Chemostat and continuous cultivation

Manfred Zinn
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Agenda

- Continuous cultivation
- The ideal chemostat
- Productivity
- Special continuous cultivation techniques

The continuous culture by Monod 1950

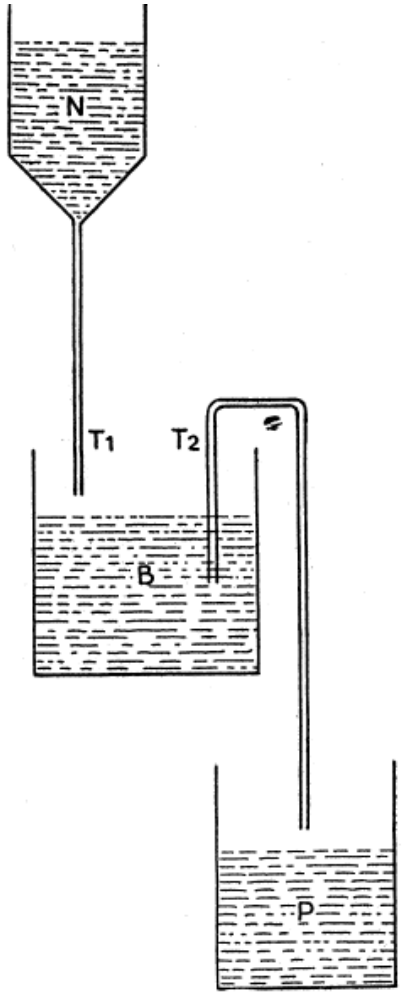


FIG. 1. — Schéma d'un appareil à culture continue.

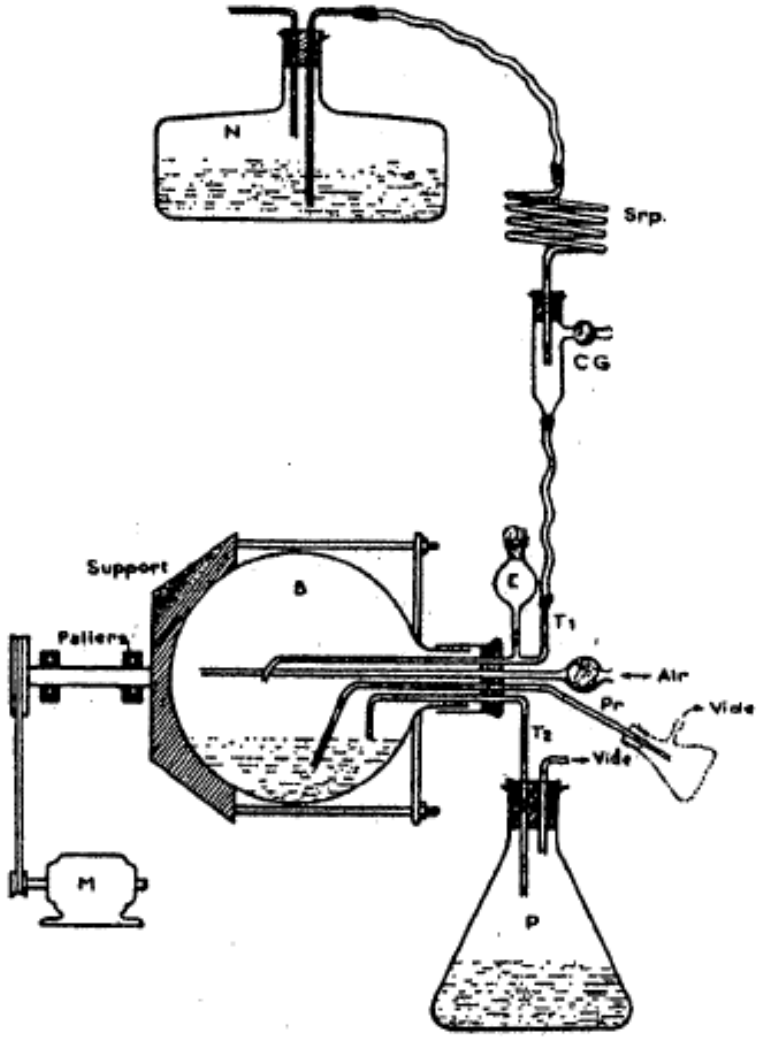


FIG. 4. — Montage d'un appareil à croissance continue. N, nourrice; Srp, serpentin capillaire; C.G., compte-gouttes; B, ballon rotatif; T_1, tubulure d'arrivée; E, tubulure d'ensemencement; Pr, tubulure de prélèvement (en pointillé, fiole de prélèvement); T_2, tubulure de niveau; P, produit; M, moteur.

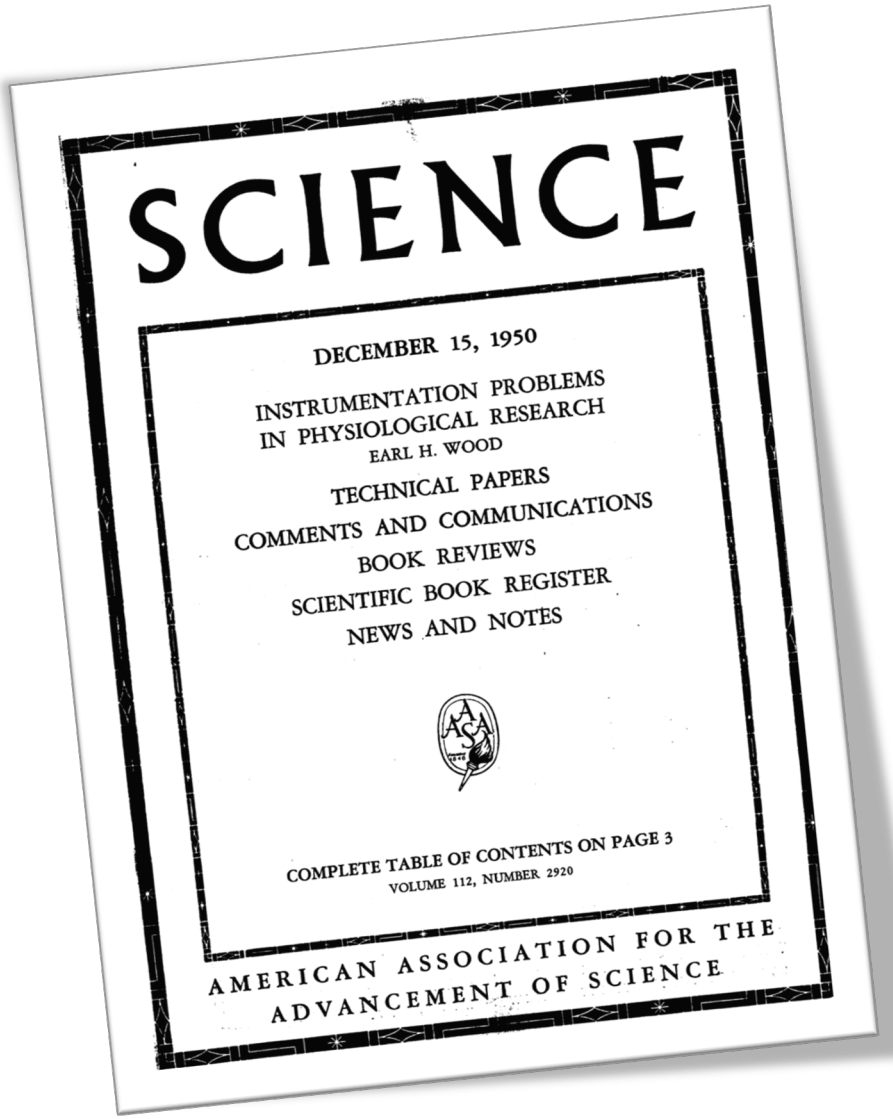
Ann. Inst. Pasteur, **79**(4), 390–410 (1950)

LA TECHNIQUE DE CULTURE CONTINUE THÉORIE ET APPLICATIONS

par JACQUES MONOD.

(*Institut Pasteur. Service de Physiologie microbienne.*)

The chemostat according to Novick & Szillard 1950



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The chemostat according to Novick & Szillard 1950

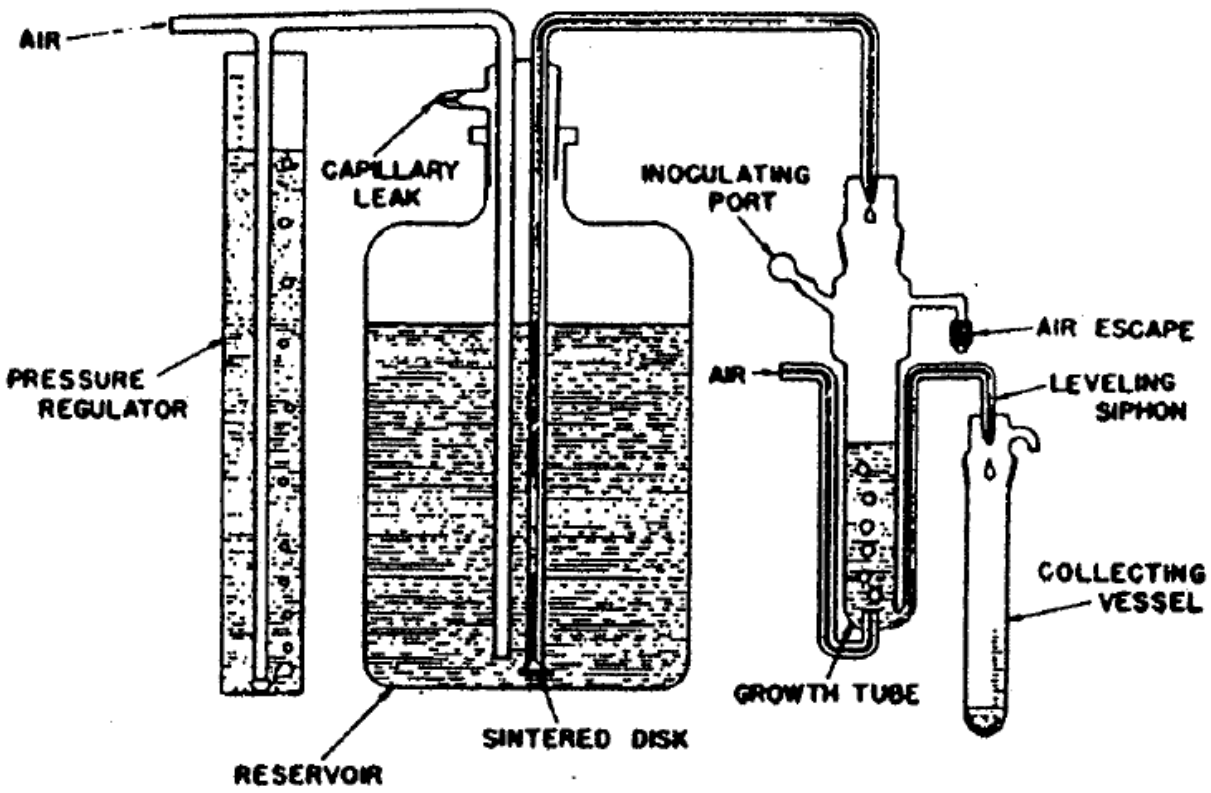


FIG. 1

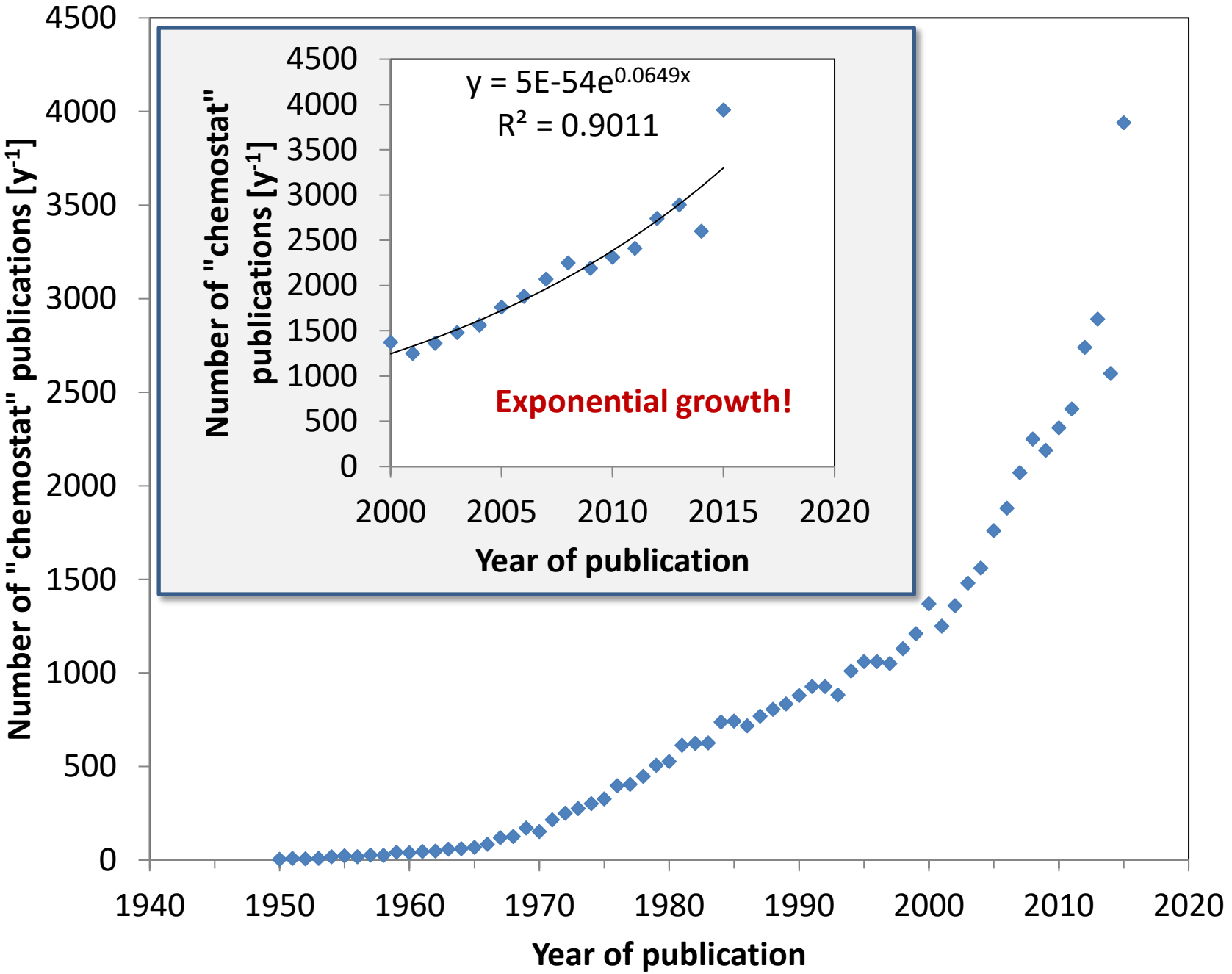
Description of the Chemostat

Aaron Novick and Leo Szilard

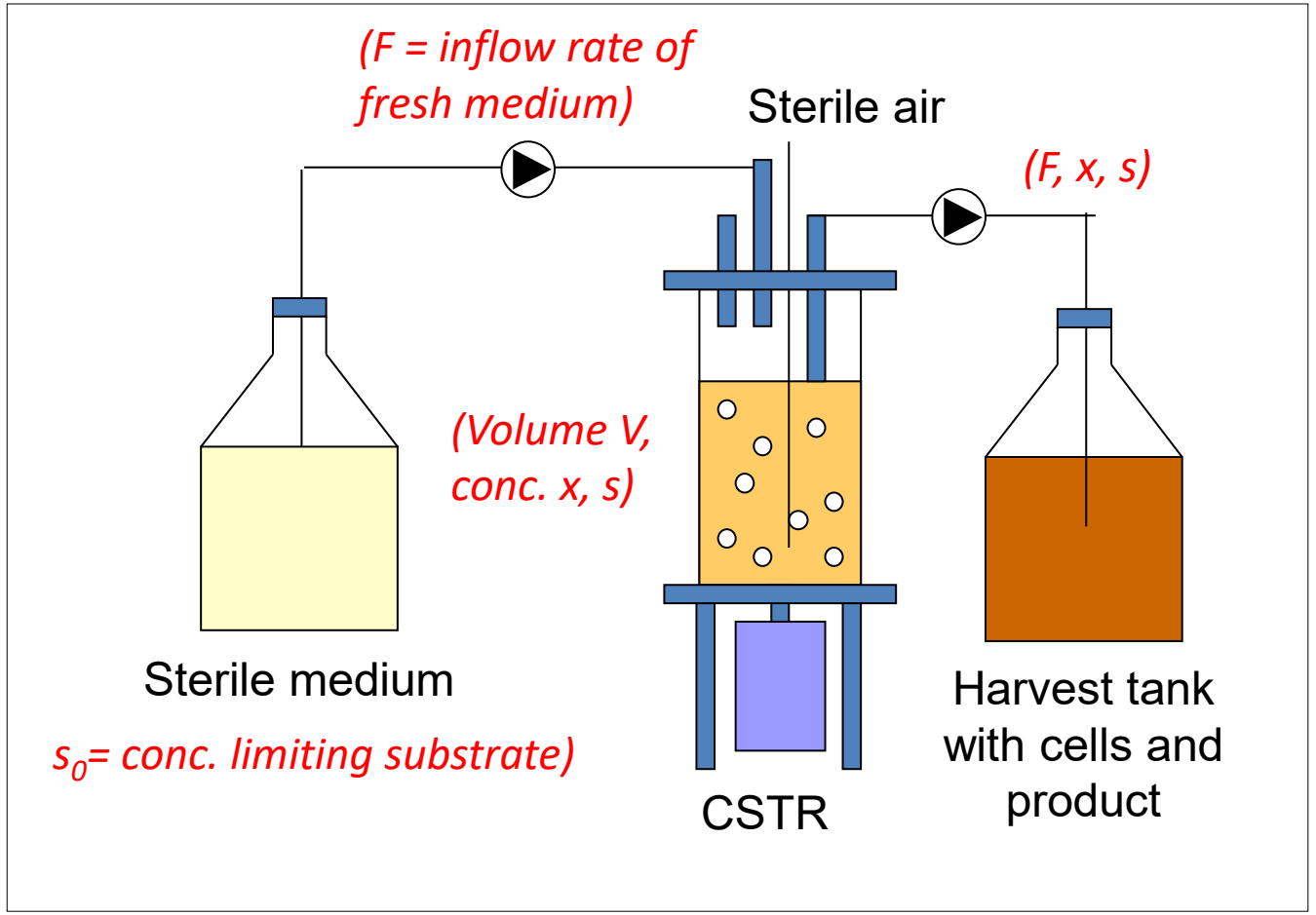
Institute of Radiobiology and Biophysics, University of Chicago

Science, **112**(2920), 715–716 (1950)

A success story!


Number of "chemostat" publications [y-1]
4500
4000
3500
3000
2500
2000
1500
1000
500
0
1940
1950
1960
1970
1980
1990
2000
2010
2020
Year of publication
Number of "chemostat" publications [y-1]
y = 5E-54e0.0649x
R² = 0.9011
Exponential growth!
2000
2005
2010
2015
2020
Year of publication

Dilution rate and specific growth rate in continuous culture



Continuous culture design

The ***chemostat*** is by far the simplest and most common mode of operation of a continuous culture.

Where contamination can be a significant problem, a pump based control system is preferred. This setup is commonly used in laboratory investigations and animal cell culture systems.

An ***overflow system*** has the advantage in that only one pump is required. However, as the effluent flow rate is determined by gravity alone, there is a greater possibility of contaminants moving up the effluent tube into the reactor. Overflow systems are, however, widely used in wastewater treatment and have been used in the large scale continuous culture of bacteria, but also combined with an effluent pump.

In other techniques, a fermenter parameter, e.g. turbidity or pH, will be monitored using an appropriate detector and the liquid flow rate will be automatically adjusted so as to maintain the variable at a constant level (closed loop control).

Examples of these types of continuous fermenters are the ***pH-stat, turbidostat and nutristat***. Apart from the pH-stat, these reactors are however rarely used as the necessary measurement-control systems are generally unreliable over long periods of time.

Continuous vs. batch culture

Advantages of continuous cultures

Continuous cultures have several advantages over batch cultures:

- In a chemostat, the cells can be maintained at a **constant physiological state and growth rate**. The growth rate can be adjusted by changing the feed flow rate. Consequently, it is easier to optimize productivity.

- It is not necessary to shut down the continuous fermenter as frequently as a batch fermenter. At the end of a batch fermentation, the reactor must be emptied, cleaned, sterilized and re-filled. The time required for these operations is known as **turnaround time**. Theoretically, a continuous fermenter could operate indefinitely without having to be shut down. In practice, however, this is not possible.

Very, very Important!

When referring to continuous culture systems, the terms lag phase, stationary phase and death phase have no meaning. This is because the system is operating continuously and growth cannot be segregated into phases. All cells are growing exponentially!

Continuous vs. batch cultivation

Advantages of continuous cultures

Continuous cultures have several advantages over batch cultures:

- Most **downstream** processing operations are most productive when operated in a continuous manner. Using a continuous culture allows the fermentation to be in-tune with other operations in the plant. Thus, overall plant productivity is easier to optimize.

- Continuous cultures thus offer the potential of **higher productivities**. As a consequence, it is possible to have smaller reactors and associated equipment and thus lower capital costs. This should lead to higher profits.

Disadvantages of continuous cultures

Despite their many potential benefits, pure culture applications of continuous cultures are not well established in industry. There are two main reasons for this:

- Risk is a major factor. The batch fermentation process is easy to understand and relatively well established in industry.
- To switch from a batch to a continuous process represents some risk; one that many managers would not take.

Continuous vs. batch cultivation

Disadvantages of continuous cultures

In the processing of pharmaceuticals, all products must be able to be segregated into batches. For this reason, unit operations in pharmaceutical processing are performed in a batch manner.

- Not all products are produced well in a continuous flow system. For full **flavor** development, some fermented foods and beverages require cellular products released from different phases of batch culture growth. Because continuous fermenters maintain the cells at a single physiological condition, the resultant product is generally inferior. Beer and soya sauce for example cannot be successfully produced by continuous culture.

- **Many commercially important products are produced only when cell growth stops**. These products are not amenable to production in a conventional continuous flow system as new biomass will not be produced to replace that removed in the effluent. Eventually the culture will be *washed out*. Note however that it is possible to use non-growing cultures in immobilized cell continuous fermenters.

- **Contamination** of a continuous fermenter can have disastrous consequences. As we shall see later, a contaminating species can cause the wash out of the resident organism and then completely take over a fermenter.

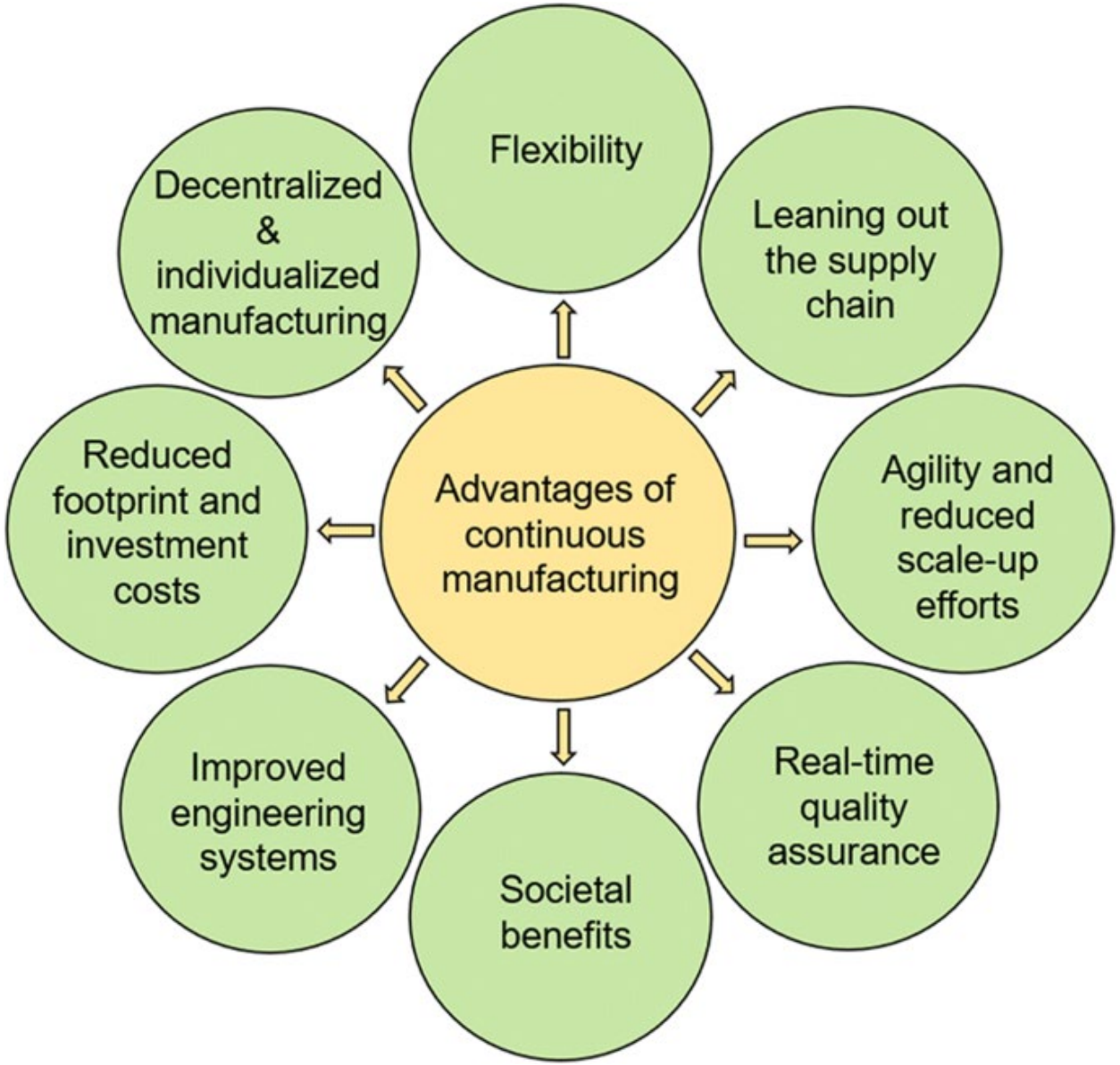
Continuous vs. batch cultivation

Advantages of continuous cultures

Continuous cultivation makes a lot of sense when the carbon sources are continuously available, which is the case for many waste streams (waste water treatment plants are continuously operated).

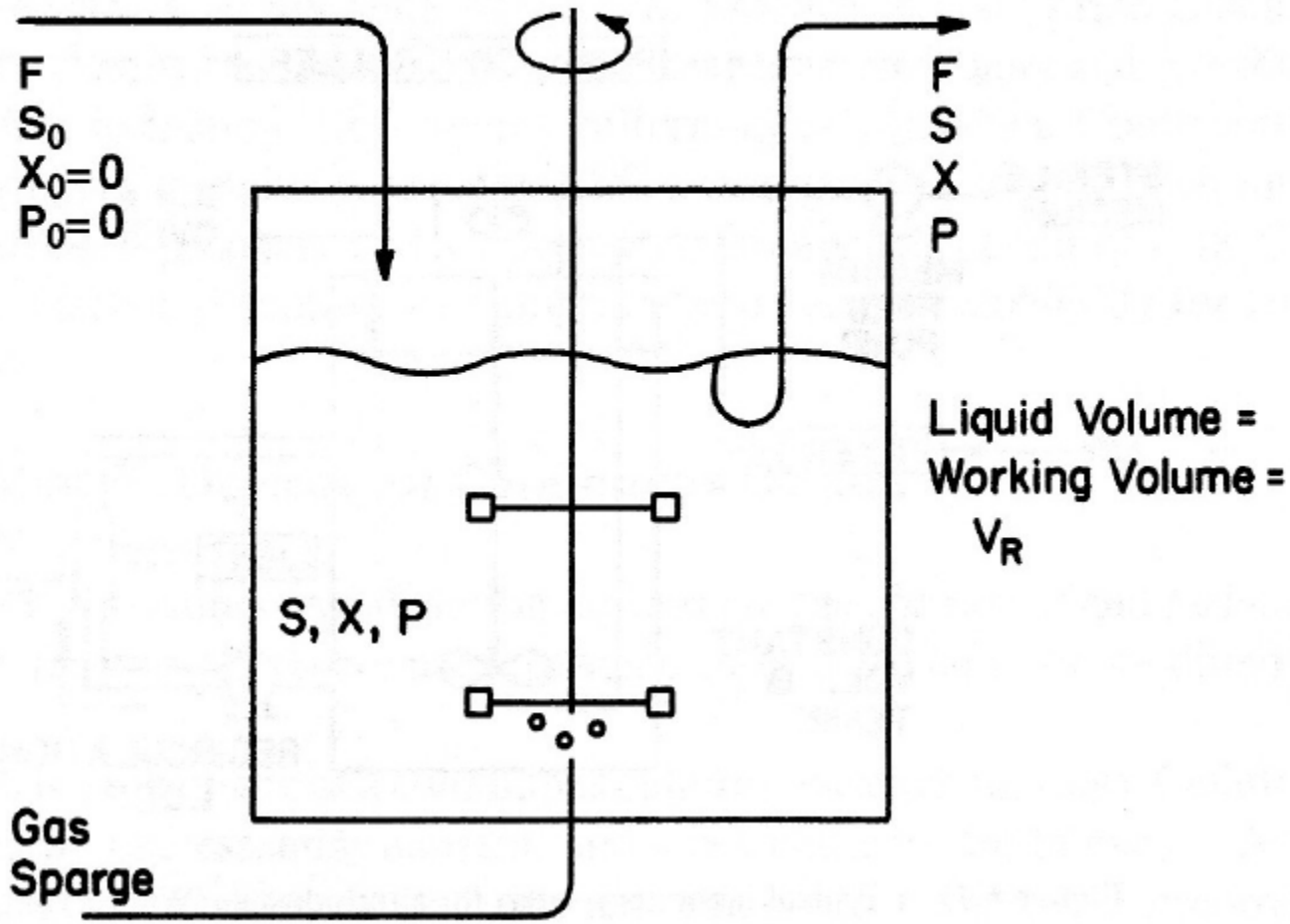
- The continuous cultivation is **more productive than batch cultivations** (see also calculations later on).
- Smaller bioreactors can be used in order to achieve the same amount of biomass, it is basically just a question of time!
- Continuous cultivations are usually highly reproducible when they are in steady-state conditions (chemostats) and therefore are considered as a plus with respect to product quality and product reproducibility. There are numerous continuous processes established in industry: Bioethanol fermentation, yeast extract production, and an increasing number in enzyme production.

Summary of advantages of continuous cultivation for pharma products



Hu, C. Reactor design and selection for effective continuous manufacturing of pharmaceuticals. *J Flow Chem* **11,** 243–263 (2021). https://doi.org/10.1007/s41981-021-00164-3

Schematic of a Chemostat



Question

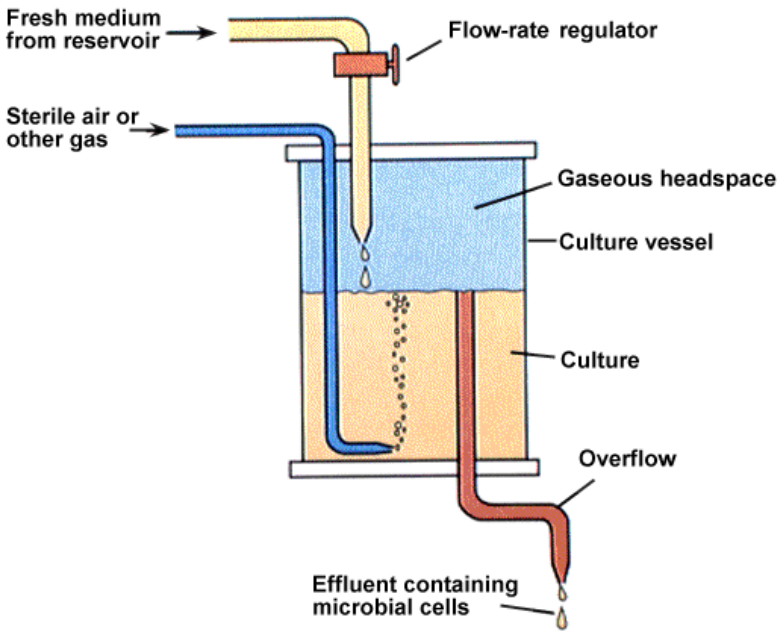
What are the definitions of a continuous cultivation, a perfusion chamber, and a chemostat?

D and μ in the chemostat

$$(1) \qquad D = \frac{F}{V} = \frac{1}{\tau}$$

D = dilution rate (h^{-1})
F = inflowing medium (mL h^{-1}; L h^{-1})
V = culture volume (mL, L)
τ = average residence time (also time for 1 volume change (h)

After 5 volume changes (VC) a steady-state is established.



Biomass in the reactor as a function of time:

change of biomass in reactor per unit of time = biomass increase from growth - loss of biomass from wash-out

$$(2) \qquad \frac{dX}{dt} = r_x + F(0 - x) = \mu X - Fx$$

where **X** is the total biomass in the system and **x** the concentration of biomass

D and μ in the chemostat

dividing (2) by the volume one obtains:

(3a) $$\frac{1}{V}\frac{d(xV)}{dt} = \mu\frac{X}{V} - \frac{F}{V}\cdot x$$

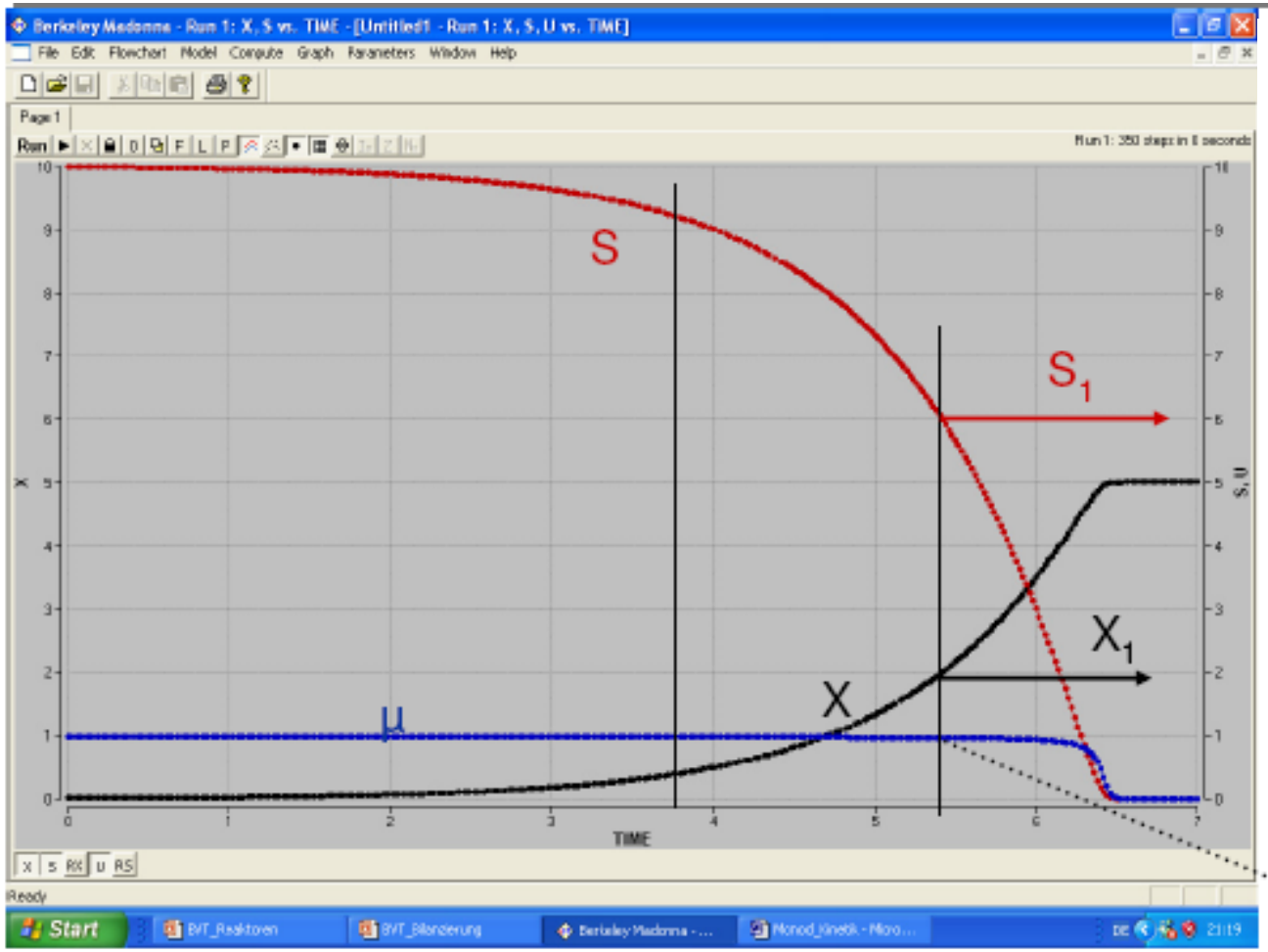
By defining the dilution rate D as $D = \frac{F}{V} = \frac{1}{\tau}$, the equation is simplified

(3b) $$\frac{dx}{dt} = \mu \cdot x - D \cdot x = x(\mu - D)$$

and if the culture is in a dynamic equilibrium (steady-state):

(3c) $$\frac{dx}{dt} = 0$$ **and therefore** $$\mu = D$$

Switch from batch to chemostat



Preculture for a chemostat Monod kinetics with

μ_{max} = 1 1/h

K_S = 0,2 g/l

S^0 = 10 g/l

X^0 = 0,01 g/l

$Y_{X/S}$ = 0,5

$D = \mu$

The switch from batch to continuous cultivation can be done at any timepoint when the dilution rate $D = \mu < \mu_{max}$.

However, when is the optimal timing for the change to chemostat cultivation?

Substrate and biomass concentration in steady-state

Balance for substrate:

change of substrate = substrate in inflow - substrate in outflow - consumption by cells

(4) $$V \cdot ds = F \cdot s_{in} \cdot dt - F \cdot \tilde{s} \cdot dt - \frac{V \cdot \tilde{x} \cdot \mu}{Y_{X/S}} \cdot dt$$

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

where $\tilde{s}$, $\tilde{x}$ are steady-state concentrations

and in steady state ds/dt = 0:

(6) $$0 = D(s_{in} - \tilde{s}) - \frac{\mu \cdot \tilde{x}}{Y_{X/S}}$$

Steady state equations

At steady state dx/dt = 0, ds/dt = 0 and μ = D:

(7) $$D = \mu_m \frac{\tilde{s}}{K_S + \tilde{s}}$$

Solving for s:

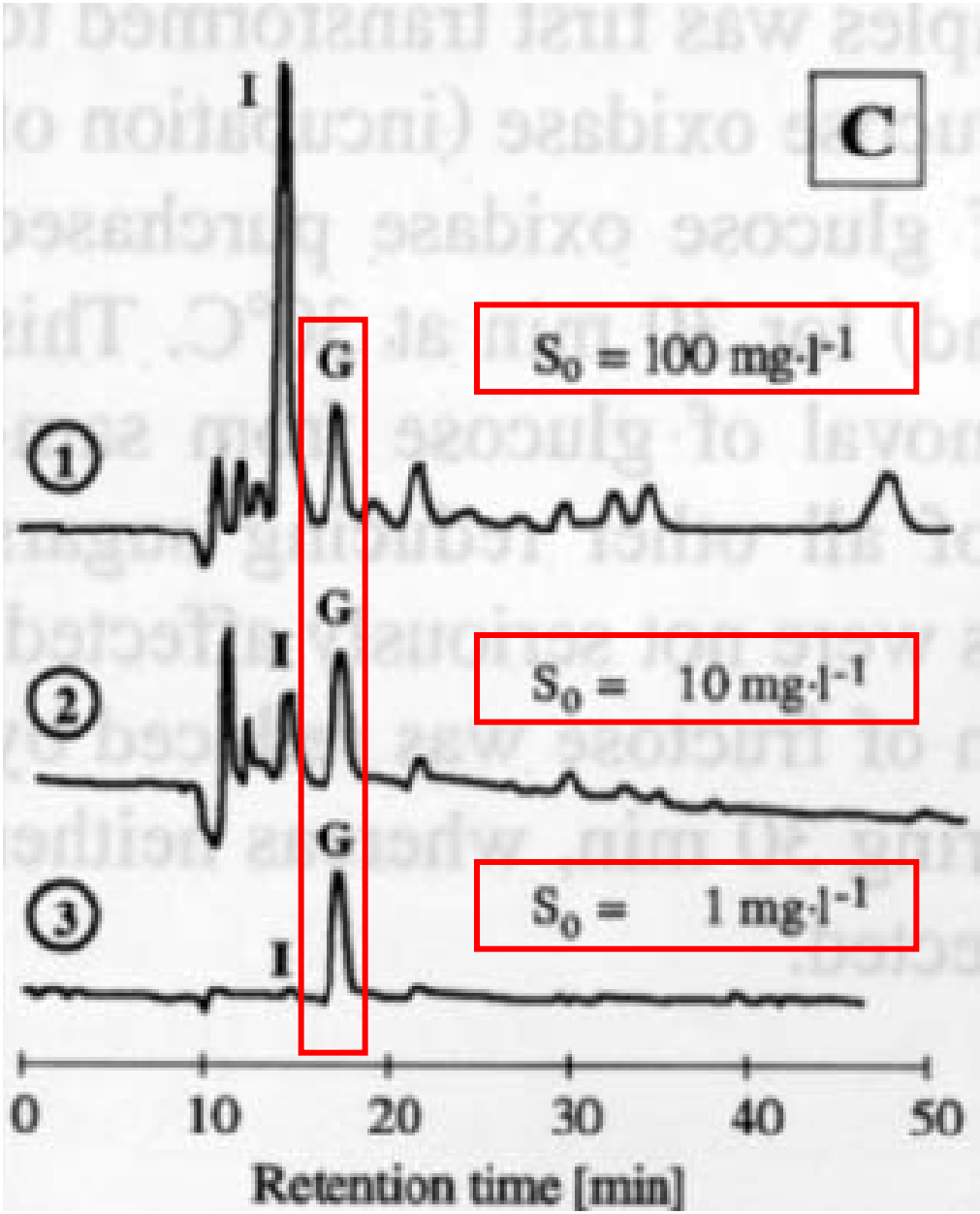
(8) $$\tilde{s} = \frac{K_S * D}{\mu_m - D}$$

Now x = $Y_{X/S}$ (s_0 -s) therefore:

(9) $$\tilde{x} = Y_{X/S}\left(s_0 - \frac{K_S * D}{\mu_m - D}\right)$$

These equations give s and x in terms of D and s_0 (μ_m, K_S and $Y_{X/S}$ being constant)

Substrate and biomass concentration in steady-state



HPLC chromatogram of steady-state glucose concentrations (G) in a culture of *E. coli* at D = 0.30 h^{-1} as a function of s_{in} (here s_0). From Senn et al. (1994)

Substrate and biomass concentration in steady-state in an ideal chemostat

Convert to concentration-based form by dividing by V

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

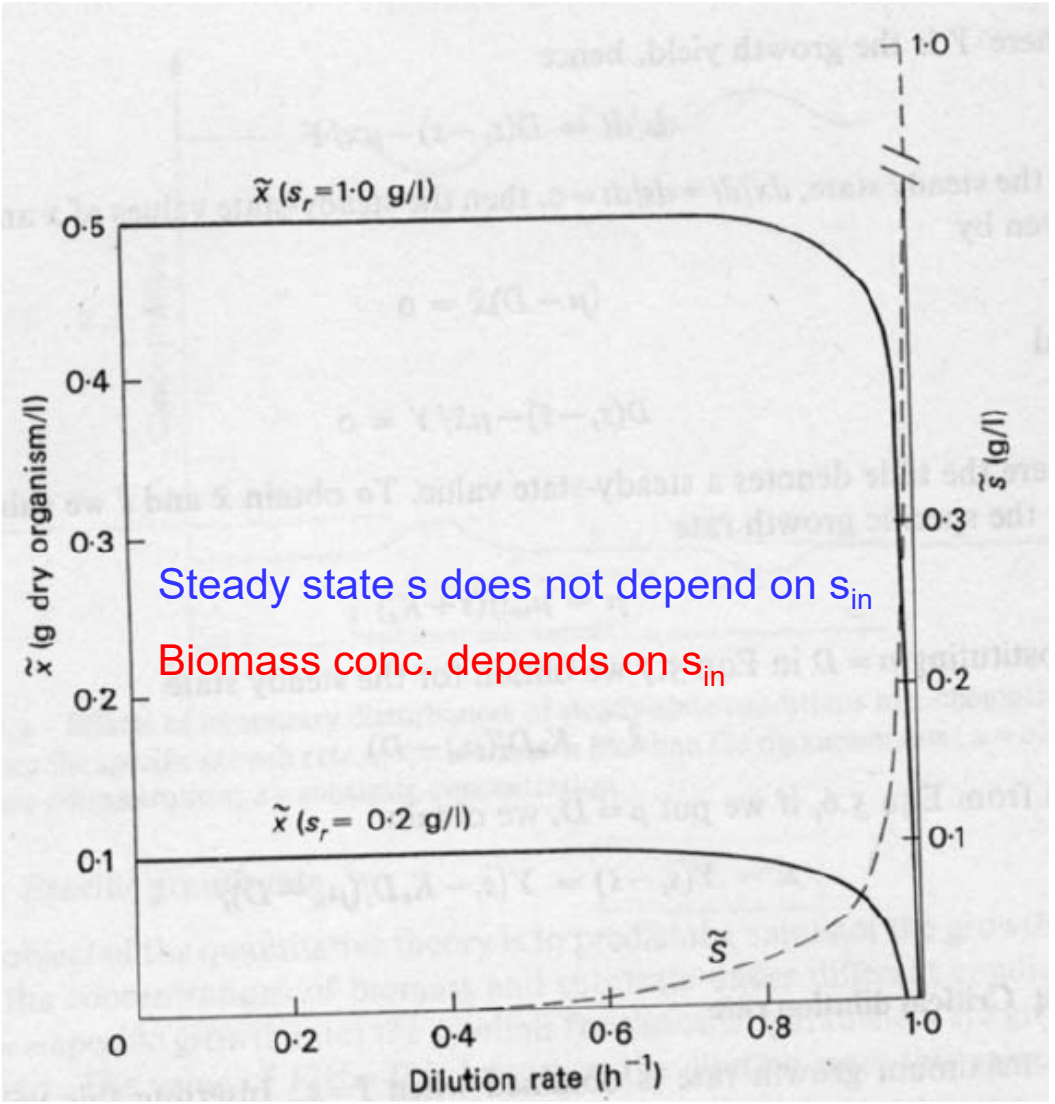
At steady state ds/dt=0 and $D = \mu$. Solving for $\tilde{x}$:

(10) $$\tilde{x} = \frac{D}{\mu}(s_{in} - \tilde{s})Y_{X/S} = (s_{in} - \tilde{s})\ Y_{X/S}$$

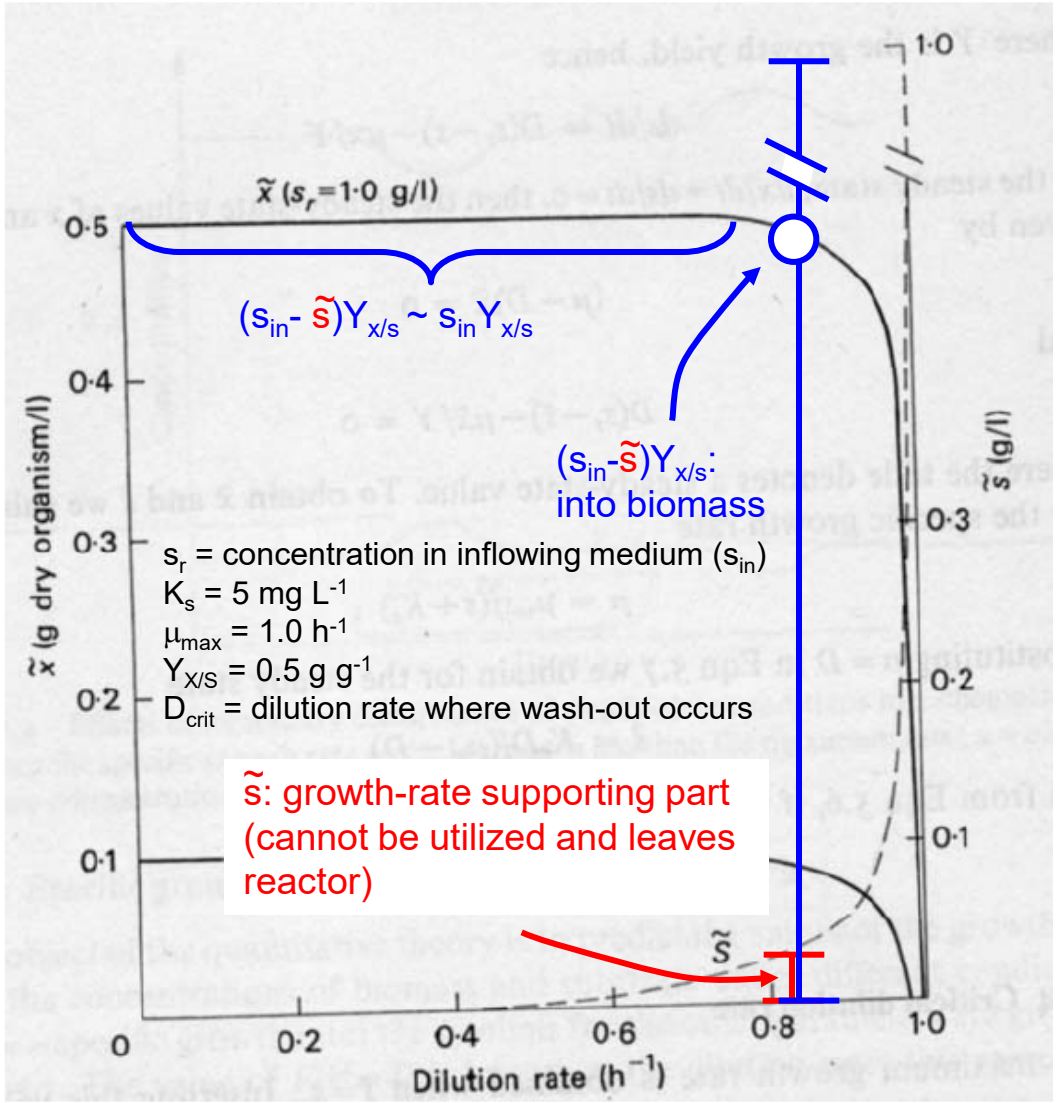
The biomass steady-state concentration is obtained from the used substrate and the growth yield.

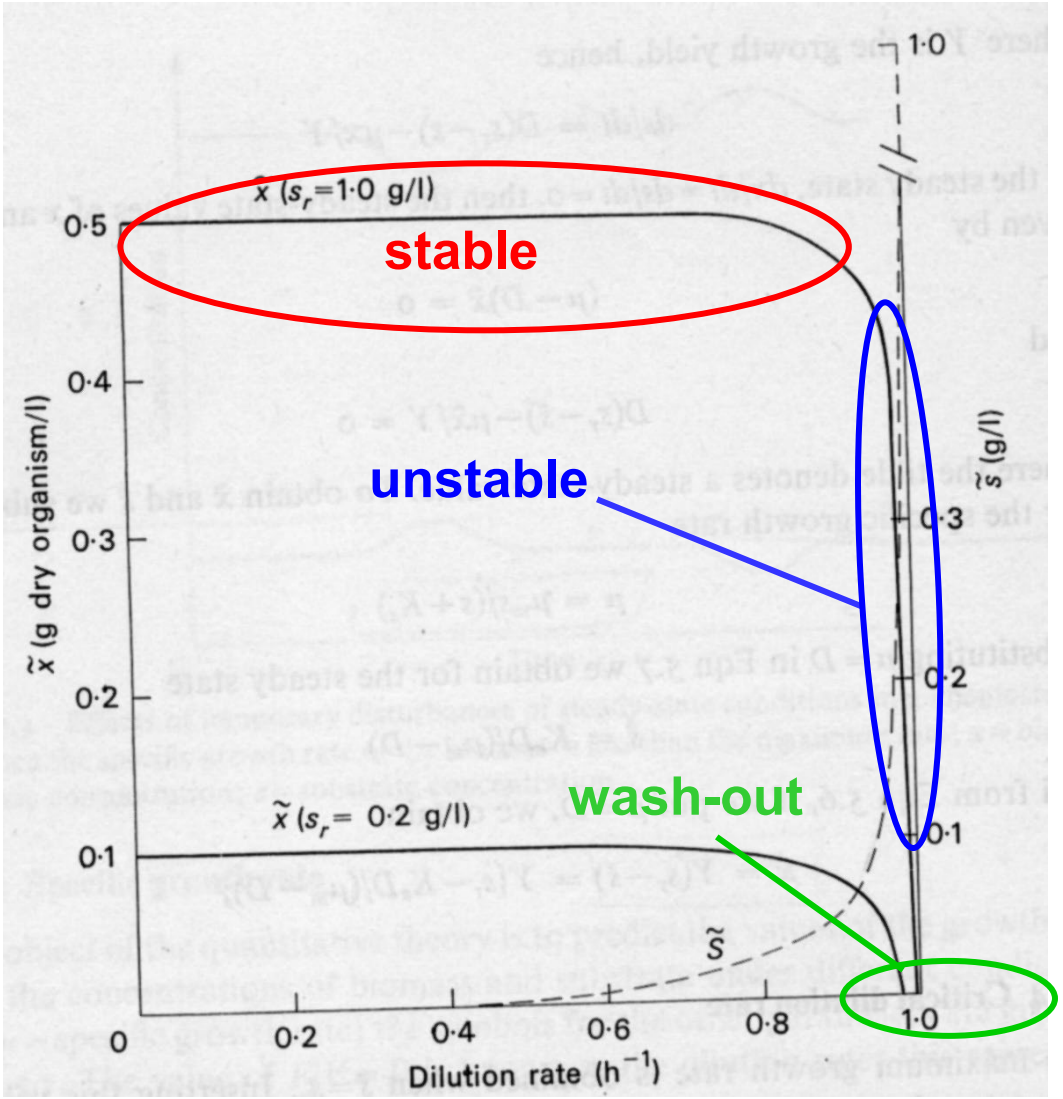
The Figure shows the steady-state biomass and substrate concentration as a function of D for two reservoir concentrations (here s_r).

s_r = concentration in inflowing medium (s_{in})
K_s = 5 mg L^{-1}
μ_{max} = 1.0 h^{-1}
$Y_{X/S}$ = 0.5 g g^{-1}
D_{crit} = dilution rate where wash-out occurs



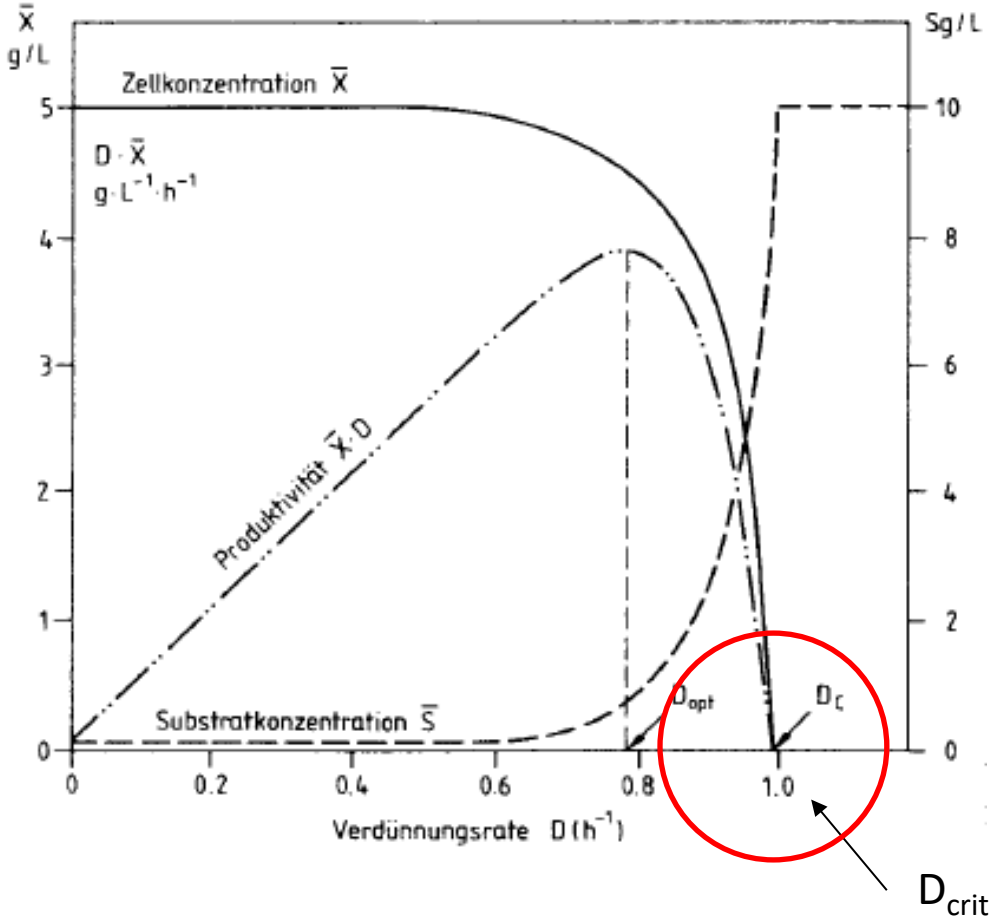
Substrate and biomass concentration at steady-state





Critical dilution rate

D_C = lowest value of D for which wash-out occurs



$D_{crit} = D_c$

In general: $D_C \approx \mu_m$ (11)

However: $D_C = \mu_m$ only when $s = s_0$

$$D_C = \mu = \mu_m \frac{s_0}{K_S + s_0} \quad (12)$$

Thus if $K_S << S_0$ then $D_C = \mu_m$

As K_S increases and s_0 decreases D_C becomes less than μ_m

$$\frac{dx}{dt} = \mu \cdot x - D \cdot x \quad (3b)$$

$$\ln x = (\mu_{max} - D) \cdot t + \ln x_0 \quad (13)$$

Question

- What is the advantage of running a chemostat?
- Is the chemostat used in industry at all?
- How fast are the cells growing during a wash-out?
- What is the reason for the wash-out?

Summary of most important points

- **Chemostat:** fixed nutrient feed rate (F) and let cells adapt their specific growth rate to this feed containing a **growth limiting nutrient.**

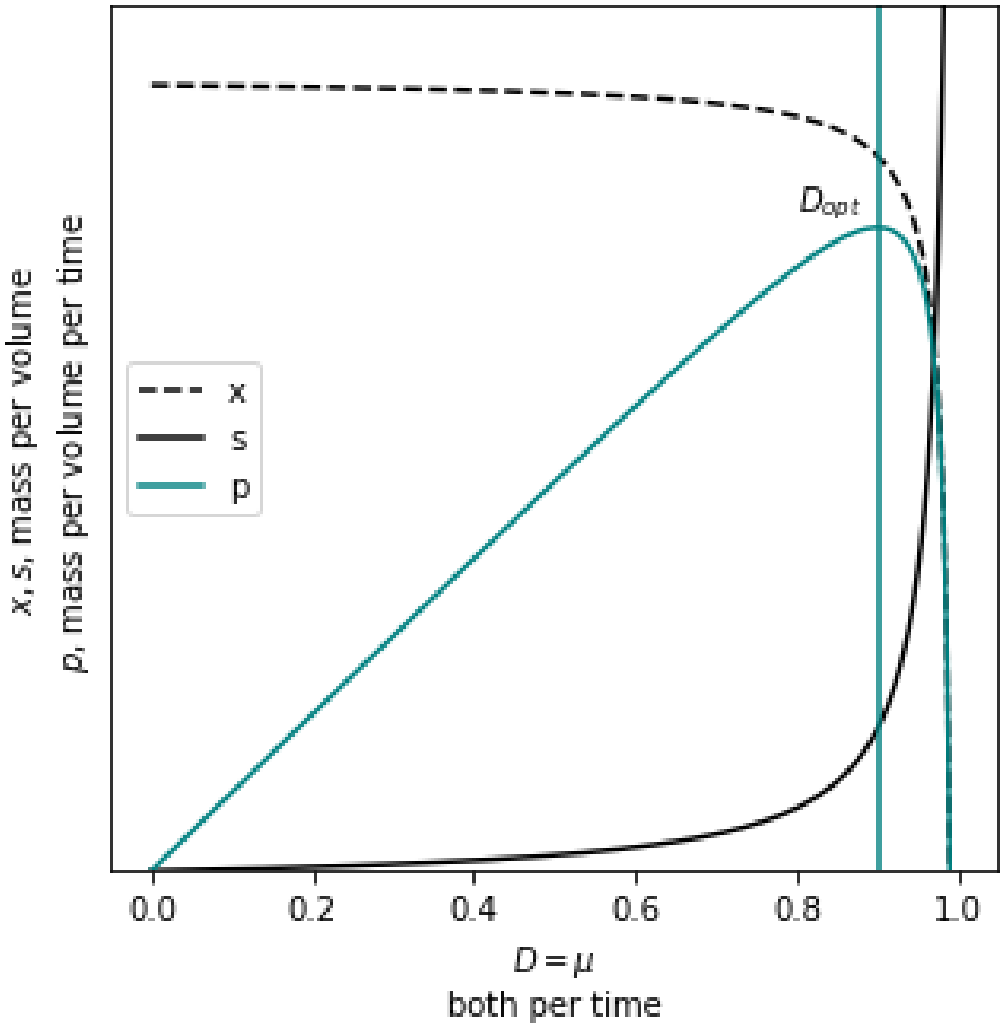
- Above D_C: $\frac{dx}{dt} < 0$ and $\mu - D < 0$, therefore cells wash out of reactor.

- **Chemostats** operate better at low D (unstable steady states approaching D_{crit})

- **Turbidostats** operate better at high D (greater changes in cell number therefore more efficient regulation)

- Other continuous cultures with feed-back mechanisms do exist: pH-stats, oxygen-stats etc.

Optimal D for max. P_x



- If D small:
  - P_X is small due to small flow out of bioreactor
- If $D \approx D_{crit}$:
  - P_X is small due to small biomass concentration in bioreactor and effluent from bioreactor.

$$D_{opt} = \mu_{max}\left(1 - \sqrt{\frac{K_S}{K_S + S_0}}\right) \quad (14)$$

Volumetric productivity

The productivity is given by the symbol P_i, r_i or Q_i (with subscript x for biomass and p for a product)

For a continous stirred tank reactor in steady-state, the **volumentric productivity** (i.e. productivity per reactor volume) is given

For a chemostat in steady-state:

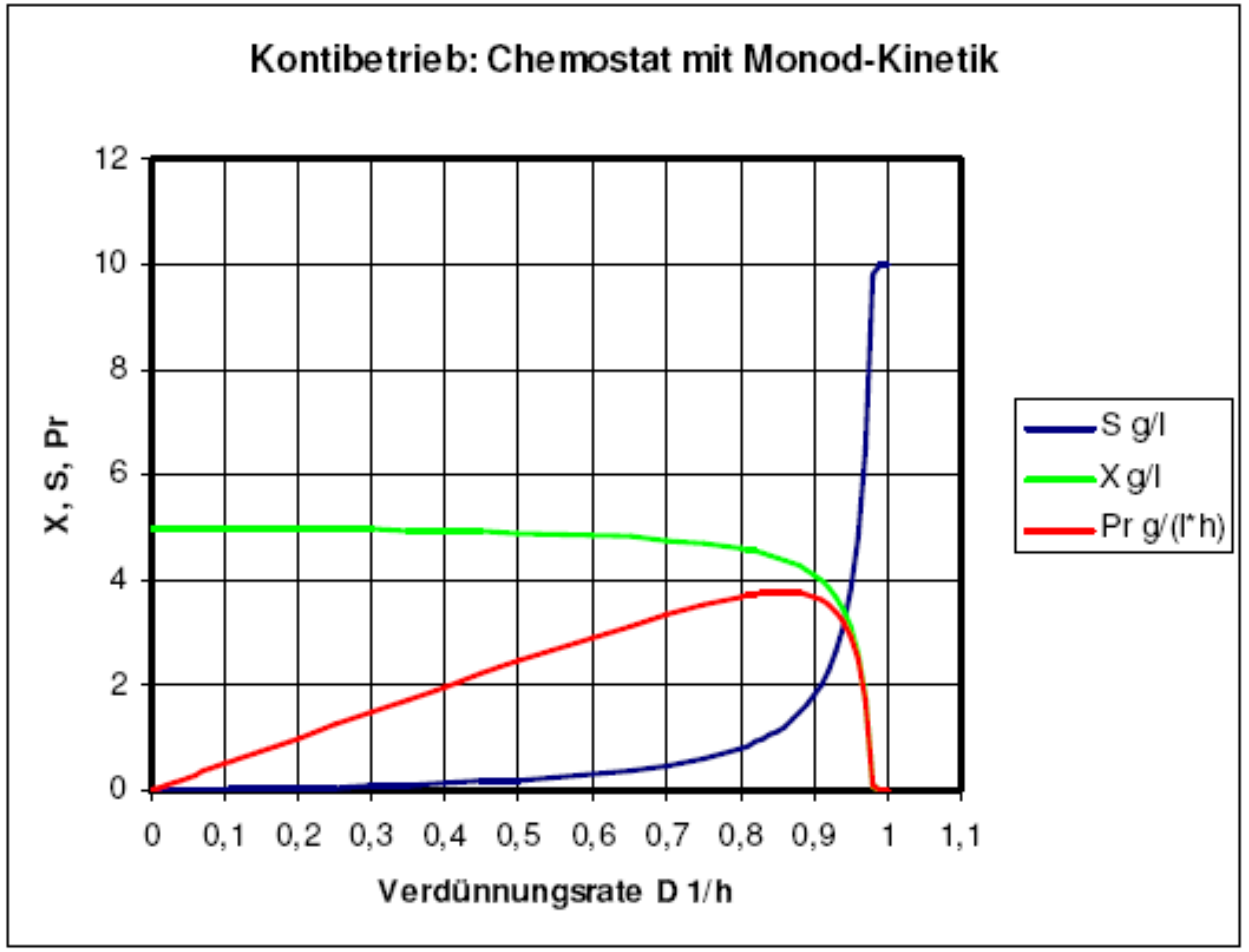
$$P_x = \tilde{x} \cdot D \quad \left[\frac{\mathrm{g}}{\mathrm{Lh}}\right] \tag{15}$$

$$\tilde{x} = Y_{X/S}\left(s_0 - \frac{K_S * D}{\mu_m - D}\right) \tag{10}$$

Consequently:

$$P_x = D \cdot Y_{x/s}\left(s_0 - \frac{K_S * D}{\mu_{max} - D}\right) \tag{16}$$

Calculations



Monod-Kinetik

mit

$\mu_{max} = 1 \ h^{-1}$

$K_S = 0{,}2 \ g \ L^{-1}$

$S_0 = 10 \ g \ L^{-1}$

$Y_{X/S} = 0{,}5 \ g \ g^{-1}$

Calculate: D_m, x (=Product) at D_m and s at D_m, max. productivity

Maximum biomass productivity

Dilution rate giving maximum P_X is D_{opt}

- But at D_{opt}: $s \gg 0$. Therefore, the conversion is not complete!
    - In a production scenario, the point of operation for a chemostat thus depends on the value of product compared with substrate costs.

Consequently, when does it make sense to use a chemostat?

- Cultures based on a single chemostat are mainly used for primary products
- Secondary products may be produced:
    - In chemostat in which carbon source is not the limiting substrate (e.g., N, P, S etc. are limiting)
    - At low dilution rates
    - Using a multi-stage chemostats (e.g., two-stage with a larger volume in the second reactor)
- Other continous culture types should also be considered (see next chapter « Continuous Cultivation »)

Effect of maintainance I

Generally, cells work as catalysts according to following growth equation:

$$C_6H_{12}O_6 + Y_{N/S} \cdot NH_4^+ + Y_{O/S}O_2 \longrightarrow Y_{X/S} \cdot C_{X1}H_{X2}N_{X3}O_{X4} + Y_{\frac{P}{S}} \cdot C_{P1}H_{P2}N_{P3}O_{P4} + Y_{C/SCO2} + Y_{W/SH2}O$$

which postulates according to Monod's third law $Y_{X/S} = \text{const.}$ **However, this is an approximation!** Instead, let's introduce a mass balance on the substrate including the substrate consumption due to the cell maintenance:

$$\frac{ds}{dt} = D(s_{in} - s) - \left(\frac{\mu}{Y_{\frac{X}{S}}} + m\right)x \tag{17}$$

This is due to **maintenance requirement m**:
Substrate is consumed for the maintenance of cell integrity, function, motility, and viability

Units: g substrate consumed per quantity of cells per hour, *e.g.* g 10^{-6} cells h^{-1} or g g^{-1} h^{-1}

Effect of maintainance II

At steady-state $\frac{ds}{dt} = 0$ and $\mu = D$:

$$0 = D(s_{in} - \tilde{s}) - \left(\frac{D}{Y_{\frac{X}{S}}} + m\right)\tilde{x} \tag{18}$$

Rearranging:

$$\frac{(s_{in} - \tilde{s})}{\tilde{x}} = \frac{1}{Y_{\frac{X}{S}}} + \frac{m}{D} \tag{19}$$

$\frac{\tilde{x}}{(s_{in} - s)}$ is the yield coefficient $Y_{X/S}^{app}$ we would obtain without including the maintenance requirements. Thus, we define $1/Y_{X/S}^{app} = \frac{s_{in} - \tilde{s}}{\tilde{x}}$:

$$\boxed{\frac{1}{Y_{\frac{X}{S}}^{app}} = \frac{1}{Y_{\frac{X}{S}}} + \frac{m}{D}} \tag{20}$$

Effect of maintainance III

- The adjusted substrate mass balance also affects the biomass concentration.
- As long as $D \gg k_d$, the maintenance consumption can be neglected.

$$\tilde{x} = Y_{\frac{X}{S}}(s_0 - \tilde{s})\frac{D}{D+k_d} \tag{21}$$

With maintenance energy: $k_d = \frac{m}{Y_{\frac{X}{S}}}$ (22)

Determination of maintenance energy

Pirt equation: $$\frac{1}{Y_{X/S}^{app}} = \frac{1}{Y_{X/S}} + \frac{m}{\mu} \quad (23)$$

$Y_{X/S}$: True growth yield on substrate s [g g^{-1}]

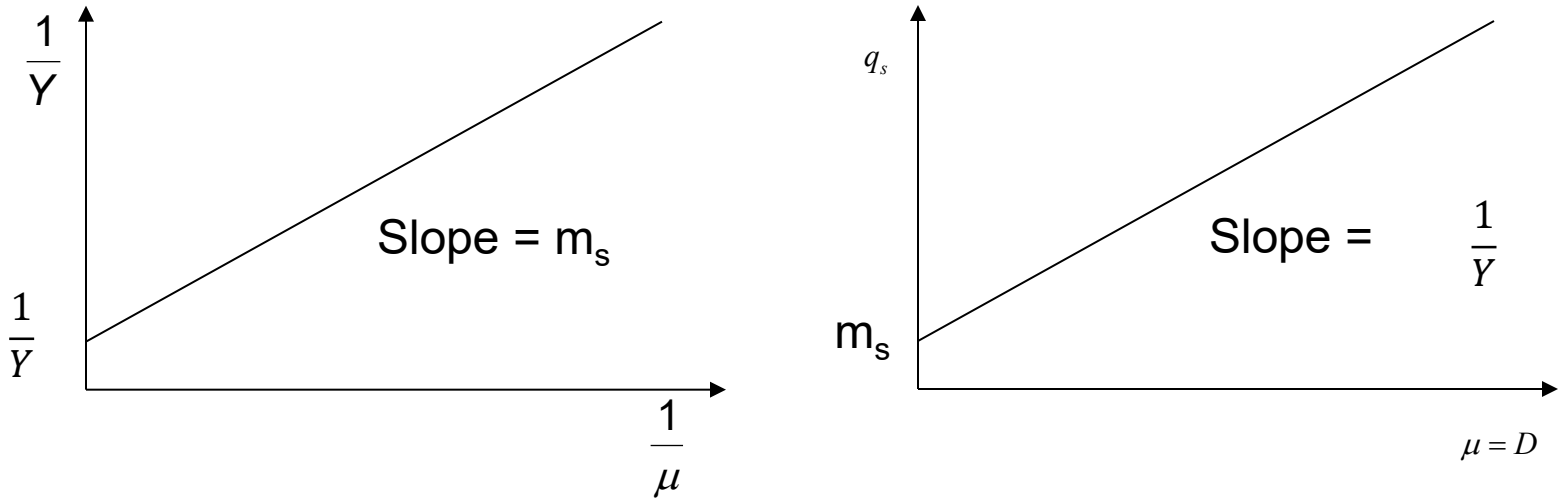
m: maintenance energy [g g^{-1} h^{-1}]

Note: Cell maintenance consumes a large percentage of the substrate in high cell-density cultures and at slow growth rates in chemostats and fed-batches.

Determination of m and Y

Presentation of the data in a graph

$$\frac{1}{Y_{X/S}^{app}} = \frac{1}{Y_{X/S}} + \frac{m}{\mu} \quad \longrightarrow \quad q_s = \frac{\mu}{Y_{X/S}^{app}} = \frac{\mu}{Y_{X/S}} + m \qquad (24)$$

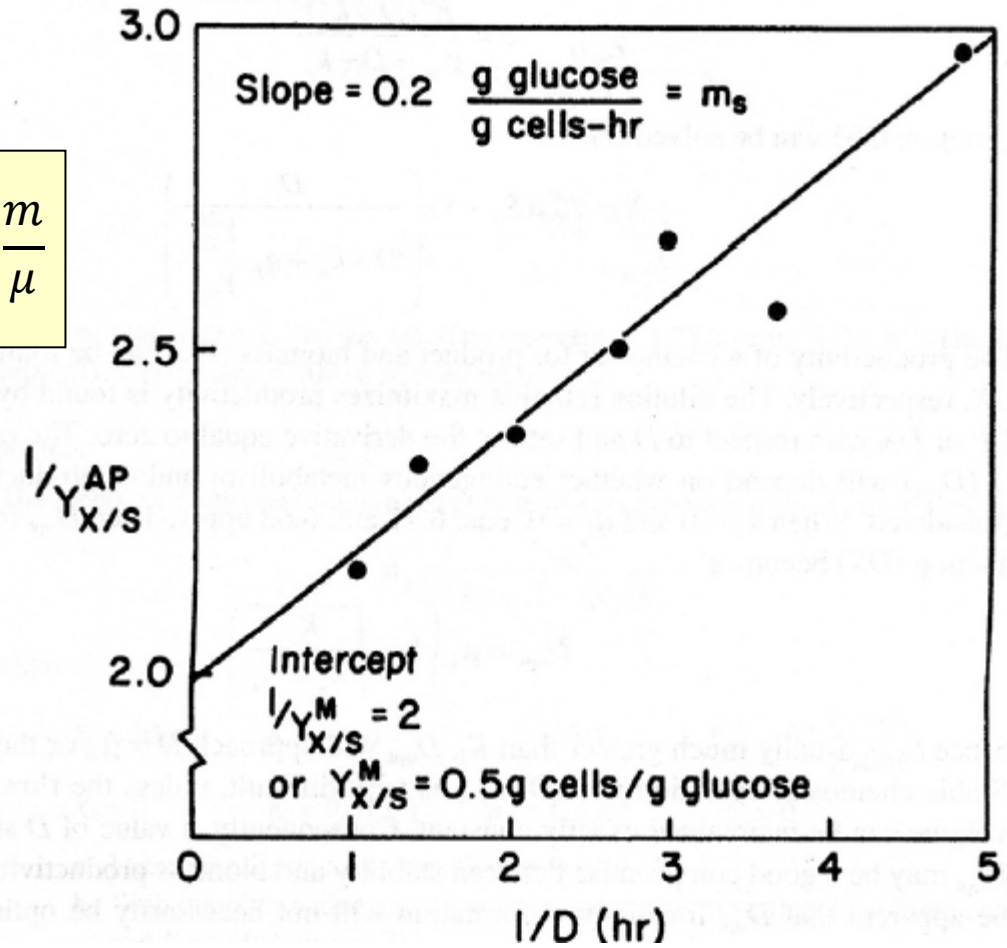


Range of values m_s: in *E. coli*: 0.072 – 0.090 g glucose g^{-1} cdw h^{-1}

Range of values Y^G: 0.35 – 0.53 g cdw g^{-1} glucose

Determination of m and Y in praxis

$$\frac{1}{Y_{X/S}^{app}} = \frac{1}{Y_{X/S}} + \frac{m}{\mu}$$

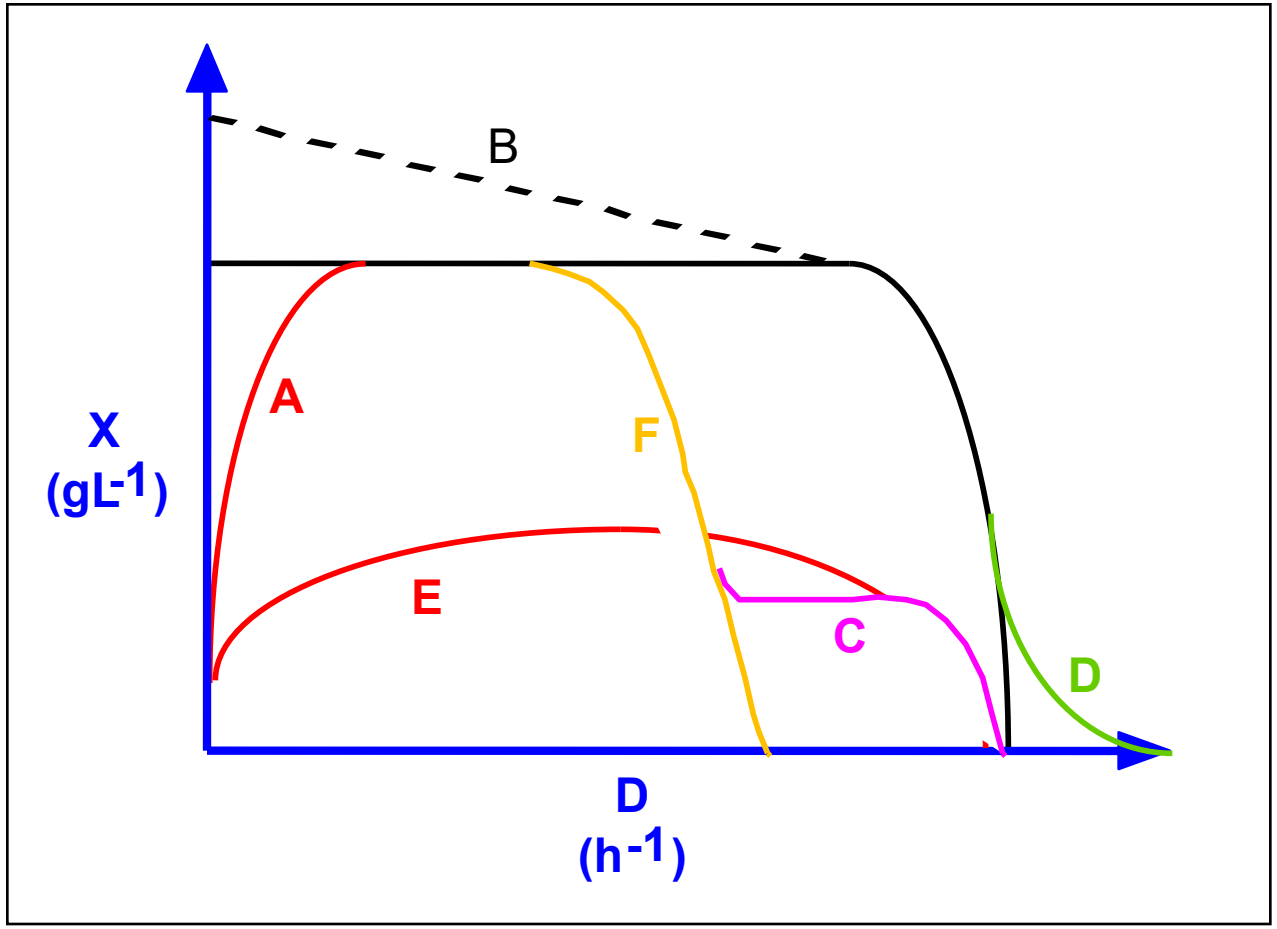


Question

- Do we always have ideal conditions in a chemostat?

- Under what growth conditions is the cell maintenance energy not neglectable?

Deviations from ideal chemostat behaviour



Deviations from ideal chemostat behaviour

A Yield factor not constant- under carbon- limited conditions yield decreases at low dilution rates due to **maintenance**

B Substrate is converted to **intracellular storage** compounds e.g. lipids, particularly when non- carbon limited e.g. N- Limited

C Variable yields due to **shifts in metabolism** at different values of D, e.g. change from purely oxidative to fermentative growth at higher values of D.

D **Imperfect mixing** in bioreactor or **cell adhesion** to walls or aggregation of cells.

E Limiting substrate perhaps **toxic** even at low levels e.g high osmotic stress leads to high maintenance at low D and substrate toxicity at high D.

F **Dc smaller than μ_{max}** due to requirement for vitamin, trace element or other growth factor which is provided by inoculum but is slowly washed out during continuous culture.

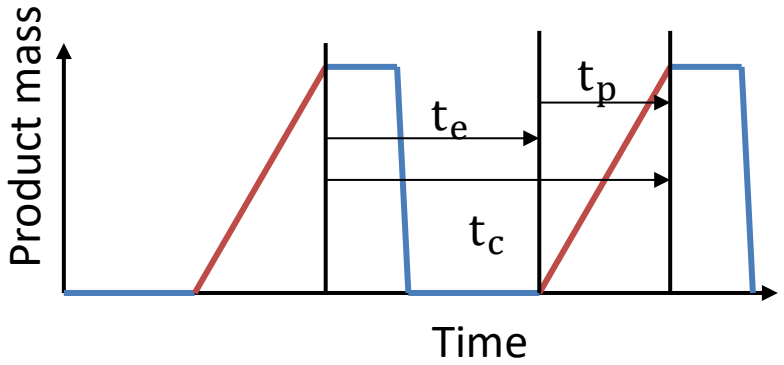
Productivity: Important definitions

Name	Definition	Calculation	Units (examples)
Global productivity	Produced product amount in reactor per time	$P^G = \frac{\Delta M_{out}}{\Delta t} = \frac{\int r_M}{\Delta t}$ (25)	g h^{-1}
Volumetric productivity	Produced product amount per reactor volume and per time	$P^V = \frac{P^G}{V}$ (26)	g L^{-1} h^{-1}
Specific productivity	Produced product per amount of biomass and per time	$P^S = \frac{P^G}{X} (= q_s)$ (27)	g g^{-1} h^{-1}

ΔM_{out}: Product mass generated by process

Δt: Time frame of consideration

Batch (idealized)



The relevant time frame needs to be defined (see next slide).

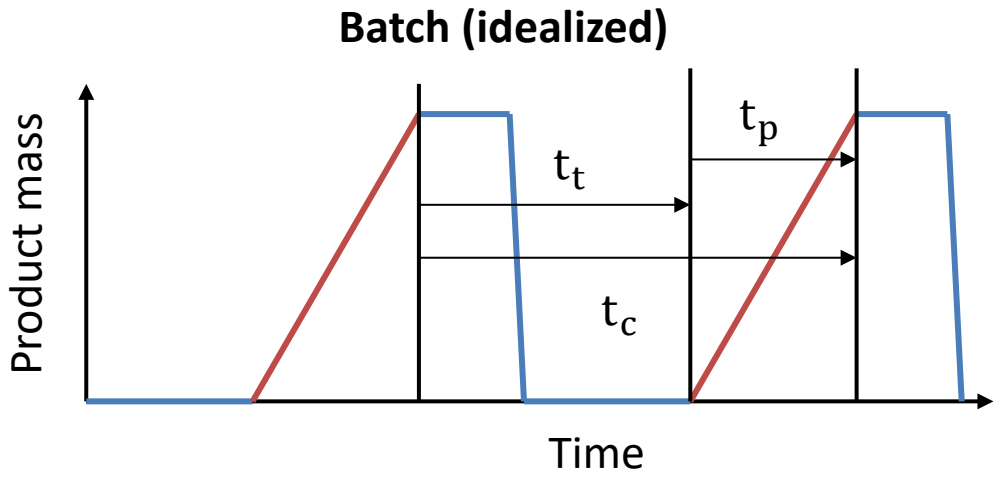
Continuous

Typically, the time in steady-state is much longer than the turn-over, start-up and shut-down time → only the productivity in steady-state is relevant.

For continuous reactors in steady-state:

$$P^G = const.$$

Batch productivity of a primary product



t_c	Total cycle time
t_p	Production time
t_t	Turnover time
M^I	Primary product mass (e.g., biomass X, ethanol, lactate)

Assumption: the cells grow exponentially during t_p.

The total batch time, t_c is given by:

$$t_c = t_p + t_t = \frac{\ln\frac{X}{X_0}}{\mu_{max}} + t_t \qquad (27)$$

The mass produced by the process (step) is given by:

$$\Delta M^I = M^I_{max} - M^I_0 = Y_{\frac{M}{S}} \cdot S_0 \qquad (28)$$

$$P^G_{batch} = \frac{\Delta M^I}{t_c} = \frac{Y_{\frac{M}{S}} \cdot S_0}{\frac{1}{\mu_{max}} \cdot \ln\frac{X_{max}}{X_0} + t_t} \qquad (29)$$

Chemostat productivity of a primary product

For a continous stirred tank reactor in steady-state, the **volumetric productivity** (i.e., productivity per volume of cell culture fluid) is given by:

$$P^V_{conti} = \tilde{m}^I \cdot D \quad [\text{g L}^{-1}\ \text{h}^{-1}] \tag{30}$$

For a chemostat in steady-state:

$$\tilde{m}^I = Y_{M/S}\left(s_0 - \frac{K_S * D}{\mu_{max} - D}\right) \tag{31}$$

Inserting:

$$P^V_{conti} = D \cdot Y_{M/S}\left(s_0 - \frac{K_S * D}{\mu_{max} - D}\right) \tag{32}$$

Chemostat productivity of a primary product

By inserting D_{opt} into the equation for the volumetric productivity of a chemostat, the optimal volumetric productivity of a chemostat is obtained.

$$P^V_{conti,opt} = D_{opt}\tilde{m} = \mu_{max}\left(1 - \sqrt{\frac{K_s}{K_s + s_0}}\right) \cdot Y_{\frac{M}{S}}\left(S_0\sqrt{K_s(S_0 + K_s)} + K_s\right) \quad (33)$$

$$= \mu_{max} Y_{\frac{M}{S}} S_0 \left(\sqrt{\frac{K_s + s_0}{s_0}} - \sqrt{\frac{K_s}{s_0}}\right)^2 \quad (34)$$

Typically, $K_s \ll S_0$. In this case, the equation simplifies to:

$$P^V_{conti,opt} = \mu_{max} \cdot Y_{\frac{M}{S}} \cdot s_0 \quad (35)$$

Remember:

$$P^G = P^V \cdot V \quad (26)$$

Volumetric productivity increase from batch to continuous

We can now estimate how much higher the volumetric productivity of a chemostat is in comparison to the batch cultivation and if run on the same substrate and used for a primary product (biomass, ethanol, lactate, etc.).

$$G = \frac{P^V_{conti,opt}}{P^V_{batch}} = \ln\frac{X_{max}}{X_0} + t_t \cdot \mu_{max} \qquad (36)$$

Note:

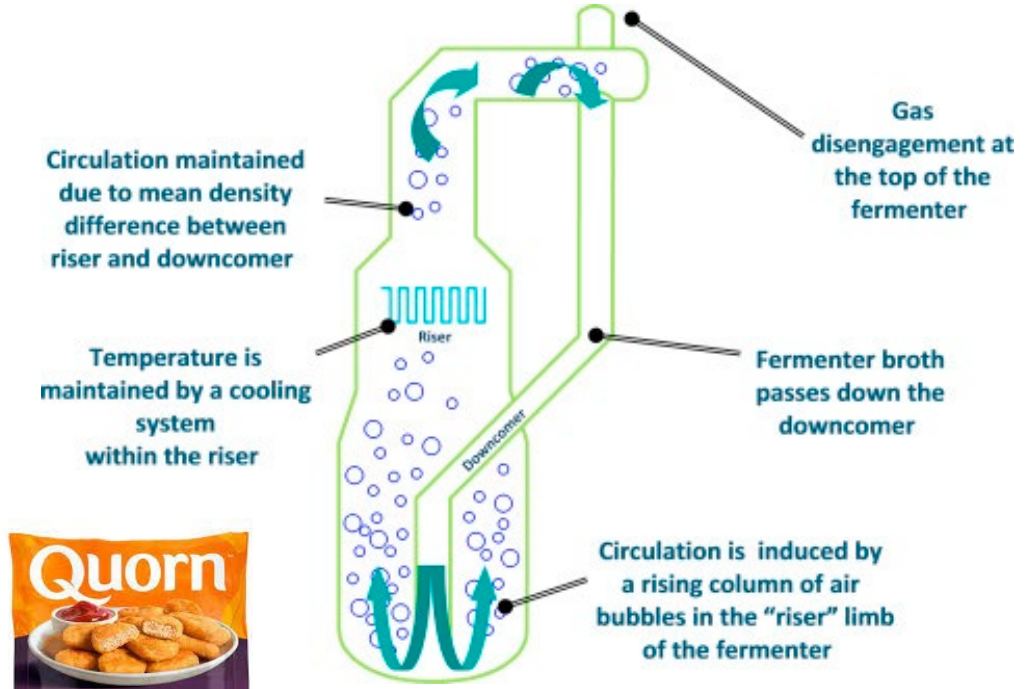
- This formula only gives a **rough estimate** in the increase of the productivity in a chemostat compared to batch culture.
- It neglects the **startup, shutdown and turnover time of the chemostat**
- It assumes production in the chemostat at the optimal dilution rate, which leaves a notable fraction of substrate unconsumed → recycling strategies may be necessary
- **Batch processes** are typically the **least productive** production scheme. The formula thus compares chemostat to the 'worst-case' scenario

Example: Volumetric productivity of the quorn process

- Quorn (a meat analog) is produced in an airlift reactor operated as a chemostat
- Quorn is processed fungi mycelia from *F. graminearum* A3/5 grown on glucose sirup, mineral salts and biotin
- Ammonia serves as nitrogen source
- Reported reactor sizes: 40 m^3 – 150 m^3
- Biomass concentration: 15 g L^{-1}

μ_{max}	$\ln \frac{X_{max}}{X_0} = t_p \mu_{max}$	$t_t \mu_{max}$	G
$0.23\ h^{-1}$	$t_p \approx 100\ h \Rightarrow$ Ca. 23	Ca. 11	**Ca. 34**

Numbers from Anthony P. J. Trinci, Microbiology, Volume 140, Issue 9, 1994 and Trevor Williamson, PhD Thesis, 1996.



https://www.sainsburys.co.uk/gol-ui/product/vegetarian-food/quorn-crispy-nuggets-300g

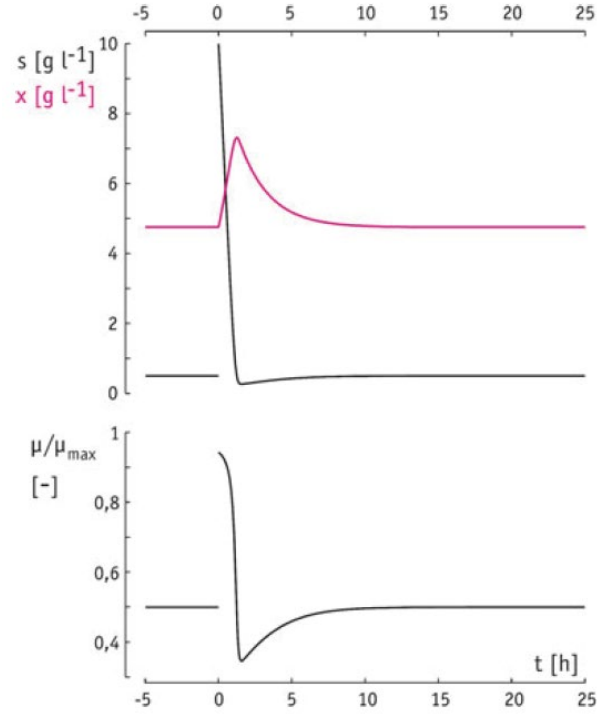
T. Finnigan, ... C. Abbott, in Sustainable Protein Sources, 2017

Questions

- What is your estimation about the chemostat productivity in comparison to **fed-**batch cultivation?

- How could you improve the performance of continuous cultivation? Think of substrate/biomass in bioreactor at D_{opt}.

Medium design: Pulse experiments



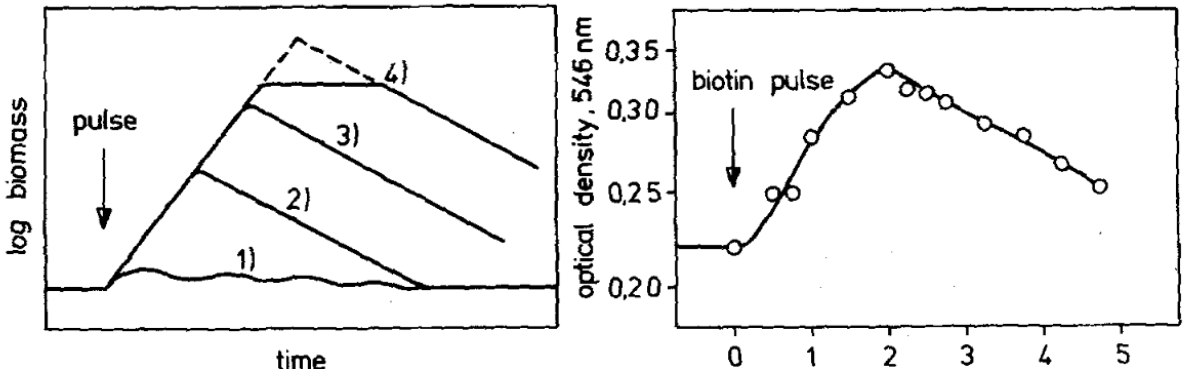
H. Chieml, 2011

- Pulse experiments are used to check, if a medium component is **limiting or inhibiting** the growth at the current steady-state.
- A pulse of concentrated substrate solution is applied by injecting the solution into the bioreactor.
- The pulse induces a transient response of the biomass concentration.
  - If a pulse with the *limiting* substrate is applied, all growth limitations concerning this substrate are lost → peak in biomass.
  - If a pulse with an *inhibiting* substrate is applied, a dip in the biomass is observed due to additional limitations.
- Dip/peak in pO_2 can be used as a fast response indicator
- The behavior can be
  - simulated with the Monod model (top figure)
  - is confirmed by real-world tests (lower figure)

Question: what does a peak in pO_2 mean?

Pulsed substrate is:

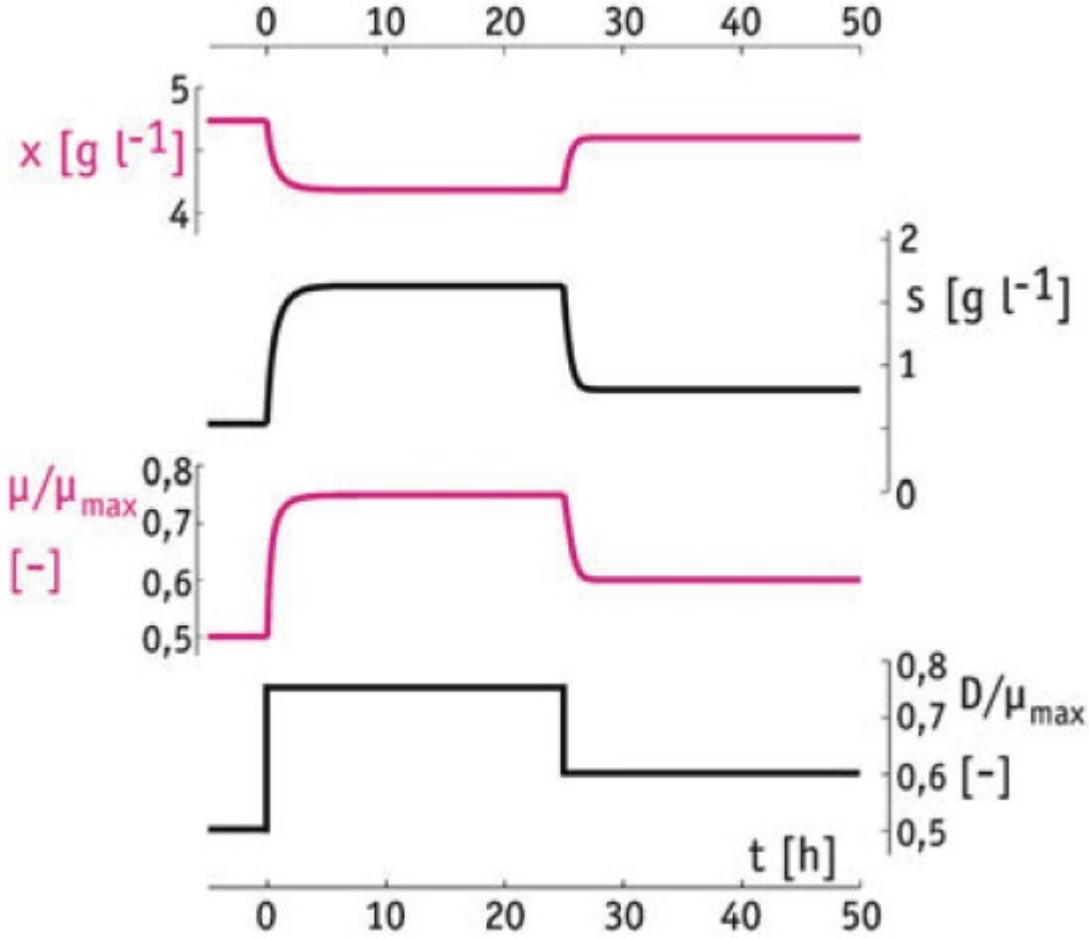
1) Not limiting substrate
2) Limiting substrate
3) Limiting substrate
4) Initially the limiting substrate, with second substrate becoming limiting during pulse.



H. Kuhn, U. Friederich, and A. Fiechter, European J. Appl. Microbiol. Biotechnol. 6, 1979.

Medium design: Shift experiments

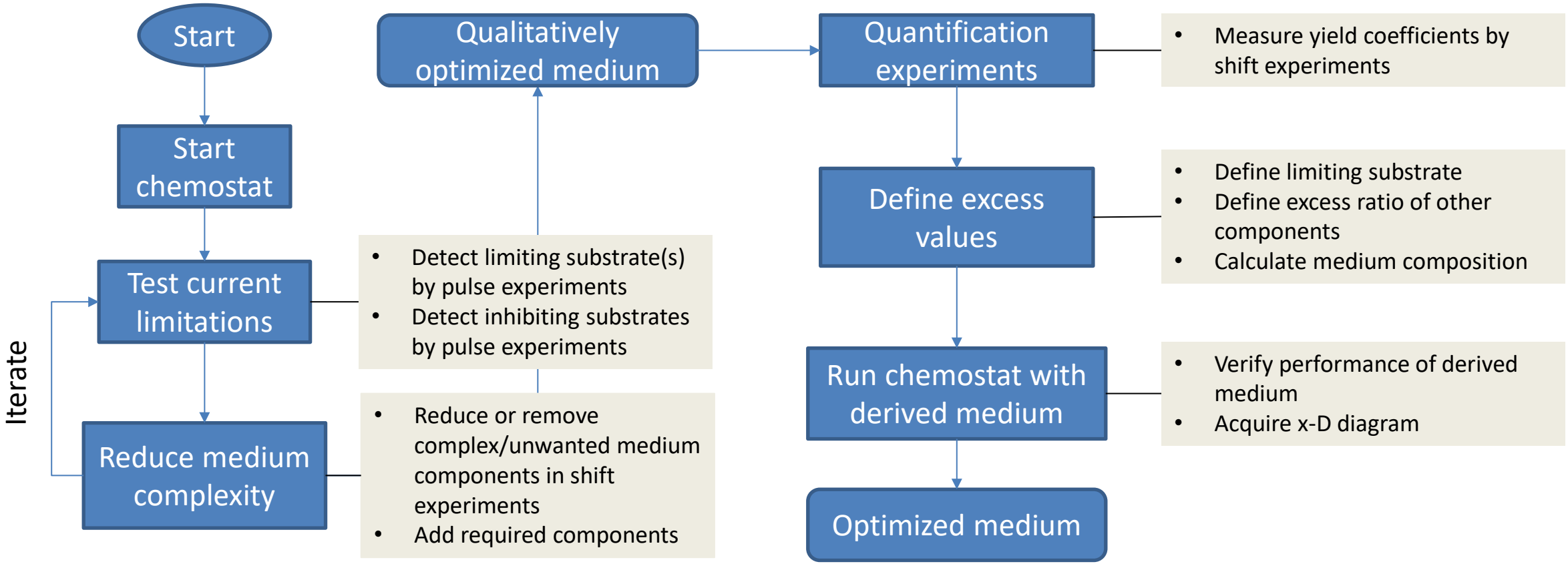
- Shift experiments change the composition of the feed or operating parameters (e.g., pH, dilution rate, temperature)
- The change in biomass upon applying the shift can be used to accurately investigate the impact of a certain process parameter on the process.
- Multiple shift experiments are used to obtain x-D diagrams.
- Inhibiting substances and shifts to high dilution rates must be done cautiously such that the chemostat is not washed out.



H. Chiemi, 2011

Medium optimization in a chemostat

- Prior to starting a medium optimization in a chemostat:
  - Define an objective function (what do we want to optimize, what is the goal?)
  - Have a medium which allows the microorganism to grow



Adapted from H. Chieml, 2011

Offgas analysis/ Respiratory Quotient

$$\text{Respiratory Quotient (RQ)} = \frac{\dot{V}CO_2}{\dot{V}O_2}$$

Carbohydrate

$C_6H_{12}O_6 + 6\ O_2 \longrightarrow 6\ CO_2 + 6\ H_2O$
(Glucose)

$$RQ = \frac{6\ \dot{V}CO_2}{6\ \dot{V}O_2} = 1.0$$

Fat

$C_{16}H_{32}O_2 + 23\ O_2 \longrightarrow 16\ CO_2 + 16\ H_2O$
(Palmitate)

$$RQ = \frac{16\ \dot{V}CO_2}{23\ \dot{V}O_2} = 0.7$$

Protein

Average $$RQ = \frac{77.5\ \dot{V}CO_2}{96.7\ \dot{V}O_2} = 0.8$$

https://www.sciencedirect.com/science/article/pii/B9780123919090500785

- The respiratory quotient (see manual of TP Chemostat) allows the determination of particular cell physiological effects
- A high RQ value indicates the consumption of highly oxidized substrates (e.g., sugars).
- Shifts in the RQ can demonstrate a change of metabolic activity (e.g., Crabtree effect).

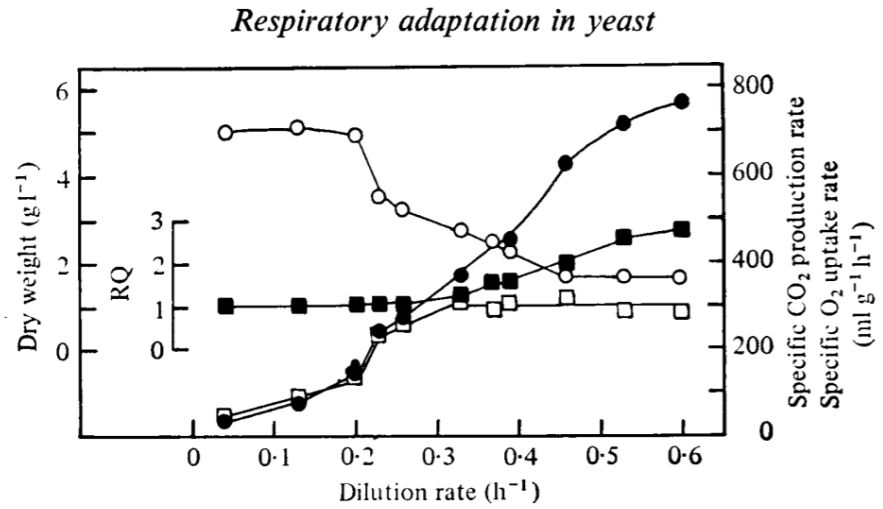


Fig. 1. Continuous culture of *S. cerevisiae* with glucose as the limiting substrate. Estimations were dry weight (○), specific carbon dioxide production rate (●), specific oxygen uptake rate (□) and respiratory quotient, RQ (■). The glucose feed concentration was 9·93 g l⁻¹.

Barford JP, Hall RJ. An Examination of the Crabtree Effect in *Saccharomyces cerevisiae*: the Role of Respiratory Adaptation. Microbiology. 1979;114(2):267-75.

Major applications of continuous culture reactors

Biopharmaceutical industry

"[Biopharmaceutical] leaders such as Genzyme, Bayer, Janssen, Merck-Serono, Novartis, and Lonza for Eli Lilly have manufactured approximately **19 marketed** recombinant protein products/monoclonal antibody (mAb) products using **perfusion or elements of continuous processing**, and these products are predominantly blockbusters with annual revenues totaling approximately $20 billion."

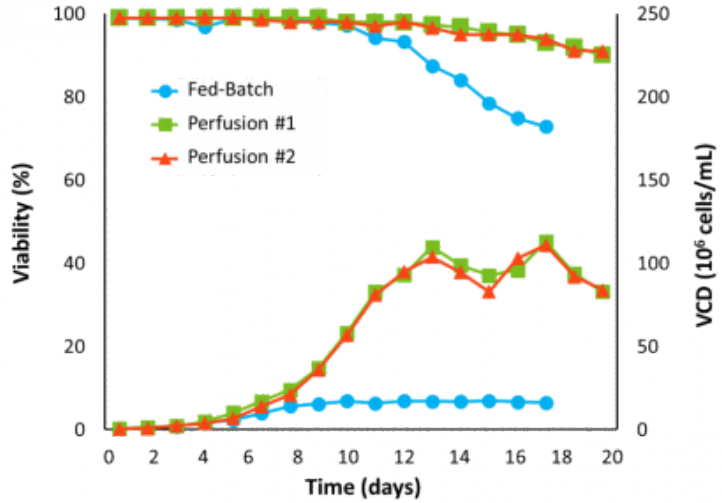
"FDA has been a **strong supporter** of continuous processing as early as 2004 when it released Pharmaceutical cGMPs for the 21st Century – A Risk-Based Approach. In addition to

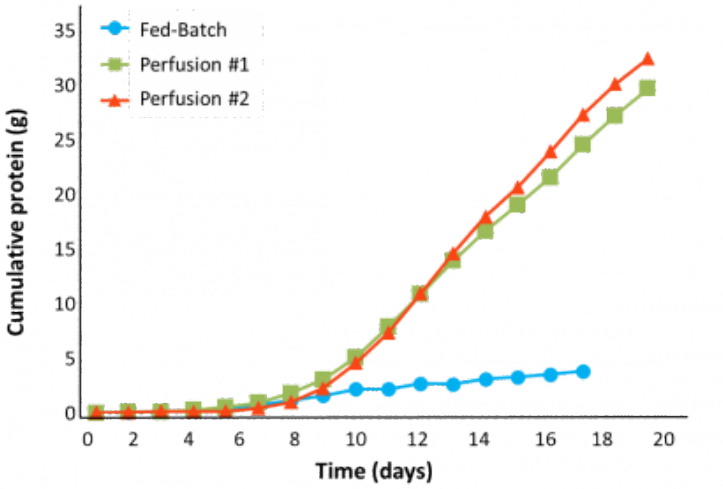
- reduced inventory,
- lower capital costs,
- a smaller ecological footprint,
- and more flexible operation,

FDA is an advocate of the fact that continuous manufacturing

- reduces manual handling of products and
- allows for better process control."

Adapted from R. Hernandez, "Continuous Manufacturing: A Changing Processing Paradigm," BioPharm International 28 (4) 2015.





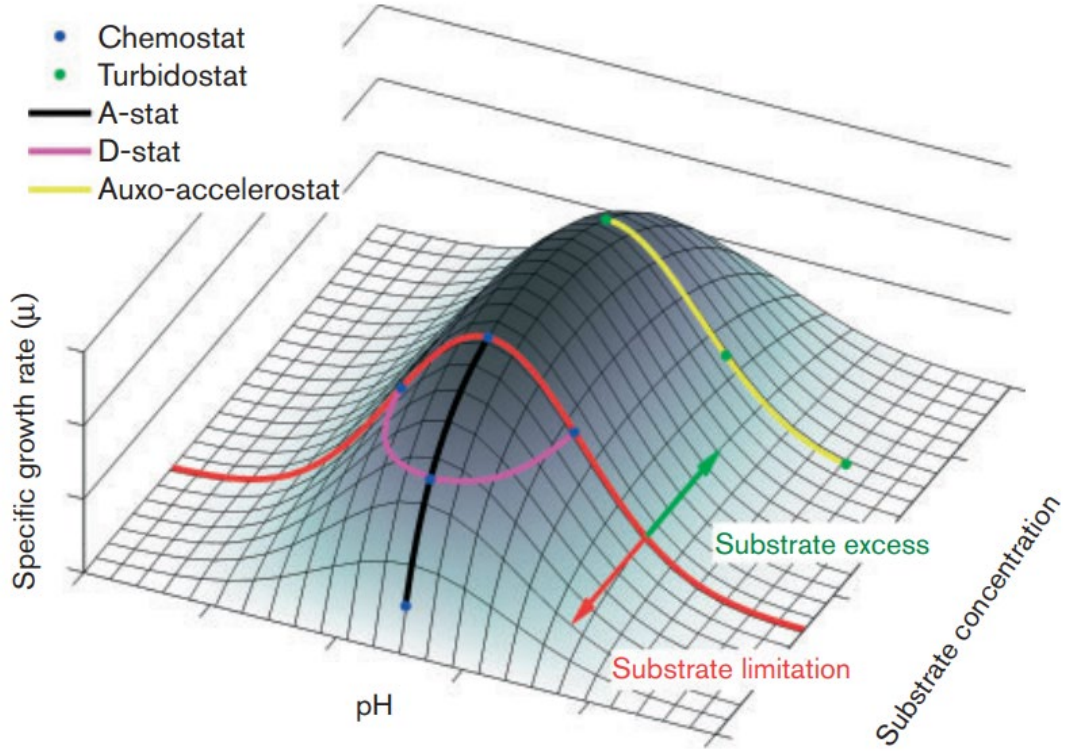
It is also worth noting, that the international regulatory alignment group (ICH) released 2021 a draft on the regulatory requirements for continuous manufacturing: ICH guideline Q13 on continuous manufacturing of drug substances and drug products

Data and plots by Repligen

Overview single-stage continuous cultures

Reactor type	Characteristics
Chemostat	• Flow into and from the bioreactor is kept constant. The biomass is not retained in the bioreactor but rather flushed out in function of the dilution rate.
Auxostat	• Flow of medium into and from the bioreactor is controlled by a feedback mechanism such as nutrient concentrations (nutristat) or constant pH values (pH-auxostat) or constant turbidity (turbidostat). • Biomass is not retained.
Perfusion	• Biomass **is retained** by e.g., continuous filtration or centrifugation. • Media perfusion is controlled by an adequate mechanism, e.g., preset flow rate, constant pH, constant nutrients.
Plug-flow and similar reactors	• Plug flow through a pipe where the bioreaction takes place. • If cells are not adherent, a continuous feed of cells is required (e.g., a chemostat). • If process is aerobic, sufficient oxygen transfer needs to be enabled.
Special purpose continuous reactors	• Continuous bioreactors for research applications (not directly associated to process development) or industrial production (open pond for microalgae) • For example, cyclic shift of steady state for strain evolution

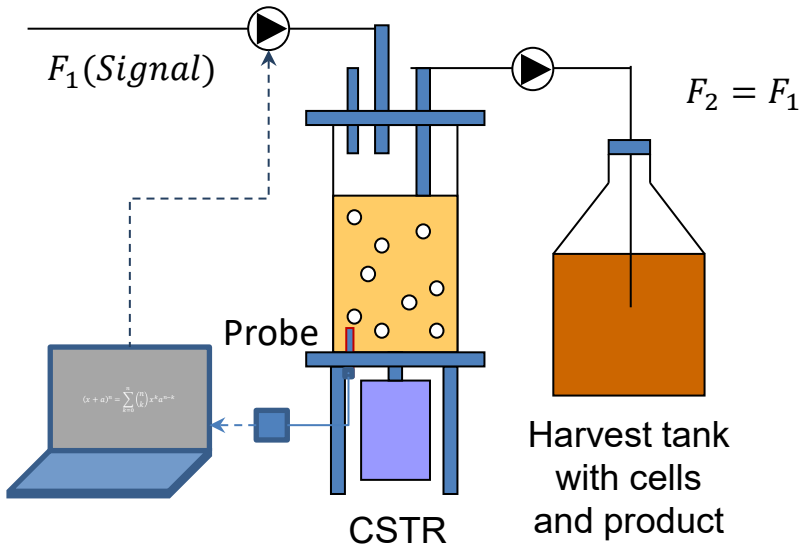
Auxostats – Actively controlled continuous cultivations



Different realizations of ‘Auxo’:

- **Turbidostat:** constant cell density based on turbidity
- **Permittistat:** constant cell density based on dielectricity (permittivity)
- **Nutristat**: limiting nutrient concentration is kept constant
- **pH-Auxostat:** growth (greek auxo) is kept constant, e.g. by assuming growth association to pH

Auxostats



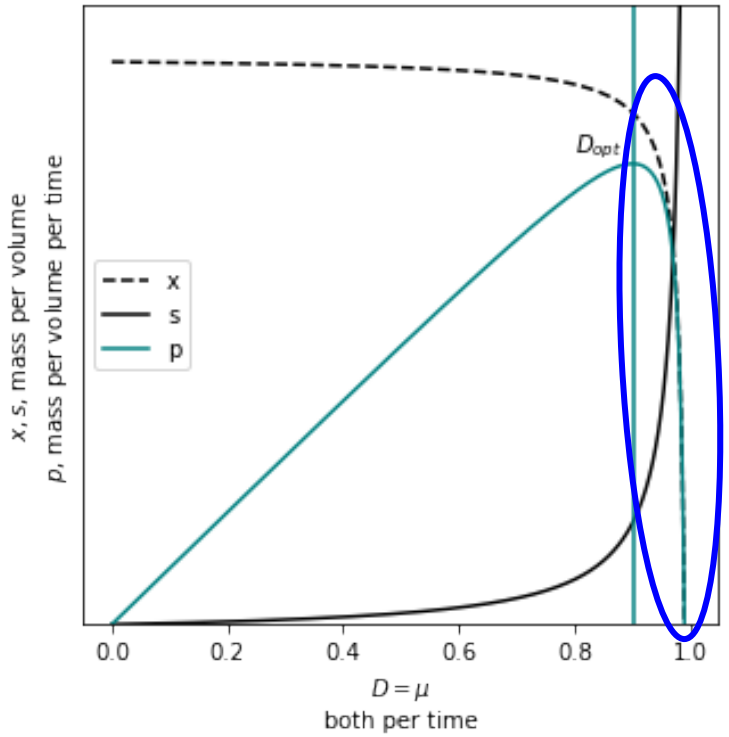
Advantages

- Cells are growing at a very fast rate ($\mu \approx \mu_{max}$)
- Cells “tell” when they are hungry
- Combination with plug flow bioreactor to consume remaining product
- Culture cannot be washed out

Disadvantages

- Fast and stable sensor needed
- Large medium usage
- Difficult to control (finetuning of thresholds)
- High concentration of unused substrate (s)

Turbidostat & permittistat – the mechanism

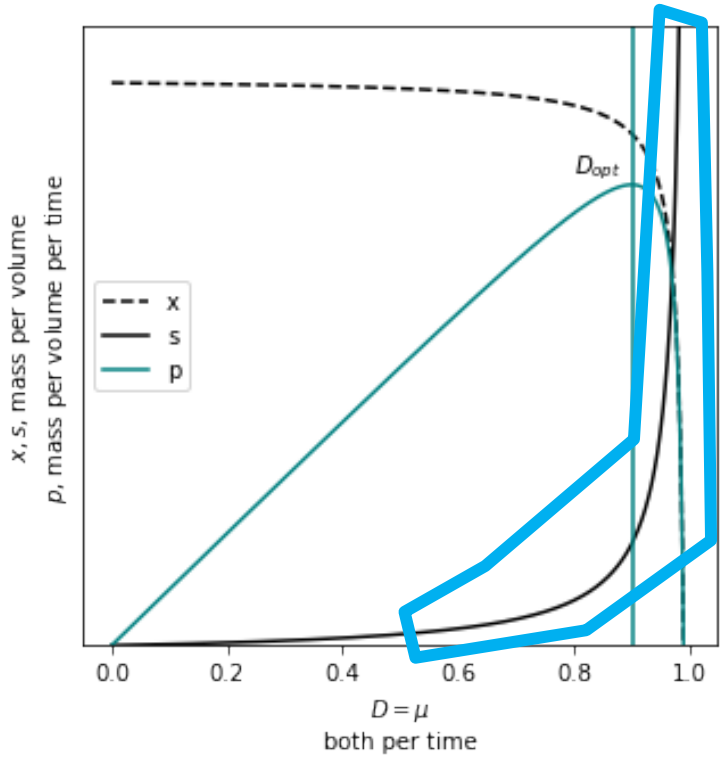


- **Turibdostat** stabilizes the turbidity:
- Assumption: $A_{665\,nm} \propto x$
- $\frac{dA_{665\,nm}}{dt} = 0 \approx \frac{dx}{dt}$

- **Permittistat** stabilizes the conductivity/permittivity
- Assumption: $\sigma \propto x$

The equations derived for the chemostat **still hold as long as** $\boldsymbol{F_{in} = F_{out} \approx const}$. The main difference is that the steady state is actively maintained.

Nutristat – the mechanism



- Turibdostat measures and stabilizes the nutrient concentration.
- $\frac{ds}{dt} = 0$

The equations derived for the chemostat **still hold as long as** $\boldsymbol{F_{in} = F_{out} \approx const}$. The main difference is that the steady state is actively maintained.

Nutristat – measurement methods

- Different methods exist for measuring the nutrient concentration in the bioreactor. For a nutristat the methods need to be:
  - Much faster than the response time of the cells
  - Accurate and precise (nutrient concentrations may be very low)
  - Typically automated

- The measurement methods may be split according to the proximity to the process:
  - Off-line: analysis done without proximity to the process (e.g. QC, not on manufacturing floor, slow)
  - At-line: close to the process (manufacturing floor) but physically separated. A manual intervention of an operator is required.
  - On-line: automated sampling system samples and analyzes sample close to the manufacturing line (either by-pass or complete withdrawal)
  - In-line: in-situ and real-time measurement in bioreactor, piping etc.

Suitable analytical equipment for auxostats

Cobas, Roche diagnostics
At-line process analytics

Other technologies:

- Mid-IR
- Near-IR
- On-line flow chemistry

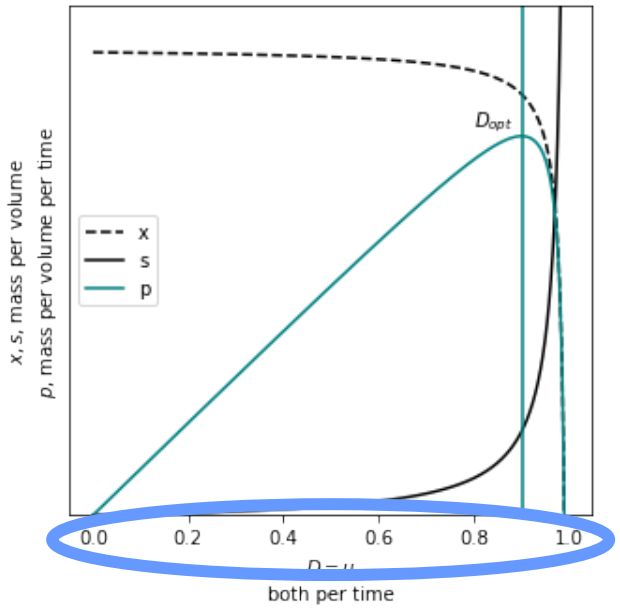
Patrol UPLC, Waters
On-line chromatography

AMA instruments
On-line gaschromatography

ReactRaman, Mettler Toledo
In-line Raman spectroscopy
@HES-SO from Jan-2022

pH-auxostat: The mechanism

$$C_6H_{12}O_6 + Y_{N/S} \cdot \mathbf{NH_4}^+ + Y_{\frac{O}{S}} O_2 \rightarrow Y_{\frac{X}{S}}^{app} \cdot C_{X1}H_{X2}N_{X3}O_{X4} + Y_{\frac{P}{S}} \cdot C_{P1}H_{P2}N_{P3}O_{P4} + Y_{\frac{C}{S}} \cdot CO_2 + Y_{\frac{W}{S}} \cdot H_2O$$



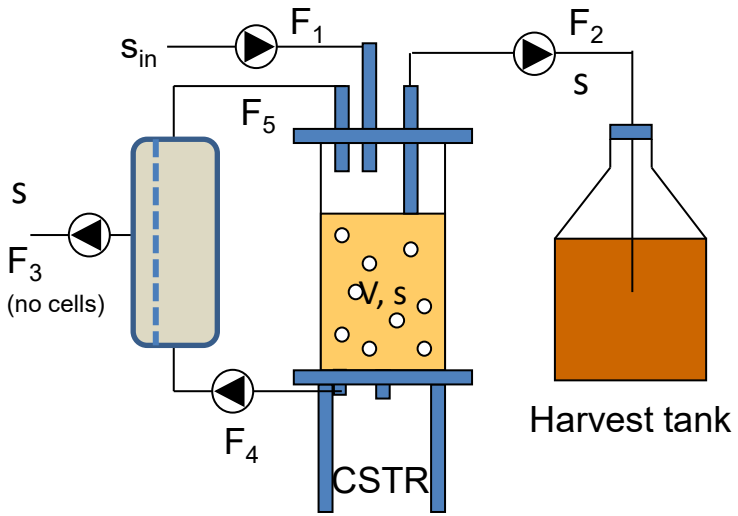
- Assumption: generation or consumption of H^+ is proportional to the growth rate
- pH is kept constant
- Substrate is fed at a rate proportional to the base/acid consumption with a proportionality constant $R > \frac{1}{Y_{H^+/S}}$
- Thus, cells are fed, when they consume substrate

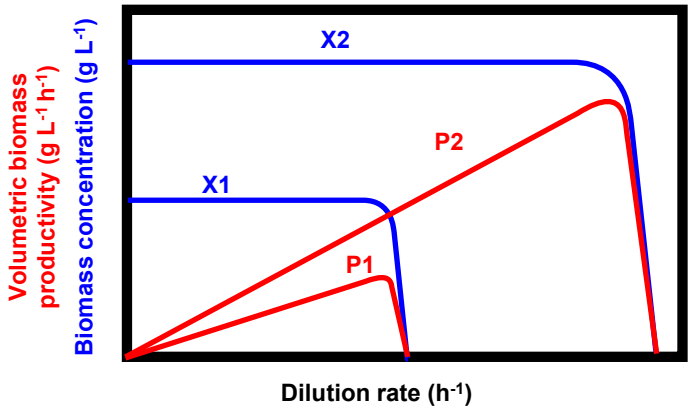
The equations derived for the chemostat **still hold as long as $F_{in} = F_{out} \approx const$**. The dilution rate D is however a result of the proportionalty constant R

Perfusion reactors

- Cells are retained in bioreactor
- Cell withdrawal in additional stream is possible (called bleeding)
- Decouples the growth rate from the dilution rate and thus enables:
  - Very high cell densities
  - Reduced specific growth rates $0 \leq \mu \leq D$
  - Higher volumetric productivities
  - Better substrate utilization

Perfusion reactors/ Retentostat





Volumetric biomass productivity in chemostat with and without cell recycle.

$$F_1 = F_2 + F_3 \quad (37)$$

$$0 \leq R = \frac{F_3}{F_1} \leq 1 \quad (38)$$

$$\frac{dx}{dt} = \mu x - (1-R)Dx \quad (39)$$

$$\frac{ds}{dt} = D(s_{in} - s) \quad (40)$$

$$\mu = (1-R)D \quad (41)$$

$$\tilde{s} = \frac{K_s * D(1-R)}{\mu_{max} - D(1-R)} \quad (42)$$

$$\tilde{x} = Y_{x/s}\,(s_{in} - \tilde{s})\,\frac{1}{1-R} \quad (43)$$

Note 1: Concentration ODEs and SS-equations only valid if the cell volume is much smaller than the total reactor volume: $x \cdot \rho_{cells} \ll V \Rightarrow R \ll 1$, with cell wet density ρ_{cells}.
Note 2: at high cell concentrations, the oxygen supply becomes challenging.

Enhanced production in chemostat with cell recycle in anaerobic fermentations

Continuous production of ethanol using a membrane bioreactor:

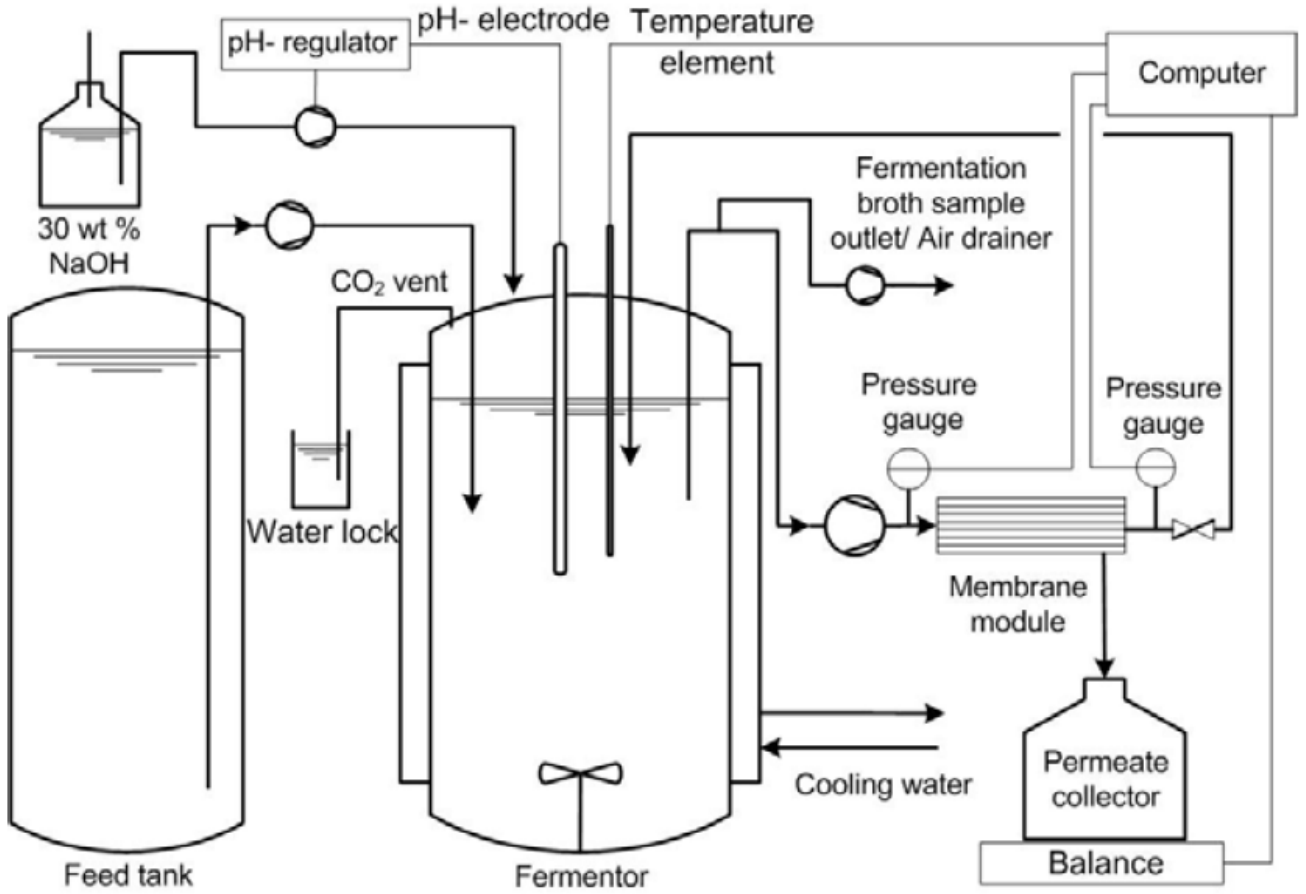


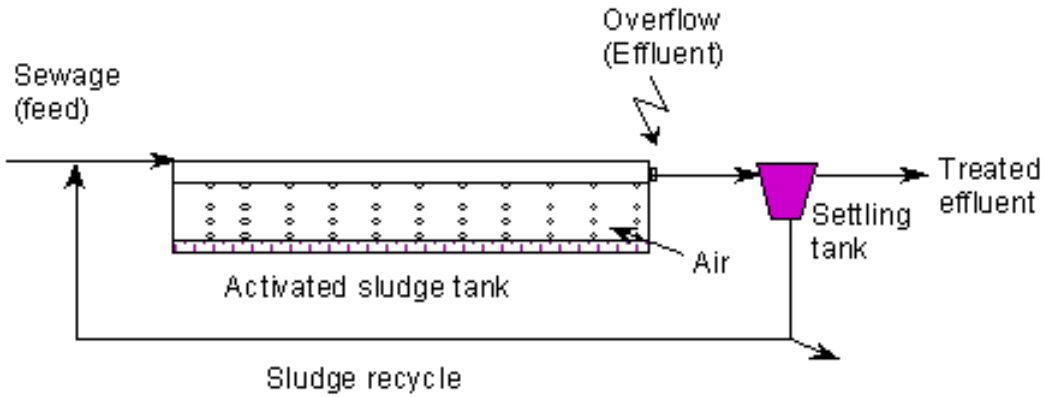
Figure 3. Equipment setup for continuous fermentation with external membranes.

http://www.chemeng.lth.se/exjobb/E641.pdf

Major applications of continuous culture reactors

The **most widespread large scale application** of continuous culture reactors is in *wastewater treatment*.

Activated sludge plants, trickle bed filters, anaerobic digester and ponds all operate in an continuous manner. Cell immobilization is also often employed to improve the efficiency of the process.



Biofilm on plastic support material

http://www.unm.edu/~schuler/researchIFAS.html

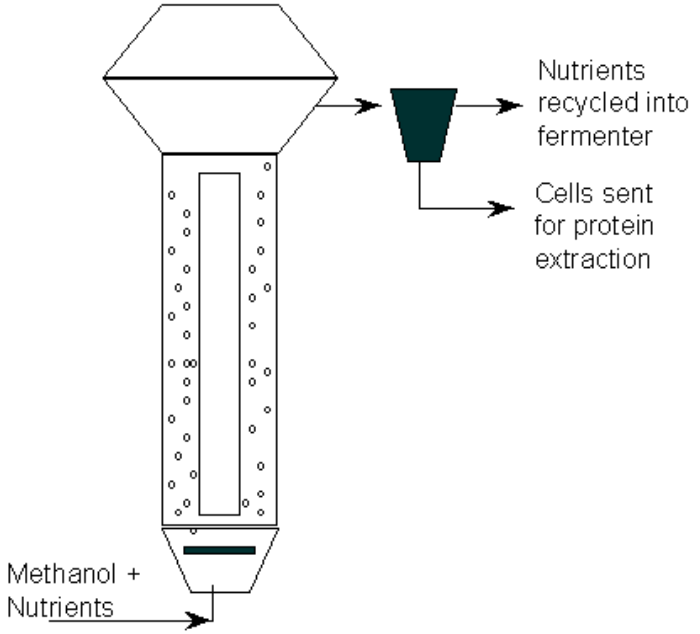
Major applications of continuous culture reactors

Continuous cultures are well established in the wastewater industry for several reasons:

- Unlike pure culture microbial and animal cell systems, contamination is not a consideration, as the wastewater feed will always contain microorganisms.
- Continuous reactors have long been used in waste treatment and their use is not considered a risk.
- Finally using batch cultures is simply not economically feasible. Wastewater flows are often measured in mega liters per hour and batch reactors simply could not cope with the load.

Major applications of continuous culture reactors

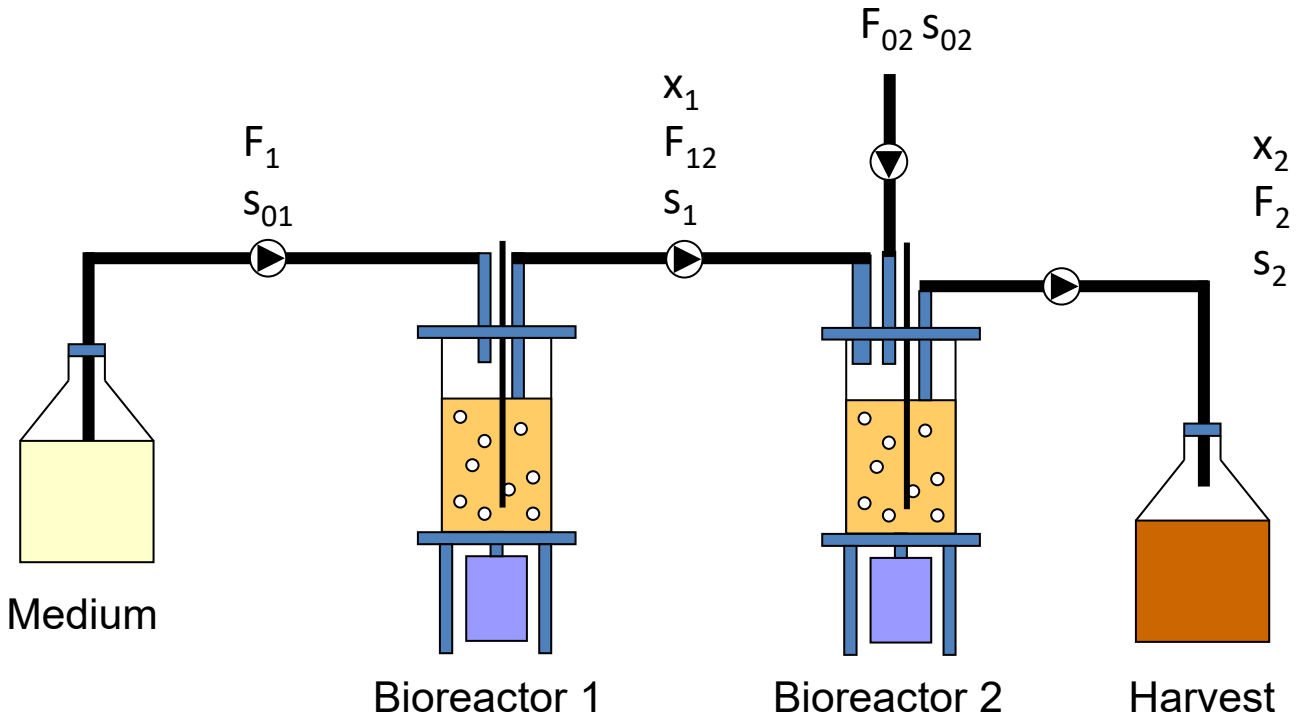
Applications of continuous cultures in other industries are limited. ICI has used a **1 Mio liter** continuous reactor to make single cell protein from animal feed (Pruteen). In this process, methanol is continuously converted to microbial biomass for sale as **single cell protein**.



Some Japanese companies use **immobilized non-growing cells** for the continuous production of certain **amides**. Despite the limit applications, there is still considerable effort being put into research and development for continuous reactors. The future widespread use of continuous reactors in industry should not be ignored.

Laboratory scale continuous reactors are also used for **simulating natural ecosystems**. For example, a continuous system can be used to study the degradation of pesticides in soils.

Two-stage chemostat theory



$$D_2 = \frac{(F_{02} + F_{12})}{V_2} = \frac{F_{02}}{V_2} + \frac{F_{12}}{V_2} = \underbrace{D_{02} + D_{12}}_{\text{Partial dilution rates}} \qquad (44)$$

Biomass balance in a two-stage chemostat

Net rate of increase = Growth rate + Input rate – Output rate

$$\frac{dx_2}{dt} = \mu_2 x_2 + D_{12} x_1 - D_2 x_2 \qquad (44)$$

$$\frac{dx_2}{dt} = 0 \qquad \text{During steady-state} \qquad (45)$$

$$(\mu_2 - D_2) x_2 + D_{12} x_1 = 0 \qquad (46)$$

$$\mu_2 = D_2 - \frac{D_{12} x_1}{x_2} \qquad \text{Consequently } \mu_2 < D_2 \qquad (47)$$

$$x_2 = \frac{D_{12} x_1}{D_2 - \mu_2} \qquad \text{As a consequence, there is no } D_{crit} \text{ for the second stage!} \qquad (48)$$

Substrate balance in a two-stage chemostat

$$x_2 = Y\left(\frac{D_{12}}{D_2}s_{01} + \frac{D_{02}}{D_2}s_{02} - s_2\right) \tag{49}$$

$$\mu_2 = \mu_{\max}\frac{s_2}{s_2 + K_s} \tag{50}$$

$$\frac{\mu_{\max} - D_2}{s_2^2} - \left\{\frac{\mu_{\max}D_{12}s_{01}}{D_2} + \frac{(\mu_{\max} - D_2)D_{02}s_{02}}{D_2} - D_{12}s_1 + K_sD_2\right\}s_2 + K_s(D_{12}s_1 + D_{02}s_{02}) = 0 \tag{51}$$

With the solution:

$$a = \mu_{\max} - D_2 \tag{52}$$

$$s_2 = \frac{-b - \sqrt{(b^2 - 4ac)}}{2a} \qquad -b = \frac{\mu_{\max}D_{12}s_{01}}{D_2} + \frac{(\mu_{\max} - D_2)D_{02}s_{02}}{D_2} - D_{12}s_1 + K_sD_2 \tag{53}$$

$$c = K_s(D_{12}s_1 + D_{02}s_{02}) \tag{54}$$

Substrate balance in a two-stage chemostat

Rate of increase = Rate of input from first stage + Rate of input of fresh medium - Outflow rate - Consumption rate

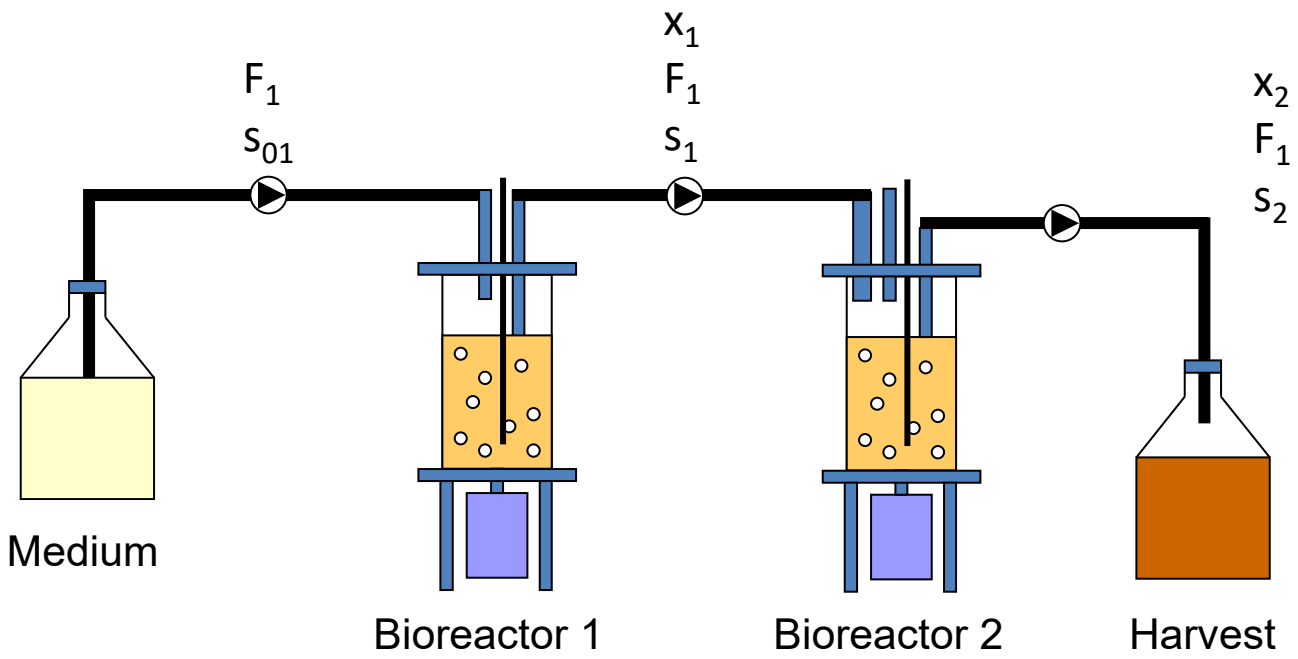
$$\frac{ds_2}{dt} = D_{12}s_1 + D_{02}s_{02} - D_2 s_2 - \frac{\mu_2 x_2}{Y} \qquad (55)$$

during steady-state:

$$0 = D_{12}s_1 + D_{02}s_{02} - D_2 s_2 - \frac{\mu_2 x_2}{Y} \qquad (56)$$

Using $\mu_2 = D_2 - \frac{D_{12}x_1}{x_2}$ (57) and $x_1 = Y(s_{01} - s_1)$ (58)

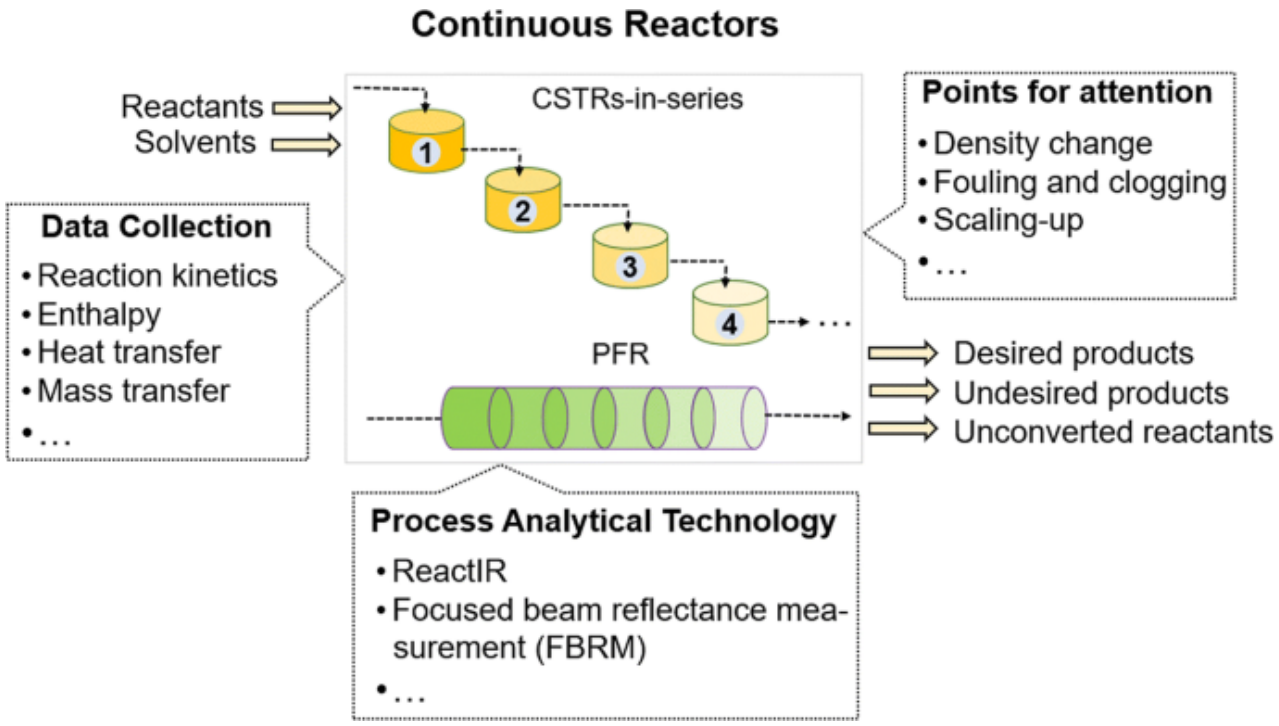
Simplified two-stage chemostat

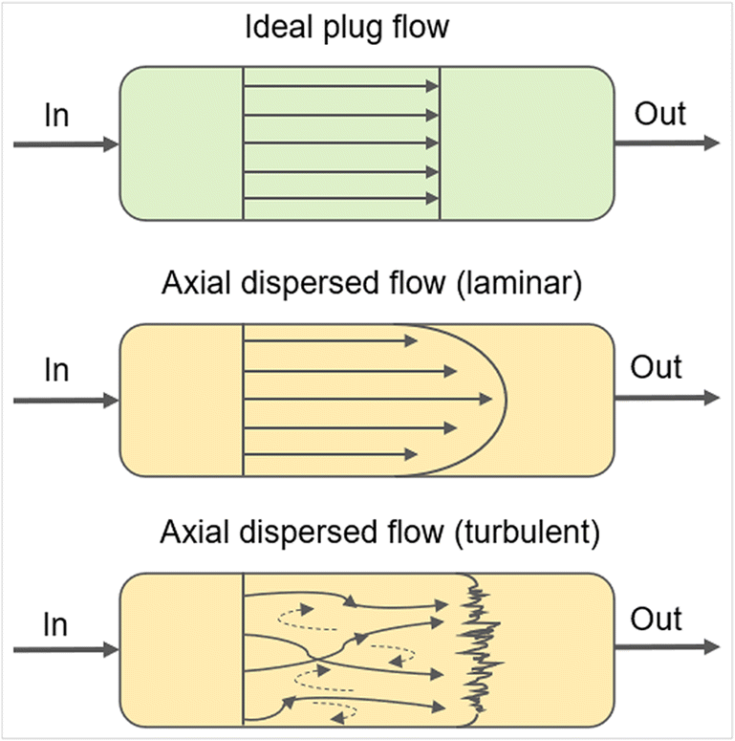


Please note in comparison to previous set-up: $D_{02} = 0$ and $D_{12} = D_2$

$$x_2 = Y(s_{01} - s_2) \quad (59) \qquad \text{and} \qquad \mu_2 = \frac{D_2(x_2 - x_1)}{x_1} \quad (60)$$

From multi-stage chemostat to plug flow reactor





- A plug flow bioreactor is a means of simulating a batch culture in an open system.
- All cells have ideally the same residence time τ

$$x = x_\alpha e^{\mu_{max} t} = x_\alpha e^{\mu_{max} \frac{v}{VD}} \qquad (60)$$

With x_α the concentration at the entrance, v the volume of liquid displaced in time t, V the total volume, and D the dilution rate of the plug flow bioreactor.

Hu, C. Reactor design and selection for effective continuous manufacturing of pharmaceuticals. *J Flow Chem* **11,** 243–263 (2021). https://doi.org/10.1007/s41981-021-00164-3

- The chemostat is the most defined way to culture cells: μ and nutrient limitations can be set.
- Steady-state conditions are time-independent.
- All cells in a chemostat are growing exponentially!
- There is an optimal growth rate that leads to the best volumetric productivity.
- Know to differentiate between specific productivity, volumetric productivity, and global productivity.
- Be able to calculate steady-state conditions for single and multi-stage chemostats with and without feed-back loop.
- Understand the principle of auxostat and plugflow bioreactor.