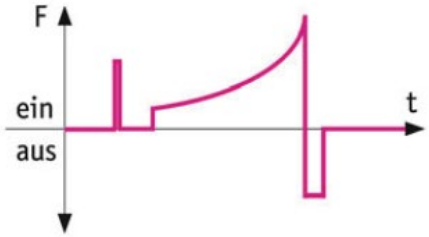
Mohammad H. A. Ibrahim, and Alexander Steinbüchel
Appl. Environ. Microbiol. 2010;76:7890-7895

Cyclic batch fermentation (CBF) for cultivation of Chelatococcus sp. MW10 under thermophilic conditions. Cultivation was conducted in a Biostat UD-30 stirred-tank reactor containing 25 liters MSM with an initial glucose concentration of 50 g/liter as the sole carbon source. The fermentor was inoculated with a 24-h-grown MSM preculture (4% [vol/vol] inoculum size). Culture temperature and pH were controlled at 50°C and at pH 6.7, respectively, during the entire course of the fermentation. Aeration and agitation rates were controlled automatically by adjusting the pO_2 at 20% saturation. Cycling of cultures was operated on a 50-h cycle by withdrawal of 92% of the cultivation medium (black arrows) and refilling with an equal volume of fresh MSM (gray arrows) with glucose (50 g/liter). During the time course of cultivation, samples were withdrawn, and the concentrations of glucose and ammonium as well as the cell dry weight (CDW) and poly(3HB) content of the cells were determined as described in the text.

4 phases of fed-batches

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

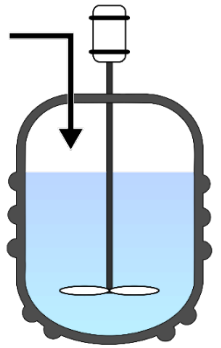




$$F_{in}(t)$$

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

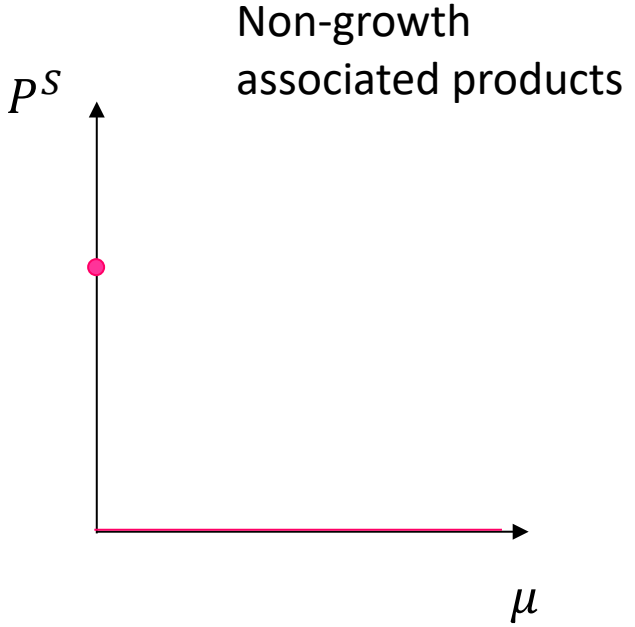
$$\frac{dV}{dt} \neq 0$$



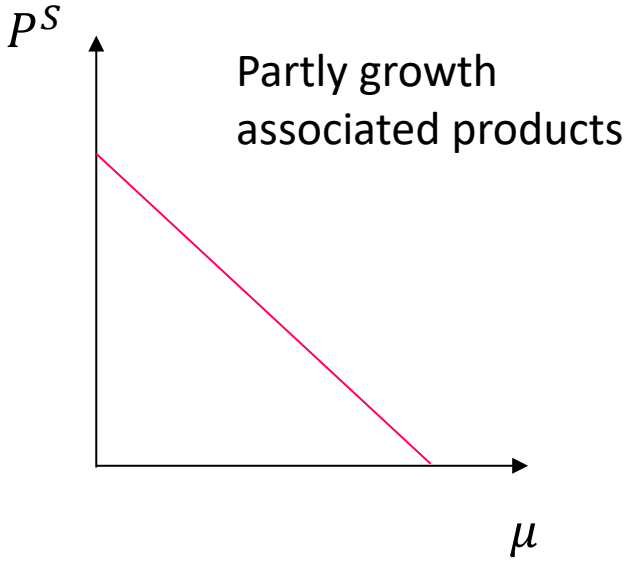
Why fed-batch cultures?

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

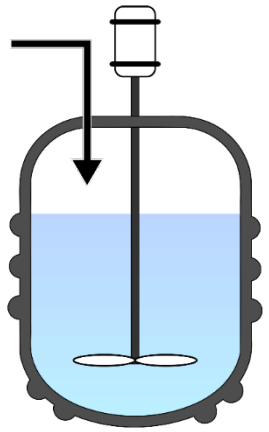
Advantages

- 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

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

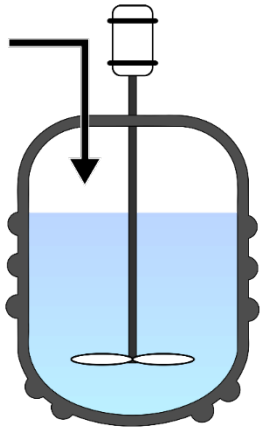
$$\frac{dV}{dt} = F(t)$$

- 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

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

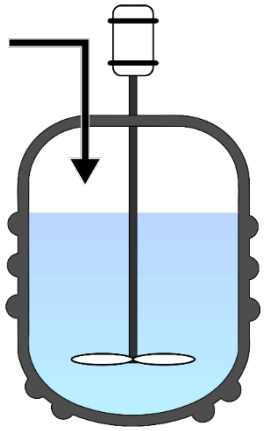
- Contrary to chemostat and batch processes, the volume in the reactor is not constant, and we cannot simply pull V out of the derivative. Instead, we apply the product rule.

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

- 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

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

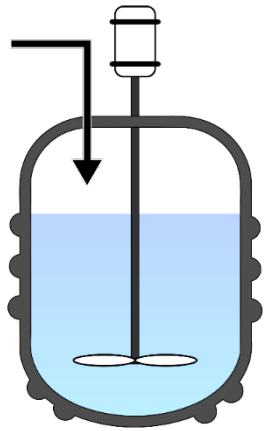
- We define once again $D(t) = \frac{F_{in}(t)}{V(t)}$ and divide the above equation by $V(t)$

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

$$\Leftrightarrow$$

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

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 energy 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:

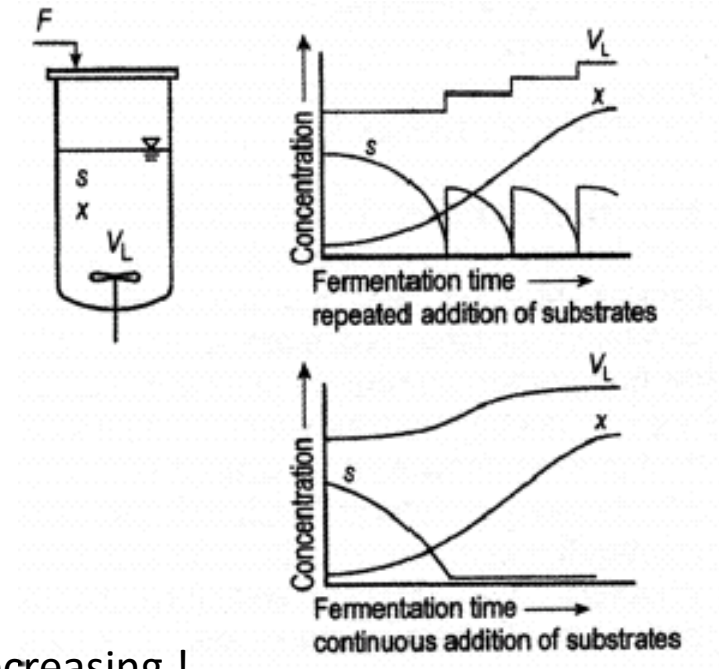
$$P = P_0 \frac{V_0}{V} + q_p X_m \left(\frac{V_0}{V} + \frac{Dt}{2} \right) t \qquad (10.17)$$

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

Summary Fed-Batch

$$\frac{d(V*X)}{dt} = \mu * X * V \quad \Rightarrow \quad \frac{dX}{dt} = X*(\mu - D)$$

$$\frac{d(V*s)}{dt} = s*\frac{dV}{dt} + V*\frac{ds}{dt} = F*s_i - \mu*x*\frac{V}{Y_{X/S}}$$



There are two choices as a feed profile:

1) F=const: First growth with μ_{max} but then steadily decreasing !

2) s=const (ds/dt=0); consequently the feed has to be **exponential**:

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

(for simplification usually s ≅ 0)

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

Potential growth inhobition by product formation:

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

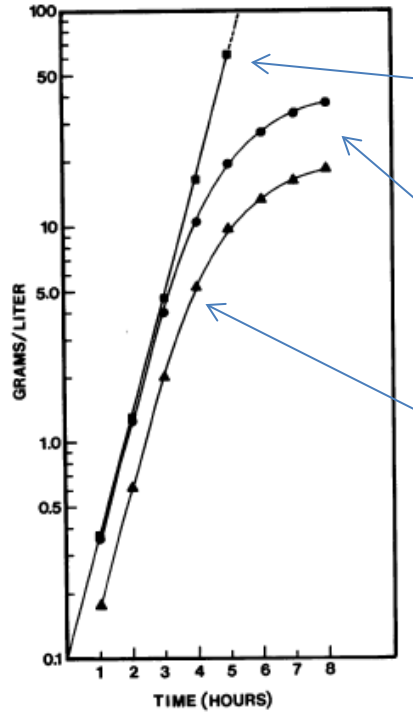
Applications of fed-batch cultures

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

GREGORY W. LULI† AND WILLIAM R. STROHL*
Department of Microbiology, The Ohio State University, 484 West 12th Avenue, Columbus, Ohio 43210

Received 18 October 1989/Accepted 11 January 1990



Growth not inhibited by acetate

Growth inhibited by acetate

Concentration of acetate during inhibition

ELSEVIER

Journal of Biotechnology 39 (1995) 59–65

journal of biotechnology

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

D.J. Korz [1], U. Rinas, K. Hellmuth, E.A. Sanders, W.-D. Deckwer *

GBF National Research Center for Biotechnology, Biochemical Engineering Division, Mascheroder Weg 1, 38124 Braunschweig, Germany

Received 31 August 1994; accepted 4 November 1994

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

High cell-density culture of non-recombinant *Escherichia coli*

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.

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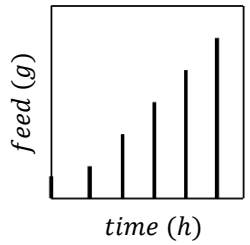
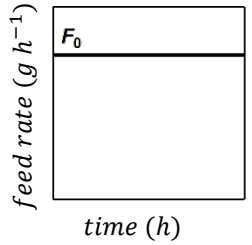
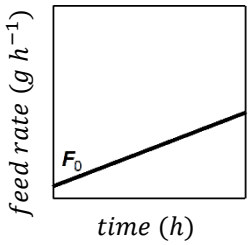
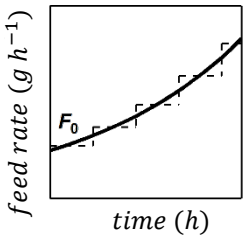
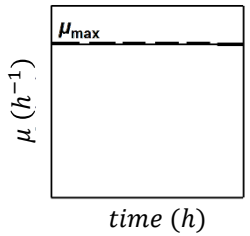
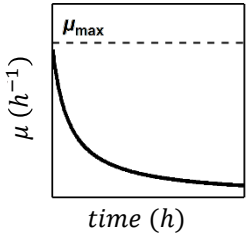
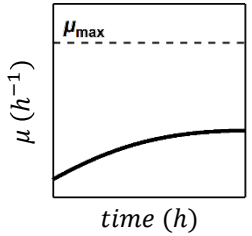
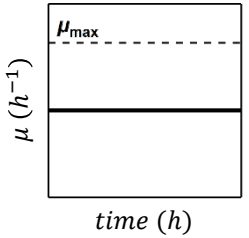
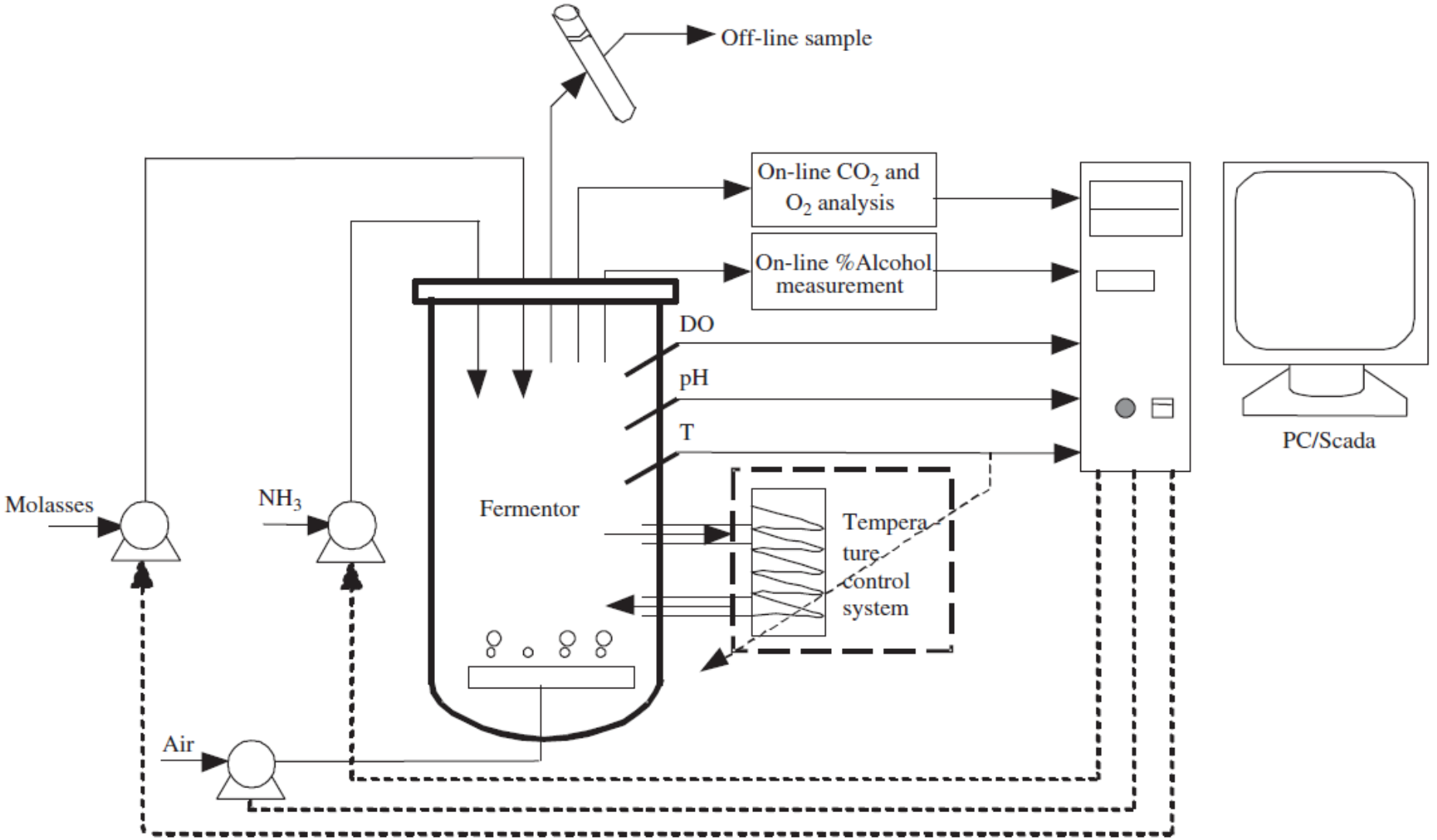
Different fed-batch feeds and their influence on μ

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

Closed loop control of *S. cerevisiae* fed-batch



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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

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}

Production kinetics

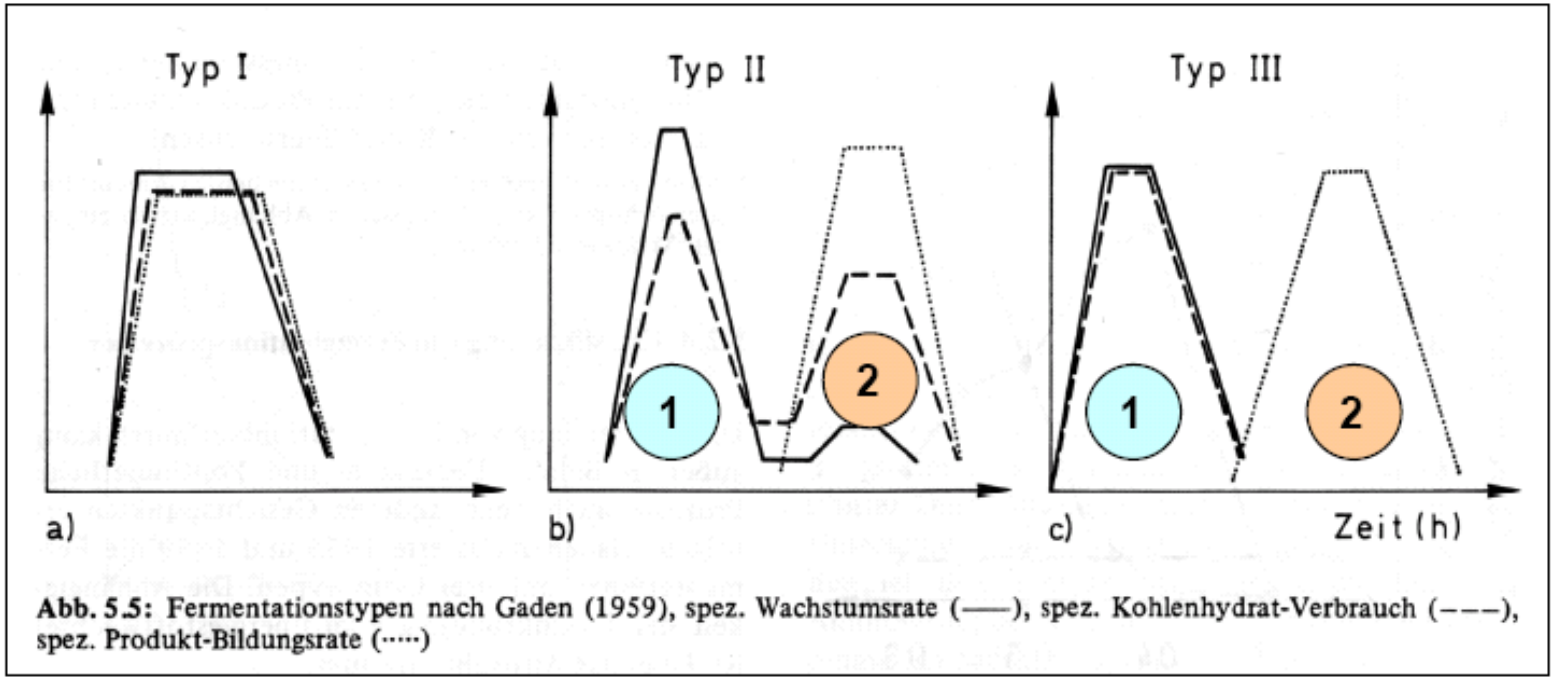


Abb. 5.5: Fermentationstypen nach Gaden (1959), spez. Wachstumsrate (——), spez. Kohlenhydrat-Verbrauch (– – –), spez. Produkt-Bildungsrate (·····)

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

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

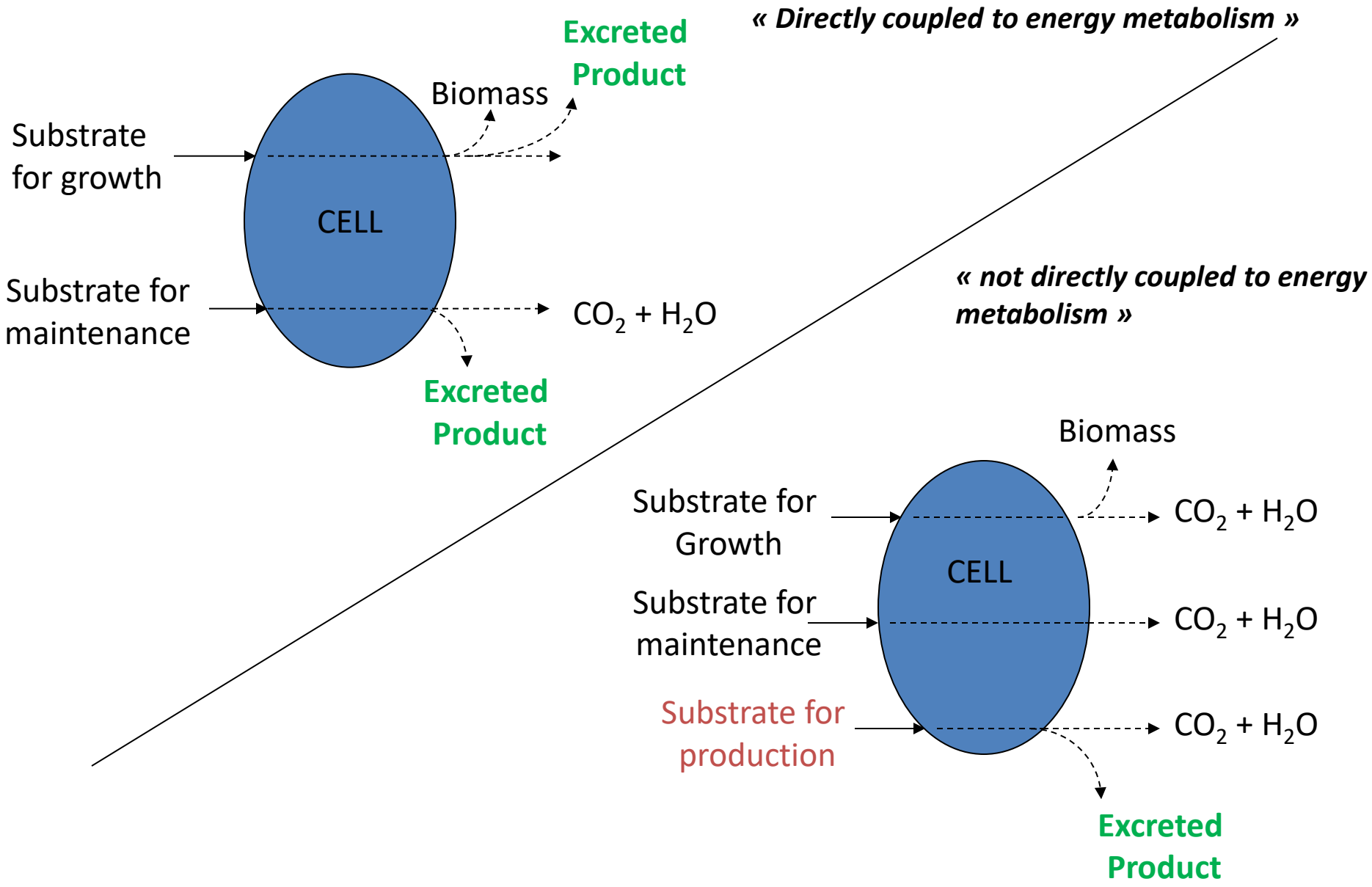
where q_P^{max} and k are constants. The formation of cyclodextrin from starch by *Bacillus macerans* and spore production by *Bacillus subtilis* are similar.

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



Summary of fed-batch technology

Positive	Negative
+ high cell density	- controlling demands require expensive infrastructure
+ control of growth / metabolism	- exponential growth needs high levels of oxygen and nutrients
+ high product titres	- change in metabolism by successively changing process strategies
+ increased space-time-yield	- technical limitations are faster reached
+ control of product quantity and quality (post-translation modification)	

Take home messages

- In biotechnology clearly batch cultures dominate the field of bioprocesses.
- Screening technology enables the selection of the optimal production strain. Direct and indirect biomass measurements simplify the follow up of biomass evolution.
- Fed-batch cultivations are very suitable for reaching high cell-densities from substrates that usually lead to substrate inhibition phenomenons.
- In fed-batch cultures it is best to calculate with total biomass [g] because the volume and concentrations change over time.
- Different feed strategies have been established (e.g., open loop, closed loop, pulsed, linear, and exponential feed).
- Depending on the type of product formation (e.g. directly coupled to growth) and the production strain particular fermentation techniques need to be applied.