Bioprocesses and Downstream Processing

ChE-437

Part 3

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Agenda

- Fed-batch exercises of last week

9. Continuous cultivation

- The ideal chemostat

10. Advanced continuous cultivation

- Multiple nutrient limited growth
- Auxostats in continuous culture

Task 12

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

V = 1000 ml dV/dt = 200 ml/h => F

s_0 = 100 g glucose/l μ_{max} = 0.3 h^{-1}

K_s = 0.1 g gluscose/l Y_{XS} = 0.5 g cells / g glucose

X_0 = 30 g (total = x_o^t)

a) Find V_0 (the inital volume of the culture) $V = V_0 + F*t \Rightarrow V_0$=0.6 L

b) Determine the concentration of growth-limiting substrate in the vessel at quasi-steady state at t = 2 h. $s=Ks*D/(\mu m-D) \Rightarrow 0.2\ g\ L^{-1}$

c) Determine the concentration and total amount of biomass in the vessel at t = 2 h (at quasi-steady state) $X=X_0+F*Y_{X/S}*S_0*t \Rightarrow 50$ g or (V_{2h}=1L) 50 g L^{-1}

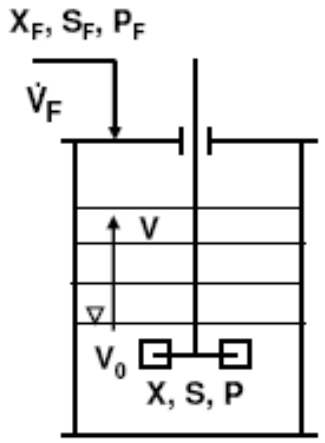
d) If q_p = 0.2 g product /g cells h, p_O = 0, determine the concentration of product in the vessel at t = 2 h

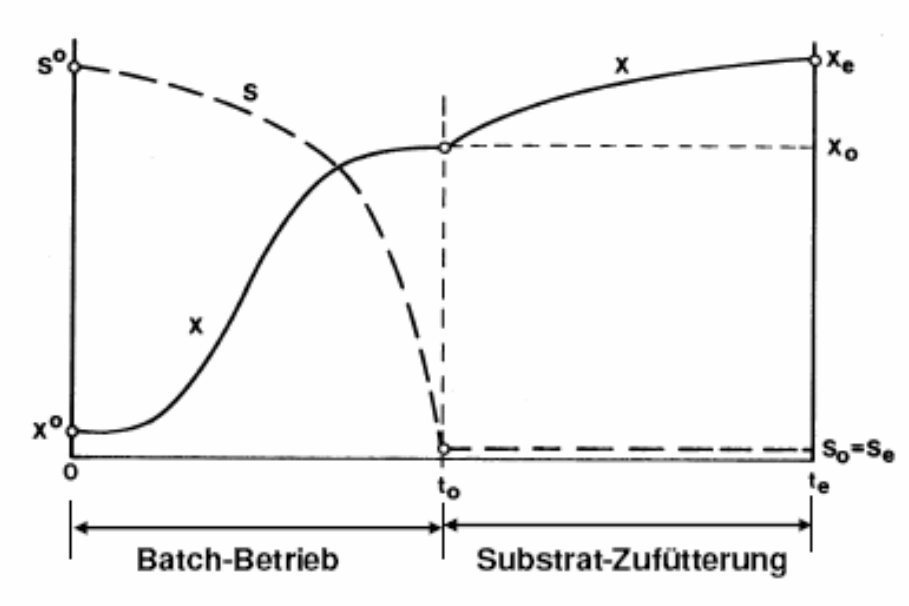
$$P = P_0 \frac{V_0}{V} + q_p X_m \left(\frac{V_0}{V} + \frac{Dt}{2} \right) t$$

16 g /1 L = 16 g L^{-1}

Task 13

The substrate concentration in a fed-batch should be kept constant at a relatively low value. How should the speed of dosage for a highly concentrated substrate solution be modified in order to reach this goal?





Subsrate should remain very low:

$d(V*X)/dt = \mu * X * V \rightarrow dX/dt = X * (\mu - D)$

$d(V*S)/dt = S*dV/dt + V*dS/dt = F*S_i - \mu*X*V/Y_{X/}$

$dS/dt = D*(Si - S) - \mu * X / Y_{X/S}$

F = const.: zunächst Wachstum mit μ_{max}; später mit sinkendem μ

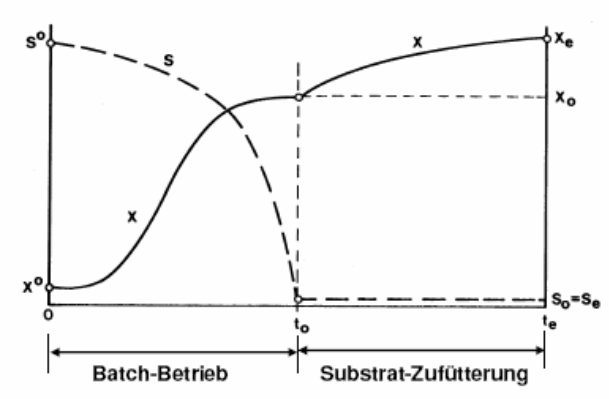
S = const ($dS/dt=0$): Zulauf des Substrates erfolgt exponentiell

$F_t = \mu*V_o*X_o*e^{\mu*t} / [Y_{X/S} * (S_i - S)]$

The feed should be exponential in order to have a constant s!

Task 14

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



Because of cell maintenance the biomass cannot increase linearly with the feed.
The substrate concentration in the culture will decrease because of decreased μ.

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

$$F_t = \mu^* V_o^* X_o^* e^{\mu^* t} / [Y_{X/S} * (S_i - S)]$$

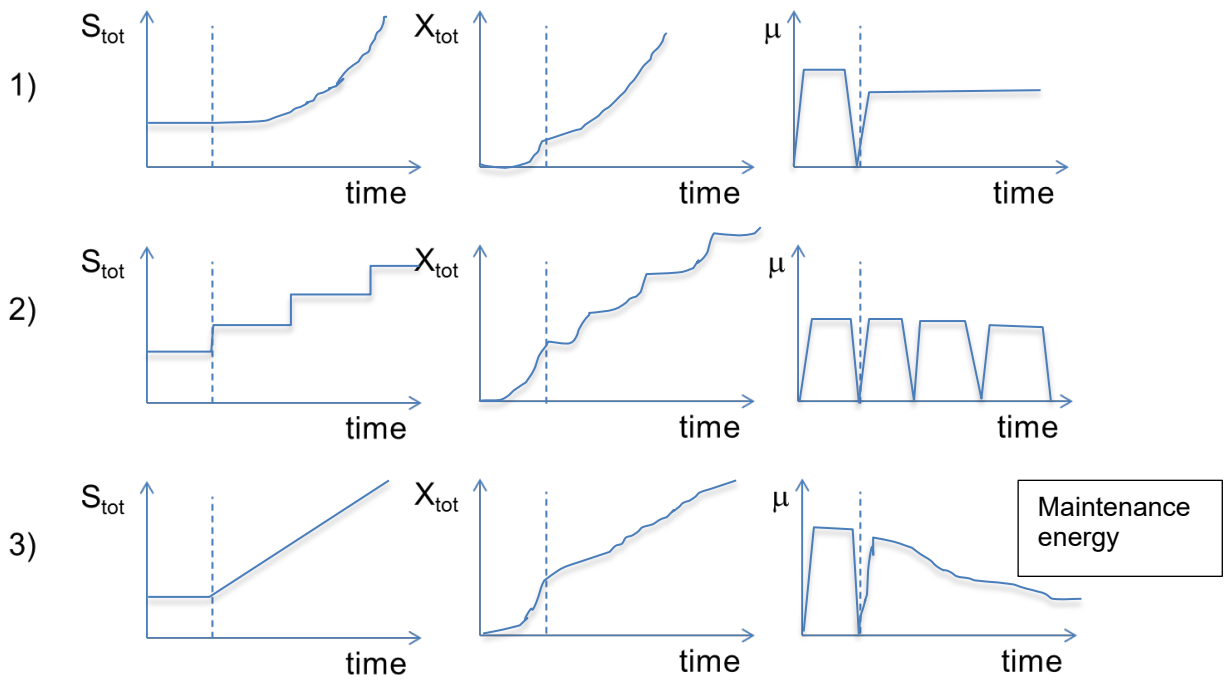
Task 15

Different feed profiles

Please complement the following figures with the trends you are expecting for the respective parameters for the following feed conditions:

1) Exponential feed; 2) Pulsed feed; 3) Linear feed

Note: Dashed line represents the end of the batch culture.



9. Continuous cultivation

The chemostat: Monod 1950

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.)

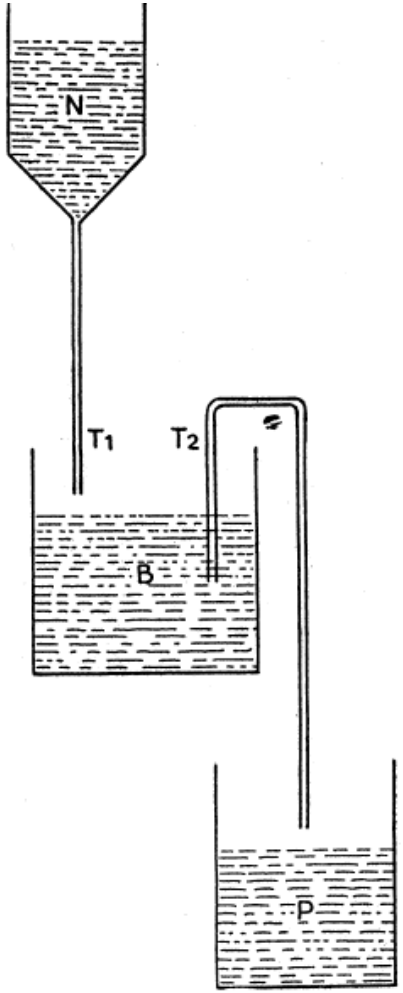


FIG. 1. — Schéma d'un appareil à culture continue.

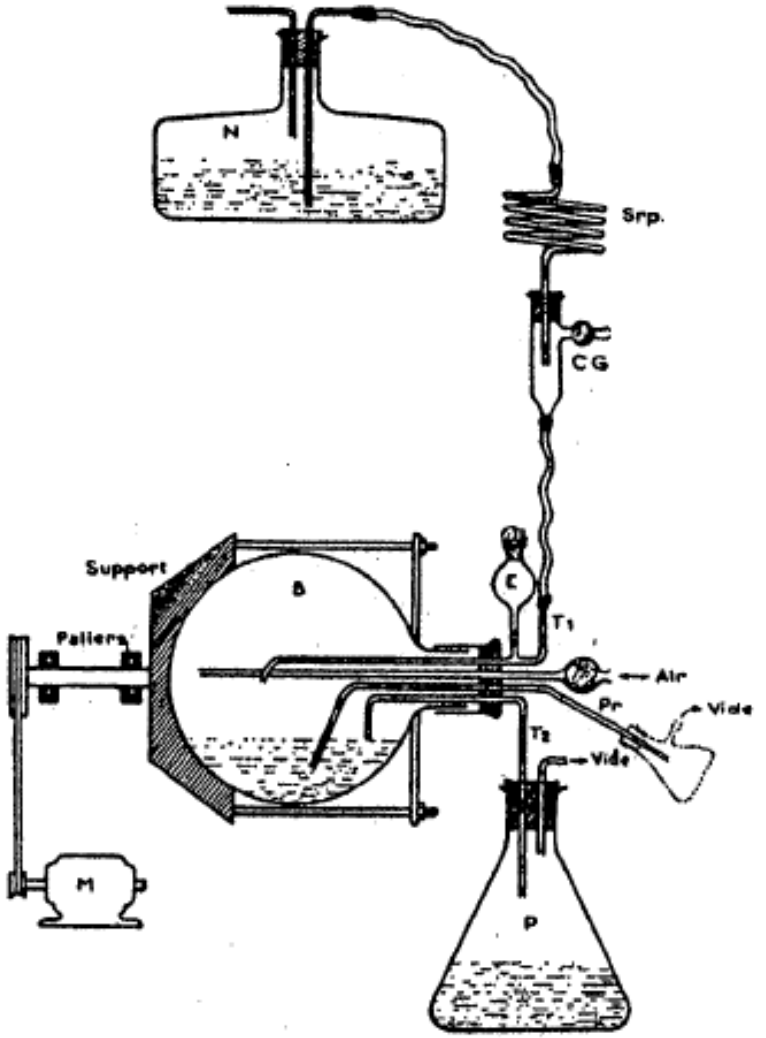


FIG. 4. — Montage d'un appareil à croissance continue. N, nourrice; Srp, serpentin capillaire; C.G., compte-gouttes; B, ballon rotatif; T_1, tubulure d'arrivée; E, tubulure d'ensemencement; Pr, tubulure de prélèvement (en pointillé, fiole de prélèvement); T_2, tubulure de niveau; P, produit; M, moteur.

The chemostat according to Novick & Szillard 1950

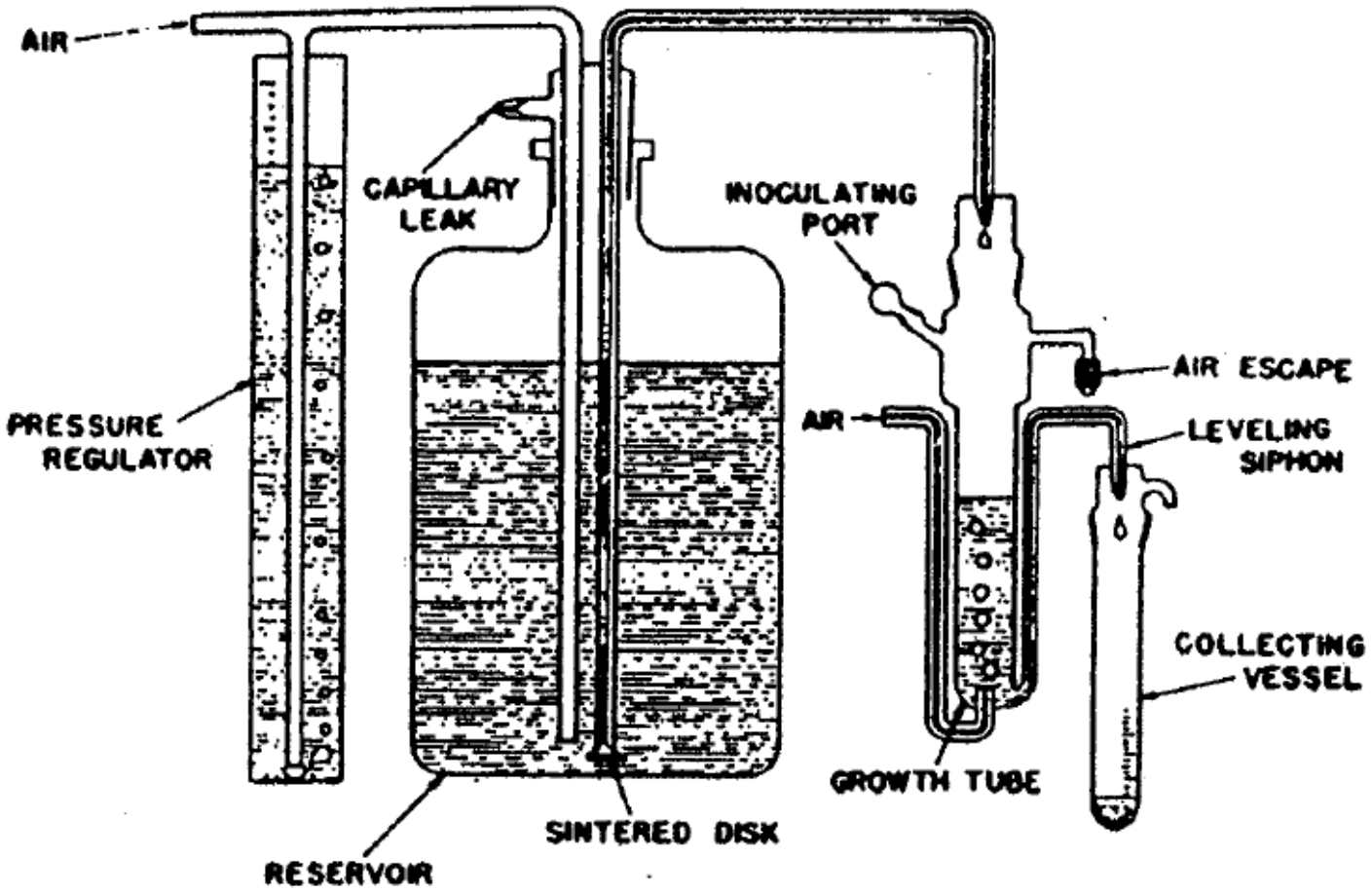


FIG. 1

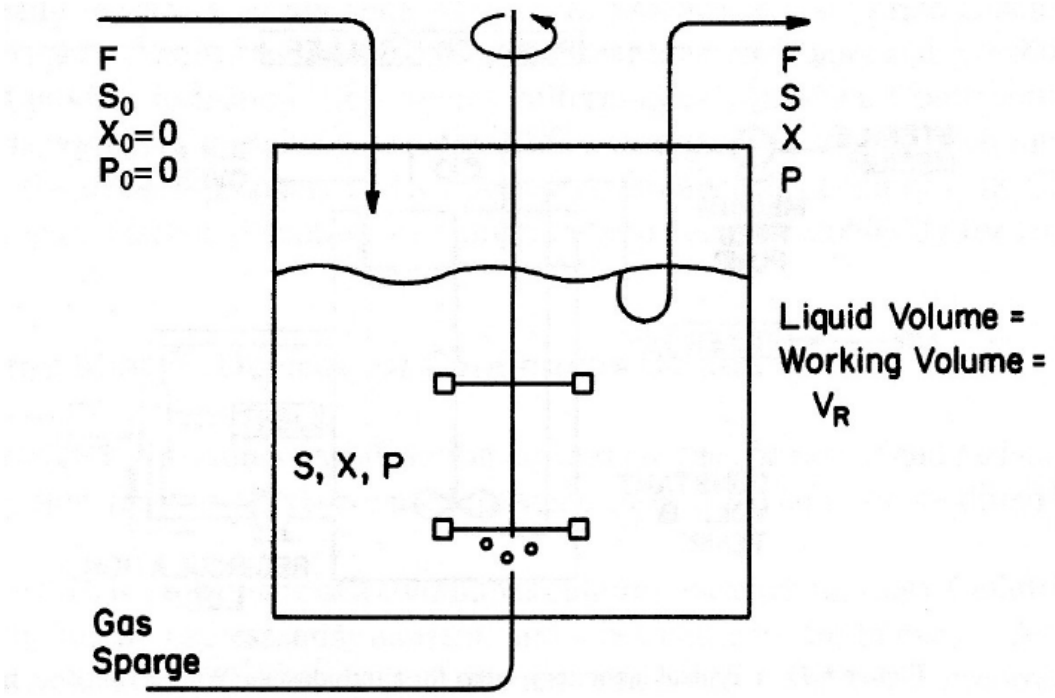
Science, **112**(2920), 715–716 (1950)

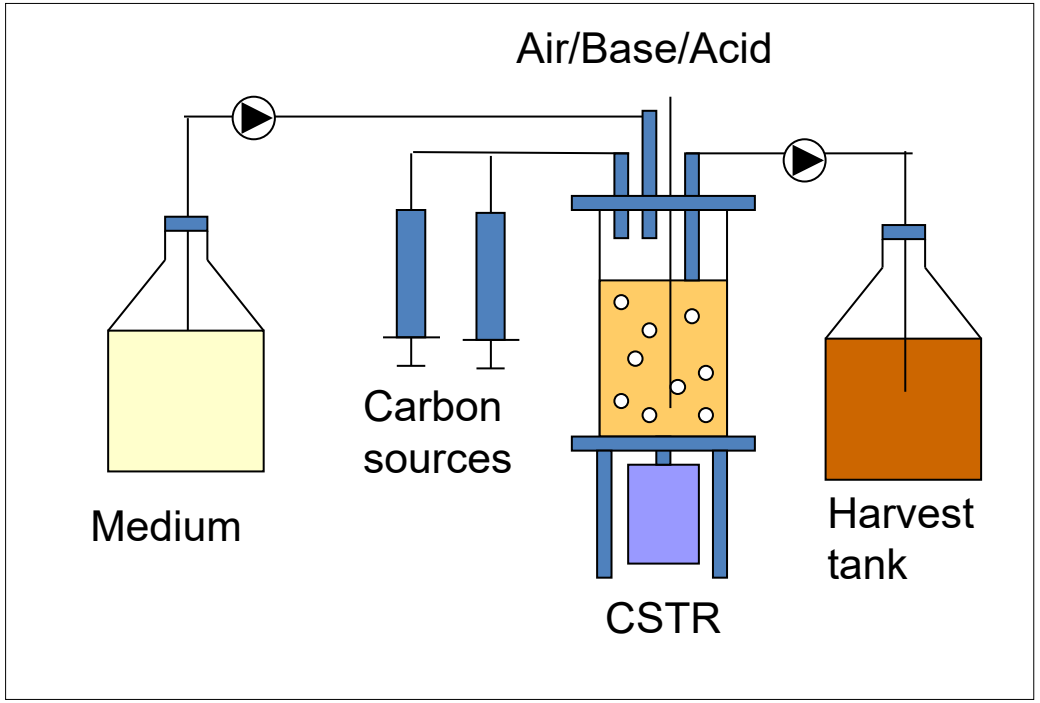
Description of the Chemostat

Aaron Novick and Leo Szilard

Institute of Radiobiology and Biophysics, University of Chicago

Set-up of chemostat culture





Continuous vs. Batch 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.

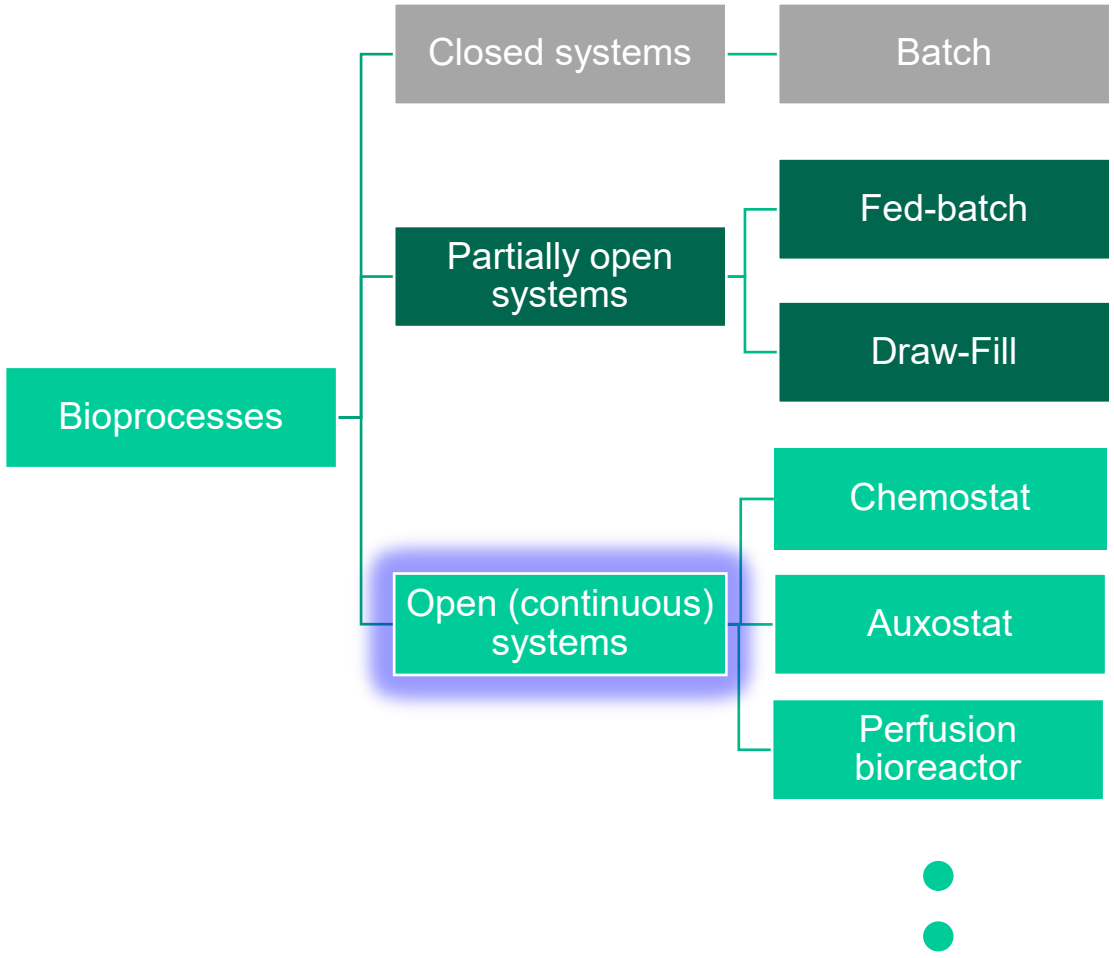
- It is not necessary to shut down the continuous fermenter as frequently as a batch fermenter. At the end of a batch fermentation, the reactor must be emptied, cleaned, sterilized and re-filled. The time required for these operations is known as **turnaround time**. Theoretically, a continuous fermenter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

- A biological phenomenon observed in batch cultures is the **lag phase**. The lag phase occurs at the beginning of the fermentation and represents a physiological adaption of the culture to the new environment. Growth during this phase is very slow and the lag phase represents a period of very low productivity. Because continuous cultures are shut down with less frequency as compared to batch reactors, there is less loss of productivity during lag phases.

Very, Very Important!

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

Categories of bioprocess modes



Closed systems:

No substrate fed; no cell culture fluid withdrawn; volume remains (approximately) constant

Partially open systems:

Intermittent withdrawal or addition of significant amounts of liquids

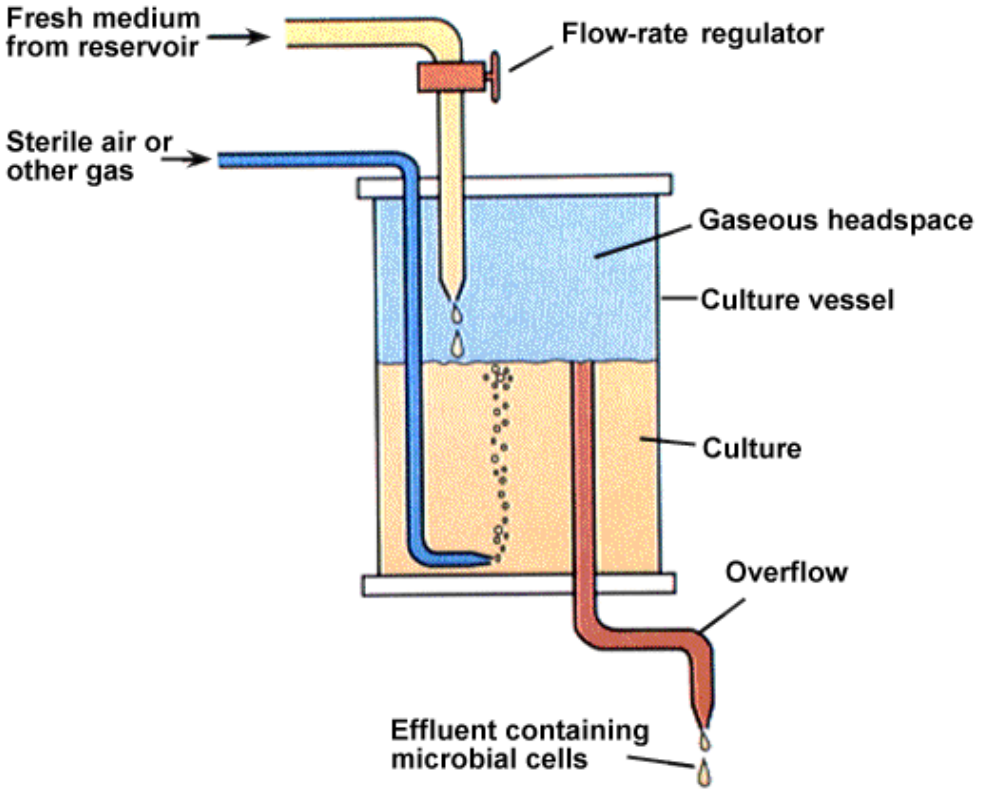
Open (continuous) systems:

Continuous addition and withdrawal of liquid

Overview on 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.F_{out}
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) • E.g., cyclic shift of steady state for strain evolution

D and μ in the chemostat



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

D = dilution rate (h^{-1})
F = inflowing medium ($L\ h^{-1}$)
V = culture volume (L)
τ = average residence time (h)

Biomass in the ractor as a function of time:

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

$$(2) \qquad \frac{dX}{dt} = \mu X - Fx$$

where **X** is the total biomass [g] in the system and **x** the concentration of biomass [$g\ L^{-1}$].

D and μ in the chemostat

dividing (2) by the volume one obtains:

$$\frac{dX}{dt \cdot V} = \mu \frac{X}{V} - \frac{F}{V} \cdot x \qquad (3a)$$

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

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

$$\frac{dx}{dt} = 0, \quad \text{hence} \quad \boxed{\mu = D} \qquad (3c)$$

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

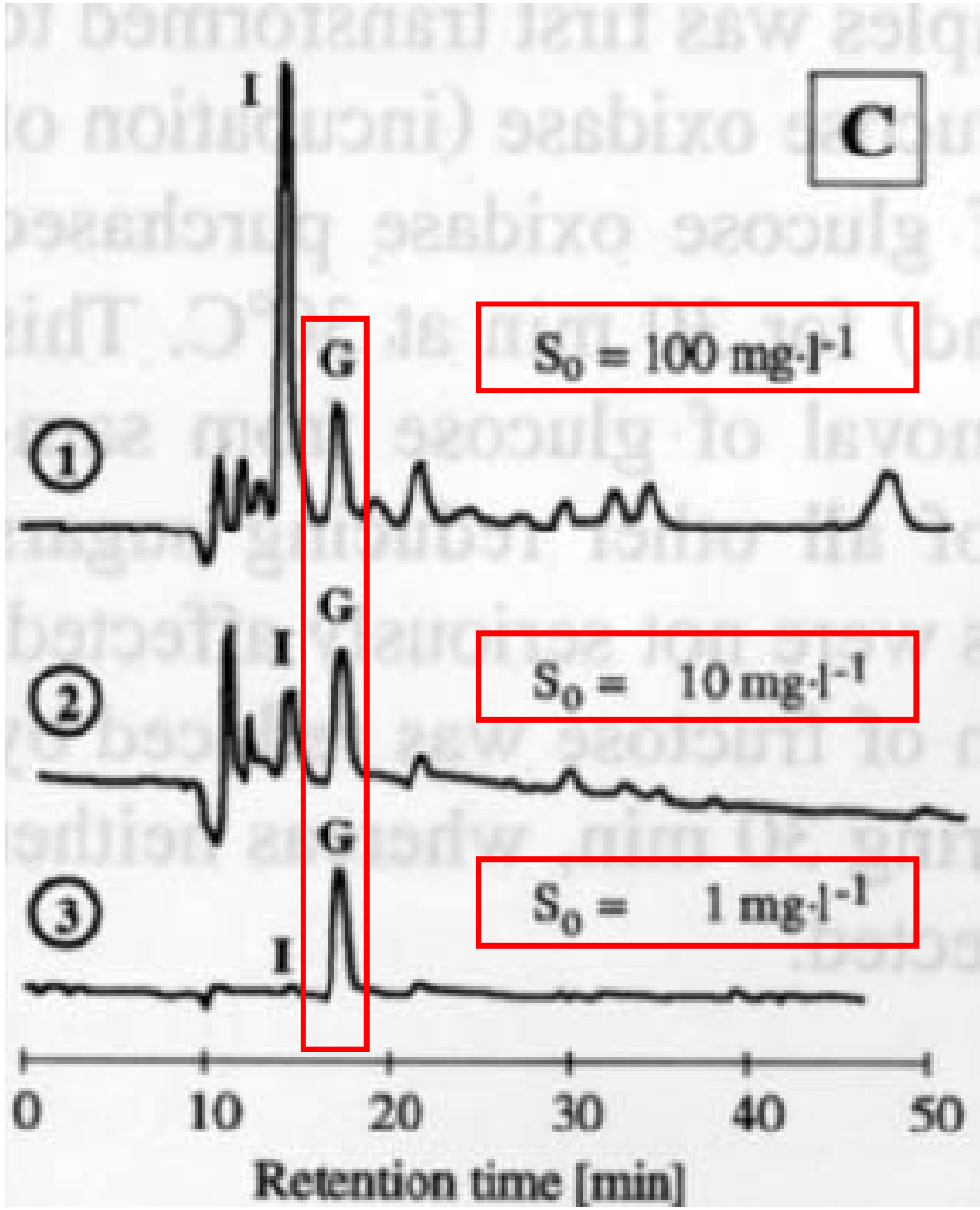
Substrate and biomass concentration in steady-state

to obtain $\tilde{s}$, we substitute μ by the „Monod equation“, multiply by $Y_{x/s}$ and set $(s_{in}- \tilde{s})Y_{x/s}= \tilde{x}$:

$$\tilde{s} = K_s \frac{D}{\mu_{max} - D} \quad (7)$$

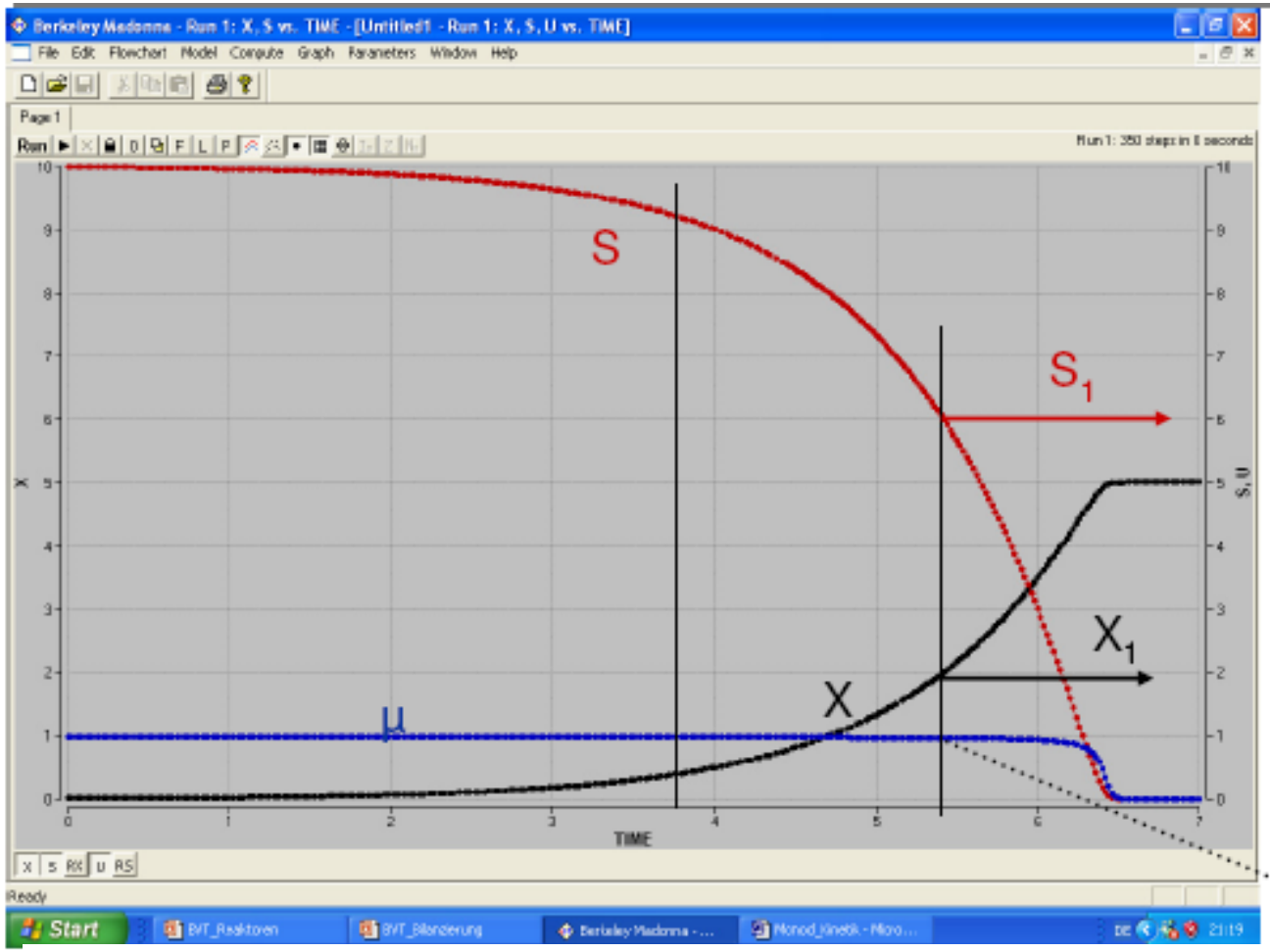
According to (7) the conc. of the limiting substrate (often called wrongly „residual substrate“) is independent of the concentration of s in the medium reservoir (and therefore also not dependent on the biomass in the reactor. It is only dependent on the dilution rate D, μ_{max} and K_s as demonstrated in figure C of next slide.

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).

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?

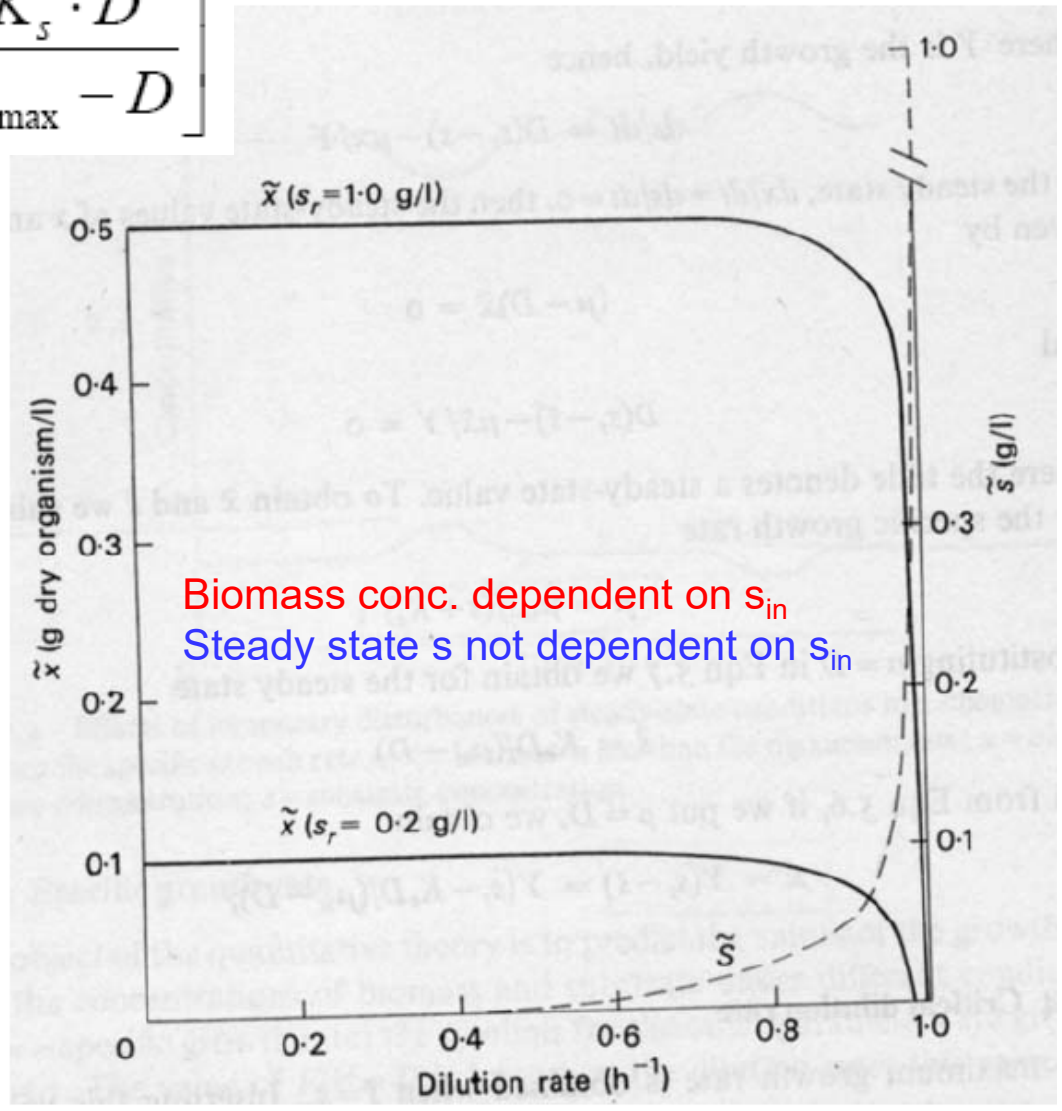
Substrate and biomass concentration in steady-state in an ideal chemostat

$$(8) \qquad \tilde{x} = Y_{X/S}(s_{in} - \tilde{s}) = Y_{X/S}\left[s_{in} - \frac{K_s \cdot D}{\mu_{max} - D}\right]$$

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

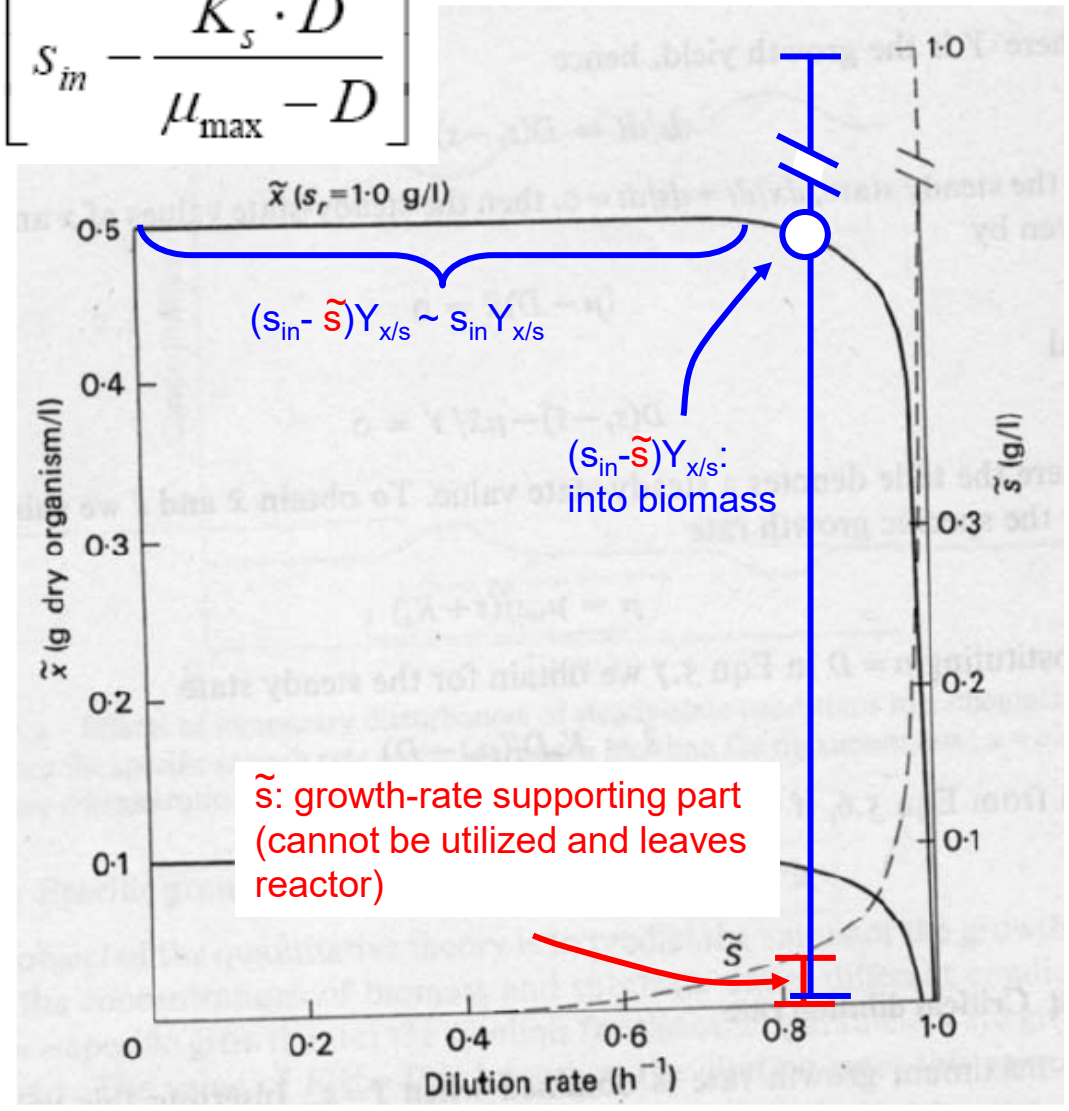
The Figure shows the steady-state biomass and substrate concentration as a function of D for two reservoir concentrations (here s_r).

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 in steady-state

$$(8) \qquad \tilde{x} = Y_{X/S}(s_{in} - \tilde{s}) = Y_{X/S}\left[s_{in} - \frac{K_s \cdot D}{\mu_{max} - D}\right]$$



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

Wash-out!

The maximum dilution rate at which the system theoretically can be run is:

$$\mu = D_{crit} = \mu_{\max} \frac{s_{in}}{K_s + s_{in}} \quad (9)$$

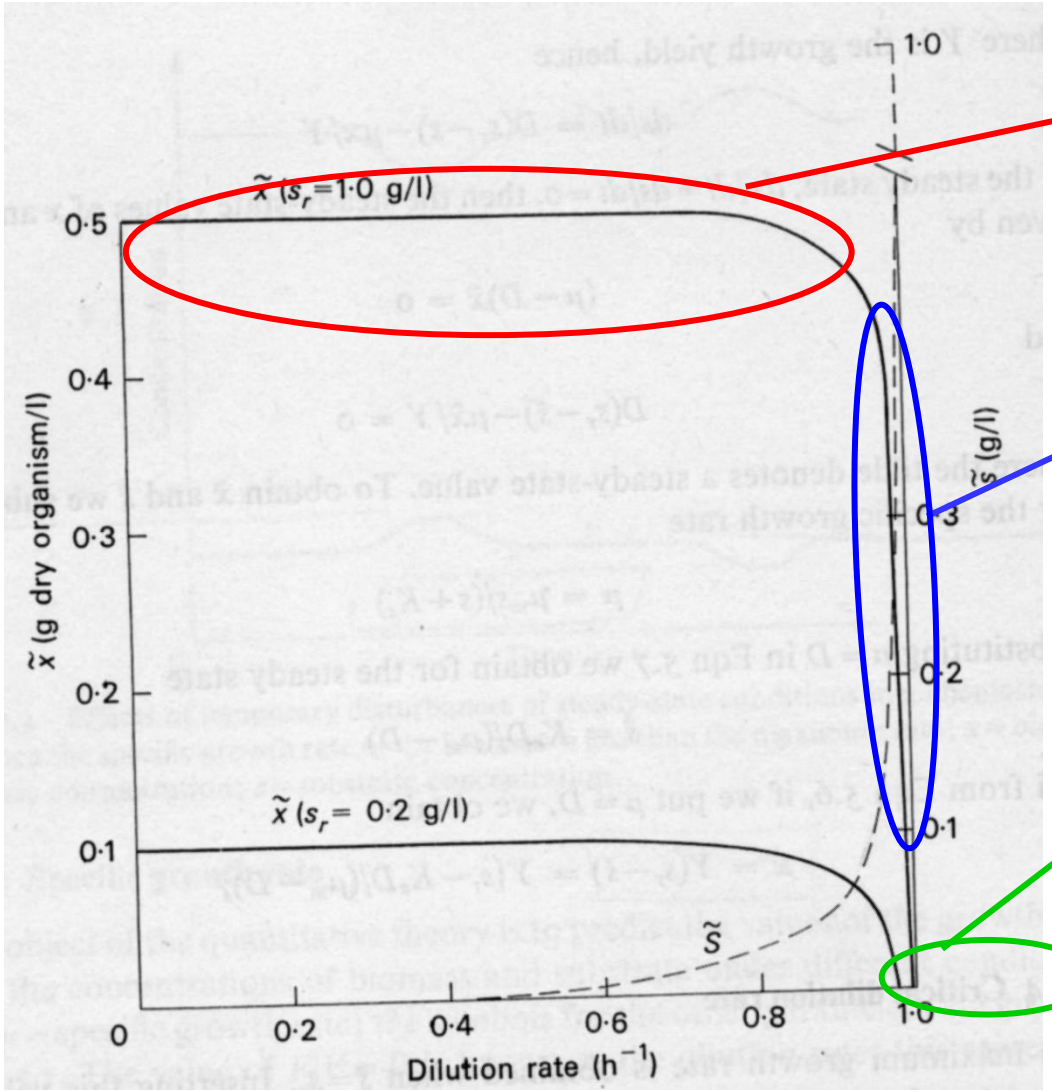
Note: D_{crit} is dependent on s_{in} !!!

If the dilution rate is higher than D_{crit}, the culture washes out and the loss of biomass from the reactor can be calculated:

$$\frac{dx}{dt} = \mu \cdot x - D \cdot x \quad (10)$$

$$\ln x = (\mu_{\max} - D) \cdot t + \ln x_0 \quad (11)$$

Summary of growth states in the chemostat



stable

unstable

wash-out

What is the physiological explanation for the wash-out of the culture?

Relationship between dilution rate D and substrate concentration s

Make a mass balance around chemostat:

$$\frac{ds}{dt} = \frac{F}{V}s_0 - \frac{F}{V}s - \frac{\mu * x}{Y_{X/S}} - mx - \frac{q_P * x}{Y_{P/S}}$$

Usually $\mu_* x/Y_{X/S} >> m_* x$ (m= maintenance)
and when little or no product formed $q_{P*}x/Y_{P/S} = 0$:

At steady state D = F/V:

$$\frac{ds}{dt} = \frac{F}{V}s_0 - \frac{F}{V}s - \frac{\mu * x}{Y_{X/S}}$$

$$\frac{ds}{dt} = D(s_0 - s) - \frac{\mu x}{Y_{X/S}}$$

Relationship between dilution rate D and s

At steady state ds/dt = 0:

$$0 = D(s_0 - s) - \frac{\mu * x}{Y_{X/S}}$$

Rearranging and since D = μ:

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

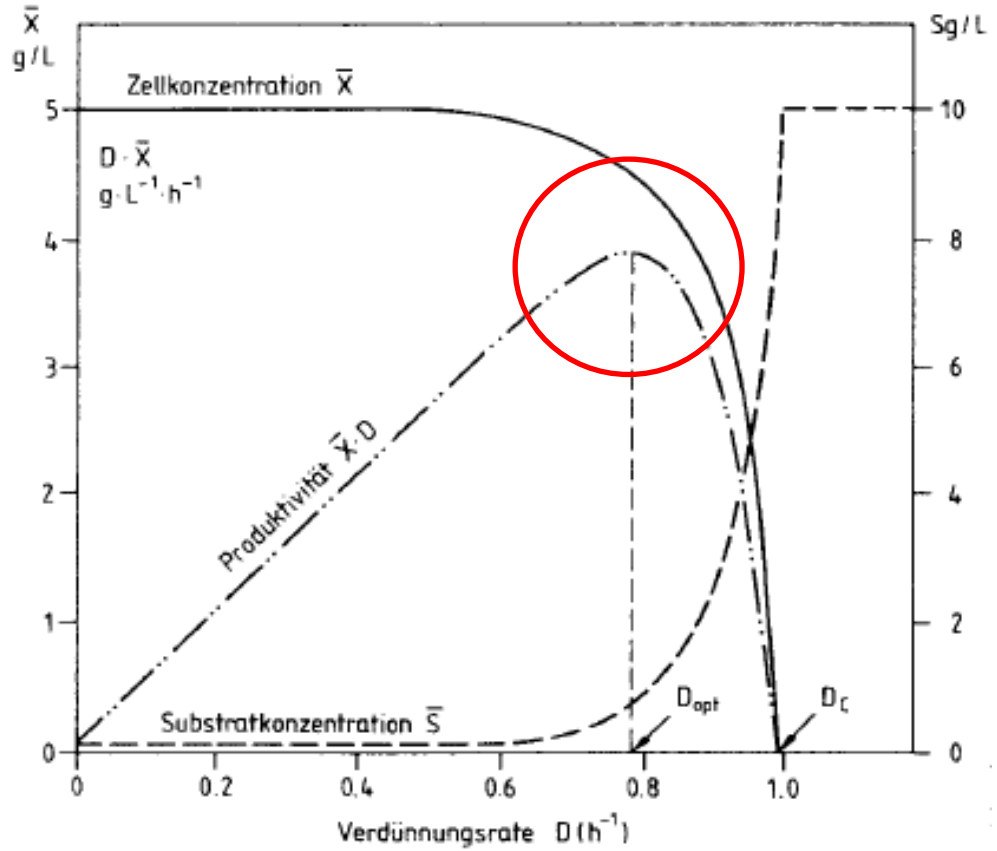
$$x = Y_{X/S}\,(s_0 - s)$$

This assumes that:

1. Cell yield ($Y_{X/S}$) is independent of growth rate- not completely true (see Pirt equation for effect of maintenance)

2. There is a single growth- limiting nutrient, and it is this which determines $Y_{X/S}$.

xD-Diagram (ideal chemostat)



Productivity in a chemostat culture

Productivity given symbol P or r_i (r_N for cell productivity, r_P for product productivity) or Q_x

P_X(or r_X) = D·x (units: g L^{-1} h^{-1})
P_P (or r_P) = D·P (units g L^{-1} h^{-1})

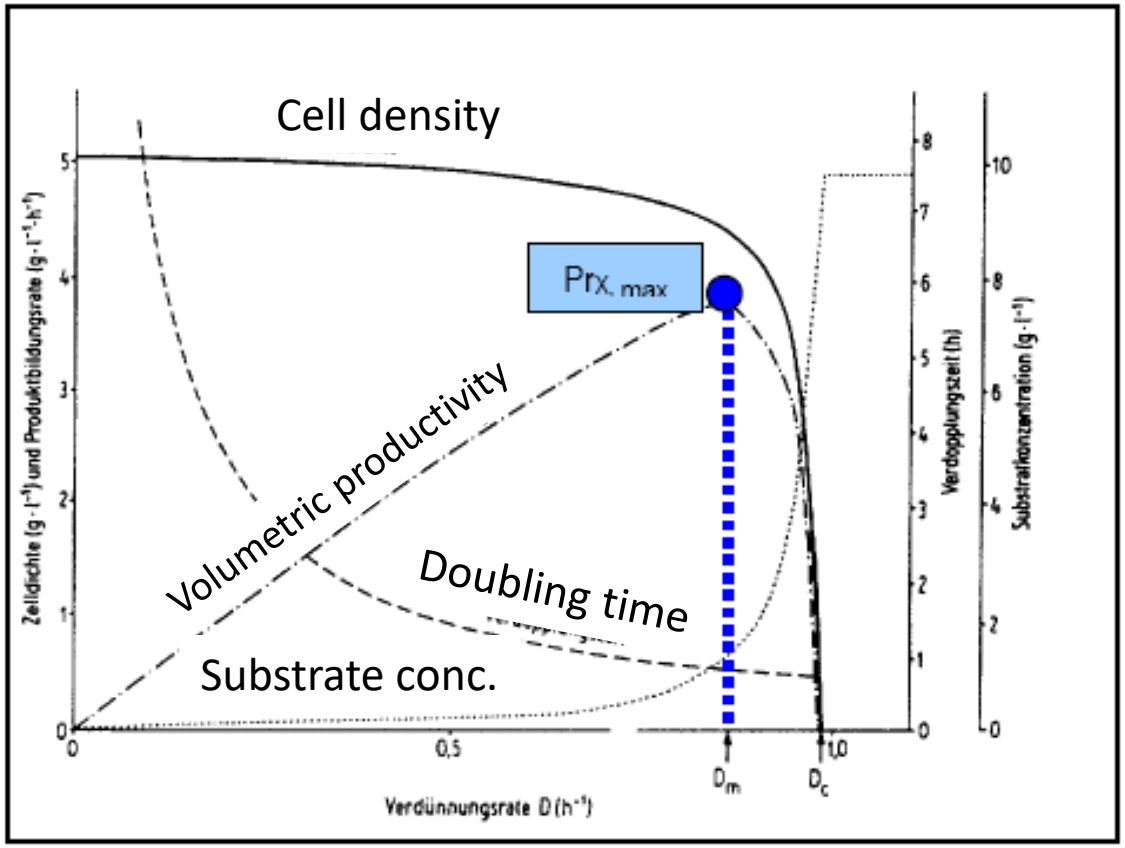
Since:

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

Then:

$$P_X = DY_{X/S}\left(s_0 - \frac{K_S D}{\mu_m - D}\right) = Q_X$$

Optimal vol. productivity in a chemostat culture



In case of $D << \mu_{max}$

$s \rightarrow 0$

$x \rightarrow$ max. ($x = Y_{X/S} * s_0$)

In case of $D \rightarrow \mu max$

$s \rightarrow s_0$

$x \rightarrow$ min.

(Wash-out of fermenter)

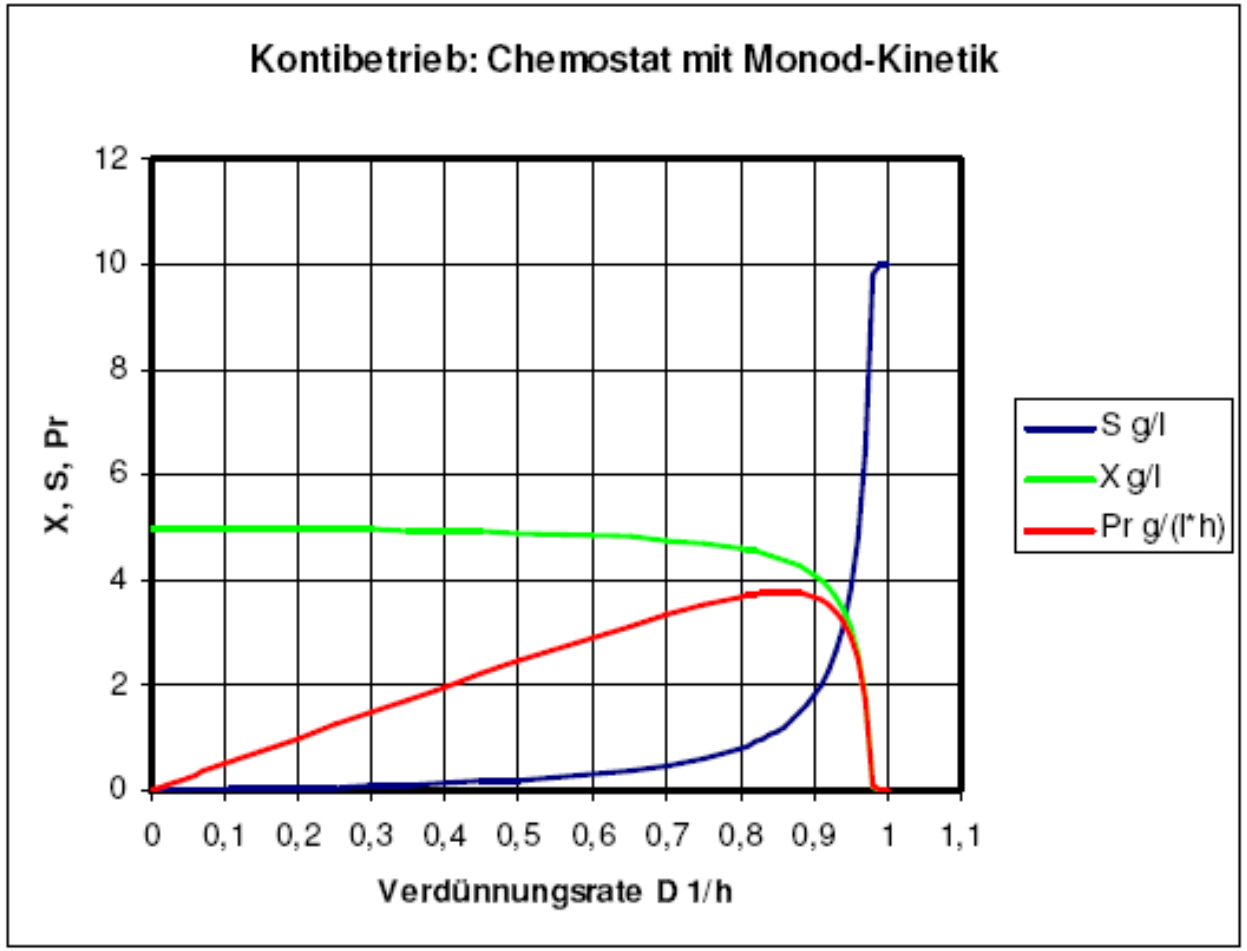
$$\boxed{D_c = D_{aus} \approx \mu_{max}}$$

D_m – max. Biomasseproduktivität

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

$D_{opt} = D_m$

Maximum productivity



Monod-Kinetik

mit

μ_{max} = 1 1/h

K_S = 0,2 g/l

S_0 = 10 g/l

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

Calculate: D_m, x at D_m and s at D_m, max. productivity

Task 16

Steady state relationship between dilution rate and biomass concentration

The growth of a strain of *Lactococcus lactis* on a medium containing glucose as the growth limiting nutrient is characterized by the following parameters:

$\mu_m = 0.6\ h^{-1}$
$K_s = 0.03\ g\ L^{-1}$
$Y_{x/s} = 0.3\ g\ g^{-1}$

Calculate the steady state [glucose] at the following dilution rates.

$D = 0.1\ h^{-1}$	s =	$g\ L^{-1}$
$D = 0.2\ h^{-1}$	s =	$g\ L^{-1}$
$D = 0.3\ h^{-1}$	s =	$g\ L^{-1}$
$D = 0.4\ h^{-1}$	s =	$g\ L^{-1}$
$D = 0.5\ h^{-1}$	s =	$g\ L^{-1}$

Task 17

Steady state relationship between dilution rate and biomass concentration

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$\mu_m = 0.6\ h^{-1}$
$K_s = 0.03\ g\ L^{-1}$
$Y_{x/s} = 0.3\ g\ g^{-1}$

Calculate the steady state [biomass] at the following dilution rates. The feed contains 1 g L^{-1} of glucose:

$D = 0.1\ h^{-1}$	x =	g L^{-1}
$D = 0.2\ h^{-1}$	x =	g L^{-1}
$D = 0.3\ h^{-1}$	x =	g L^{-1}
$D = 0.4\ h^{-1}$	x =	g L^{-1}
$D = 0.5\ h^{-1}$	x =	g L^{-1}

Task 18

Steady state relationship between dilution rate and biomass concentration

The growth of a strain of *Lactococcus lactis* on a medium containing glucose as the growth limiting nutrient is characterized by the following parameters:

$\mu_m = 0.6\ h^{-1}$

$K_s = 0.03\ g\ L^{-1}$

$Yx/s = 0.3\ g\ g^{-1}$

Lactate (P) is produced in a growth associated manner and the yield coefficient for lactate formation is:

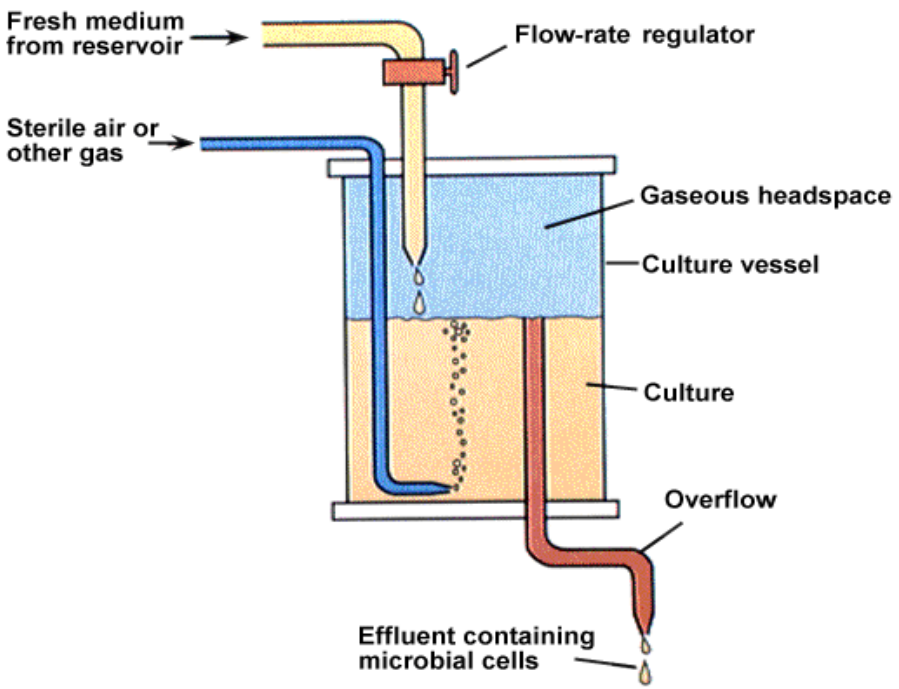
$Y_{p/s} = 0.8\ g\ g^{-1}$

Calculate the steady state lactate concentration at the following dilution rates.

The feed contains 1 g L^{-1} of glucose:

$D = 0.1\ h^{-1}$	P =	g L^{-1}
$D = 0.2\ h^{-1}$	P =	g L^{-1}
$D = 0.3\ h^{-1}$	P =	g L^{-1}
$D = 0.4\ h^{-1}$	P =	g L^{-1}
$D = 0.5\ h^{-1}$	P =	g L^{-1}

Explanation of continuous culture

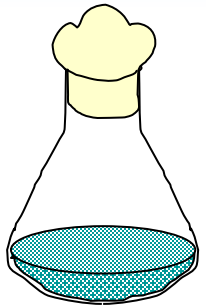


A journey from batch to chemostat

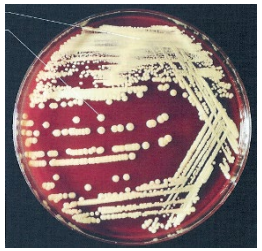
Thomas Egli, Eawag

Growth in closed and open systems
(in the lab)

Closed systems:
No nutrients added after inoculation
(except for oxygen for aerobes)
Growth stops after a while

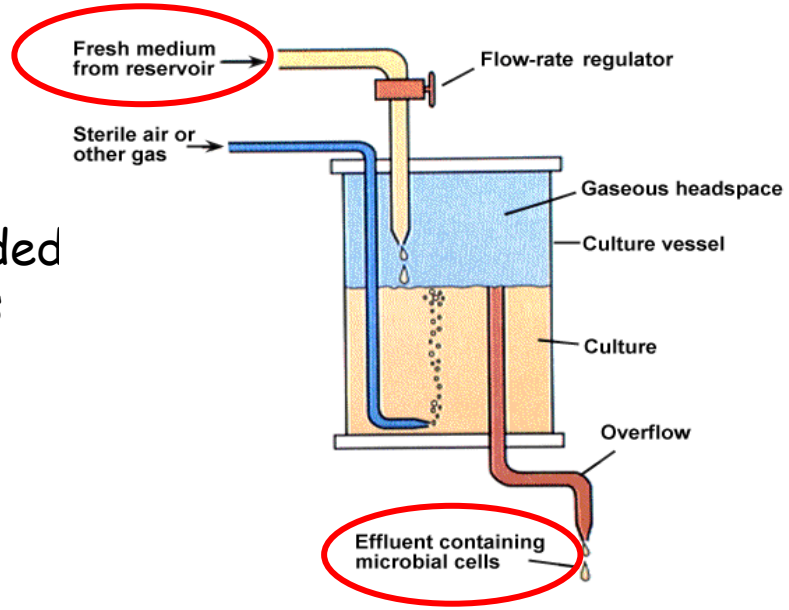


shake flask



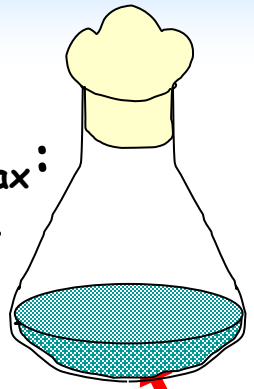
agar plate

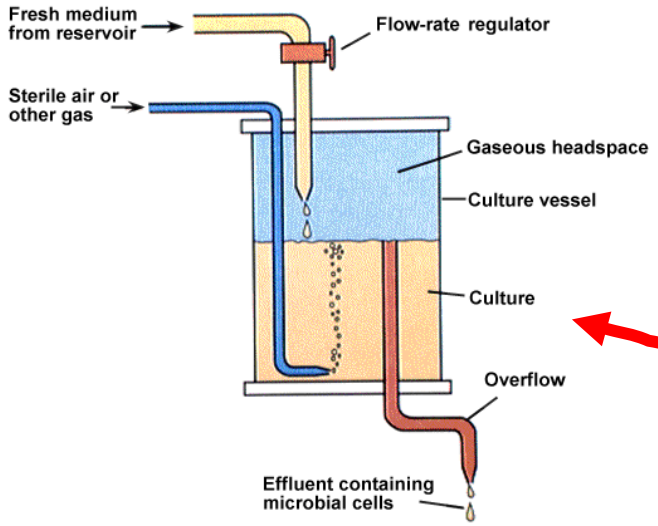
Open systems:
Nutrients continuously or discontinuously added after inoculation, growth continues as long as fresh medium is added.
Flow-through system where the rate of addition determines growth state



Choice of appropriate technique to grow cells

growth at μ_{max}:
batch culture





growth at < μ_{max}:
chemostat

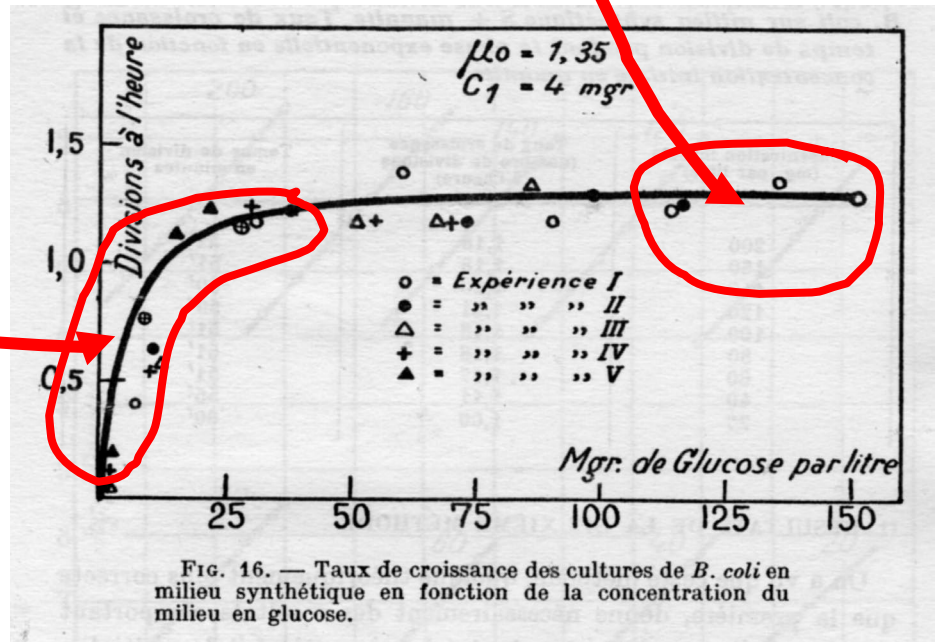
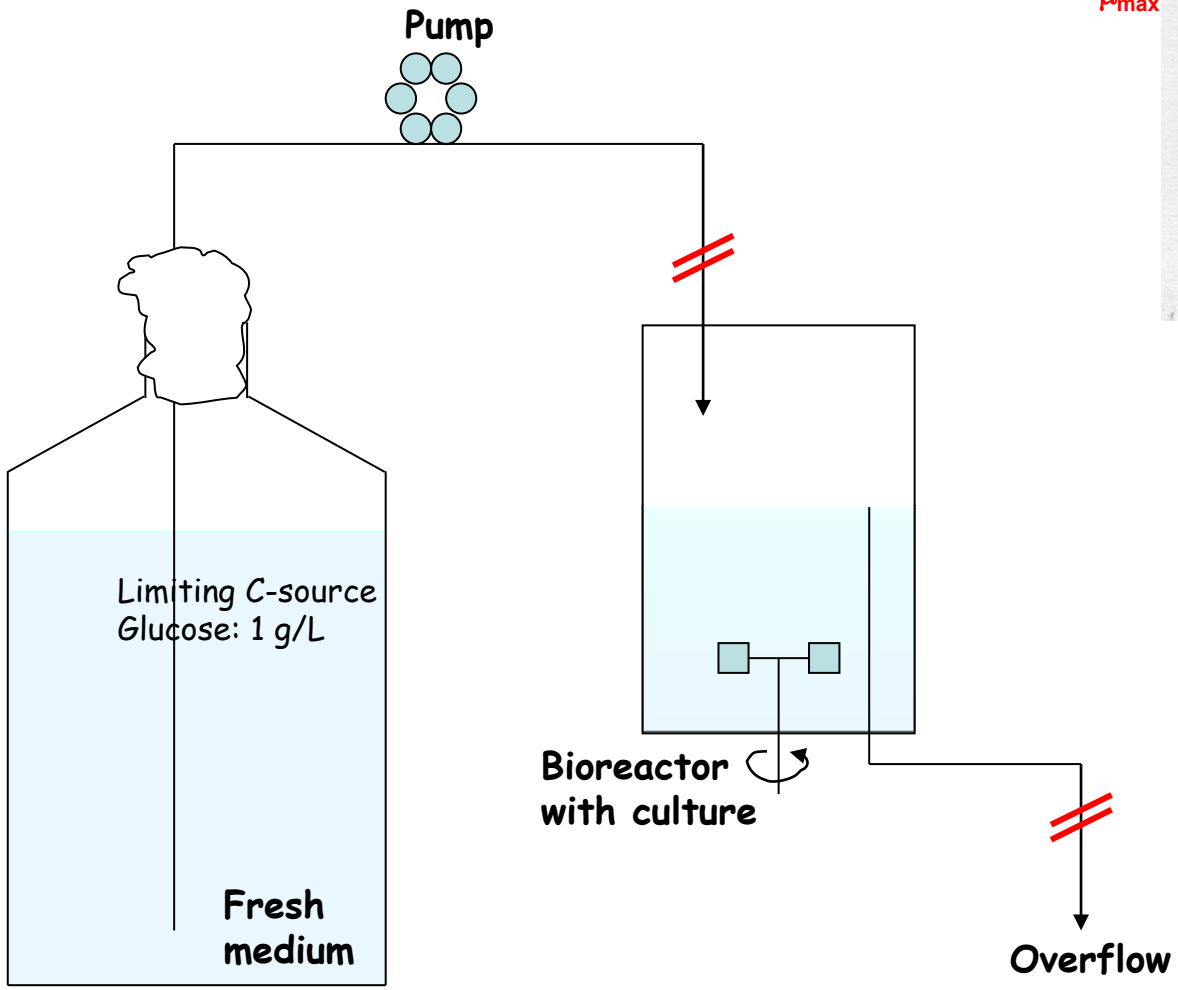


FIG. 16. — Taux de croissance des cultures de *B. coli* en milieu synthétique en fonction de la concentration du milieu en glucose.

Starting a continuous culture

Phase 1:
Batch mode



Y = 0.5
μ_{max} = 1.0/h
K_s = 4 mg/L

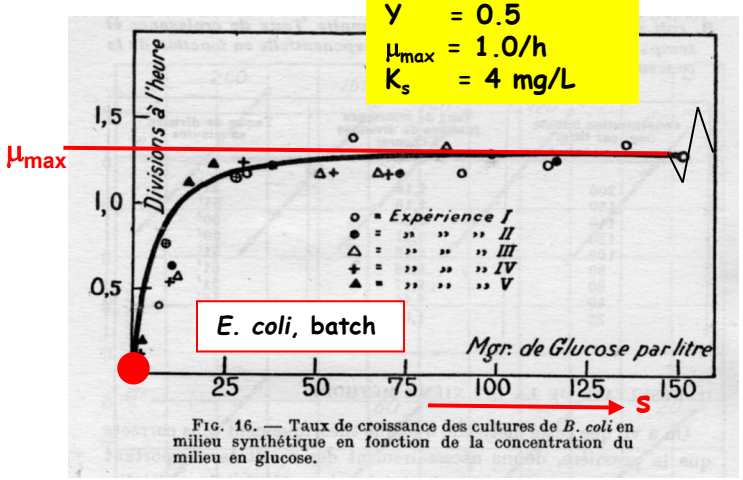


FIG. 16. — Taux de croissance des cultures de *B. coli* en milieu synthétique en fonction de la concentration du milieu en glucose.

Starting a continuous culture

Phase 1:
Batch mode

T = 0 h
fresh medium
s = 1.0 g/L
x = 0.0 g/L

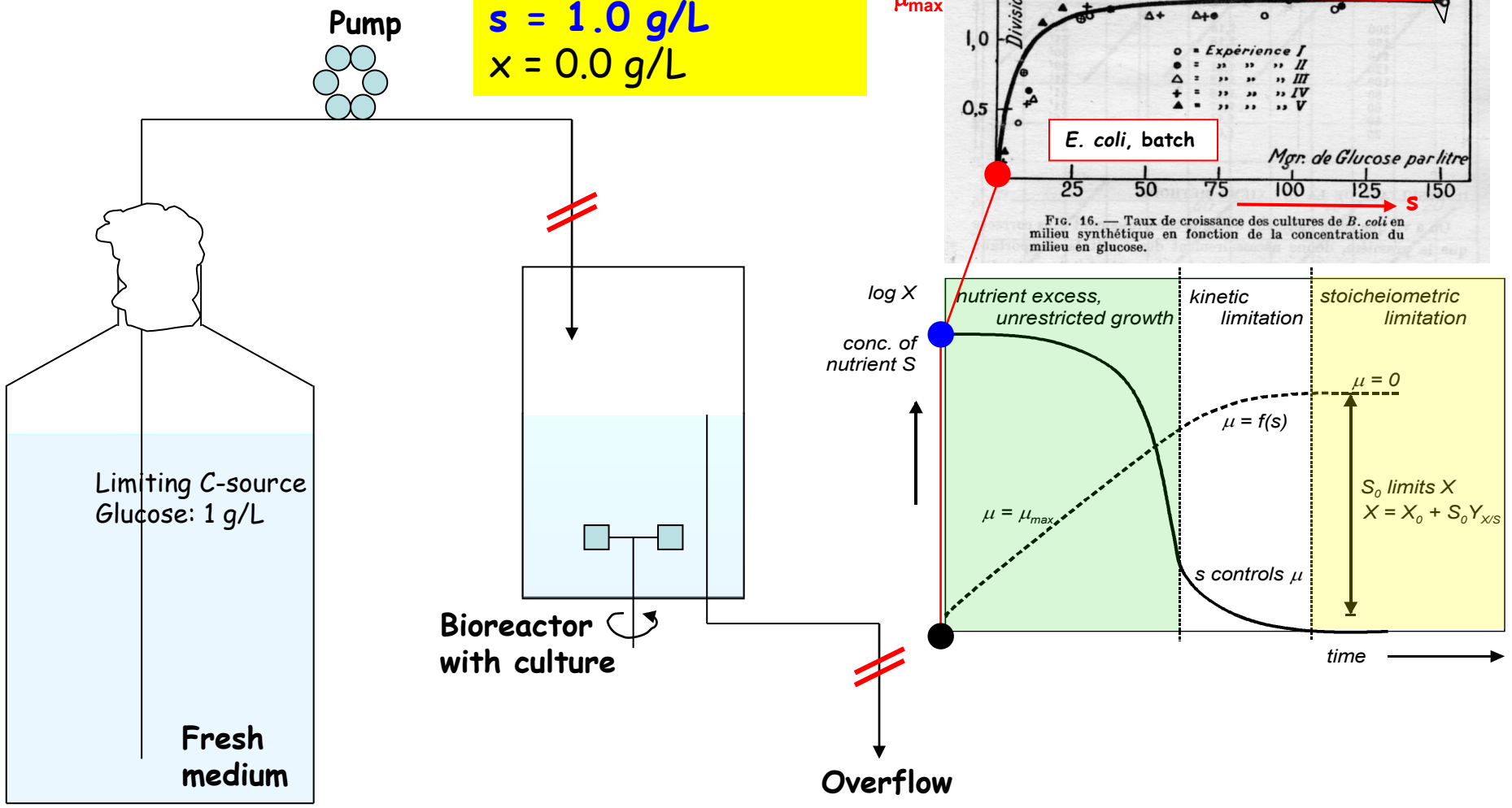


FIG. 16. — Taux de croissance des cultures de *B. coli* en milieu synthétique en fonction de la concentration du milieu en glucose.

Starting a continuous culture

Phase 1:
Batch mode

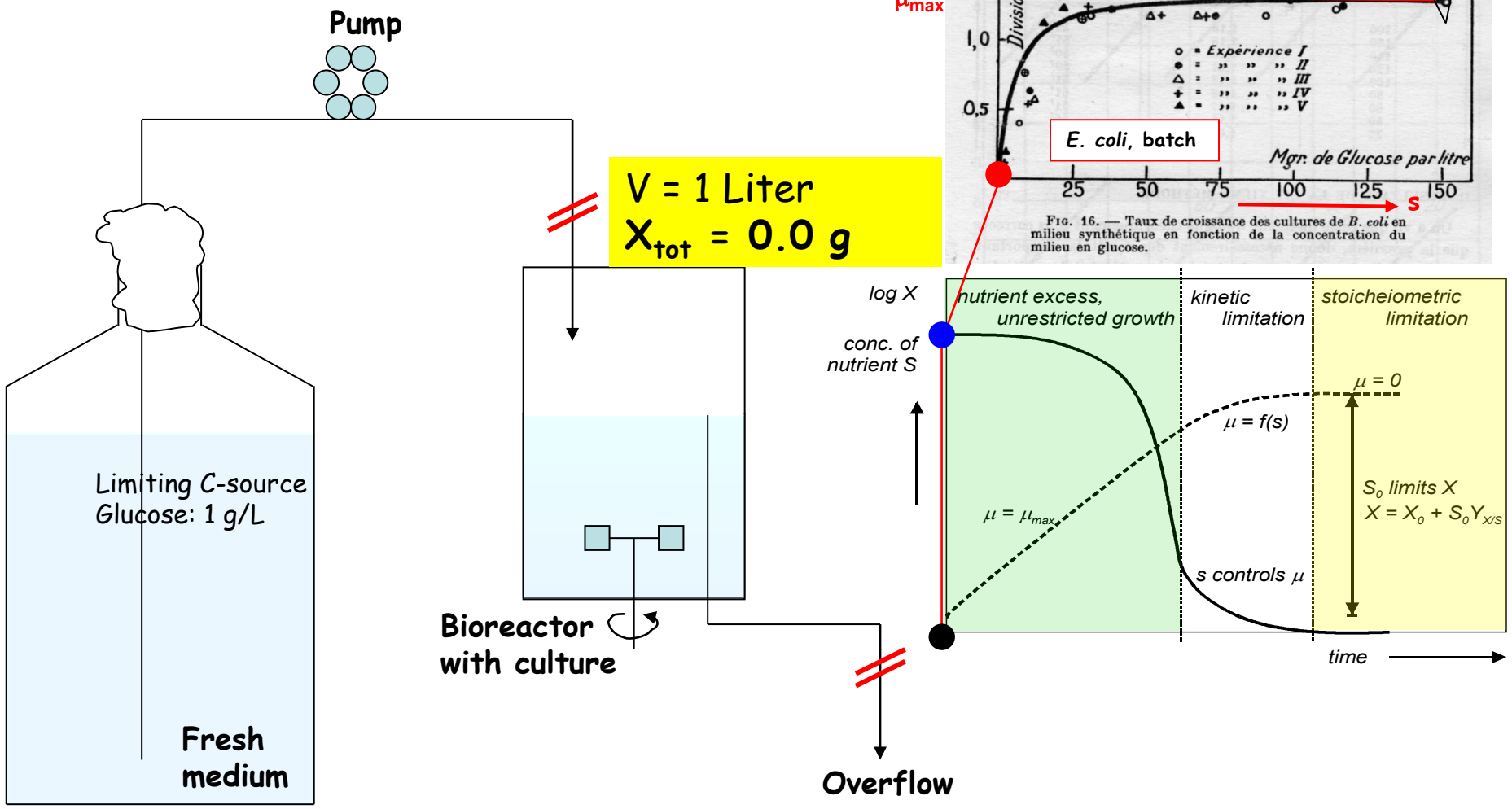


FIG. 16. — Taux de croissance des cultures de *B. coli* en milieu synthétique en fonction de la concentration du milieu en glucose.

Starting a continuous culture

Phase 1:
Batch mode

T = 0 h
fresh medium
s = 1.0 g/L
x = 0.0 g/L

Y = 0.5
μ_{max} = 1.0/h
K_s = 4 mg/L

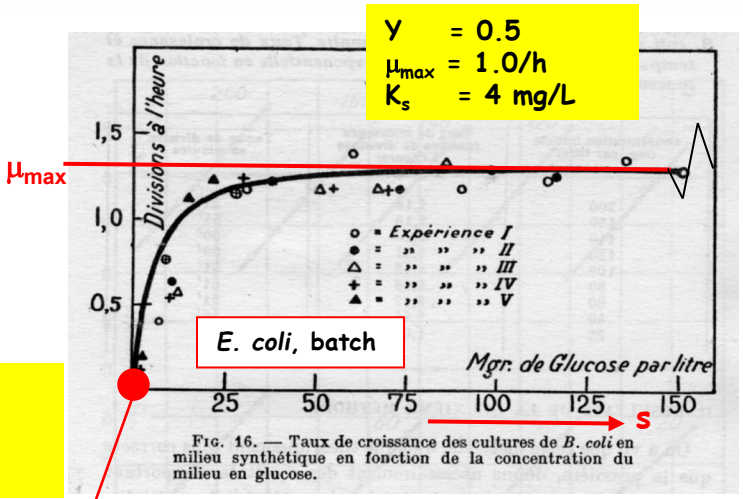


FIG. 16. — Taux de croissance des cultures de *B. coli* en milieu synthétique en fonction de la concentration du milieu en glucose.

Pump

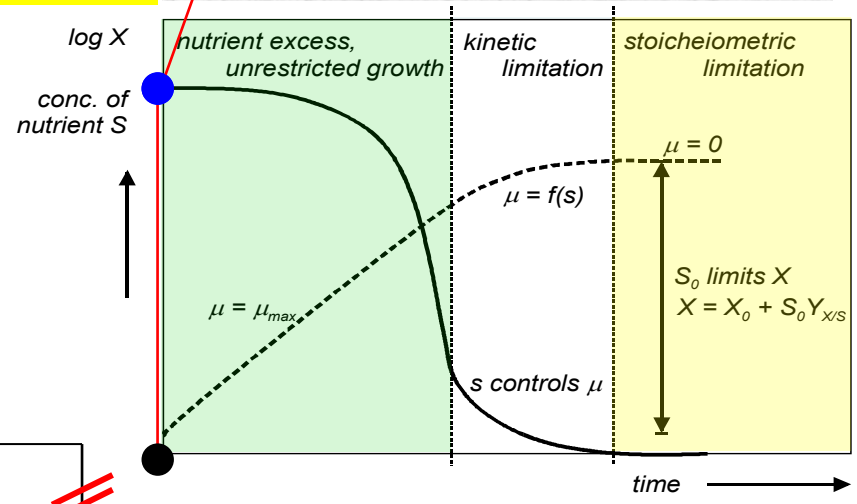
V = 1 Liter
X_{tot} = 0.0 g

Limiting C-source
Glucose: 1 g/L

Fresh medium

Bioreactor with culture

Overflow



Starting a continuous culture

Phase 1:
Batch mode

T = 0 h
inoculated
s = 1.0 g/L
x = 0.05 g/L

Y = 0.5
μ_{max} = 1.0/h
K_s = 4 mg/L

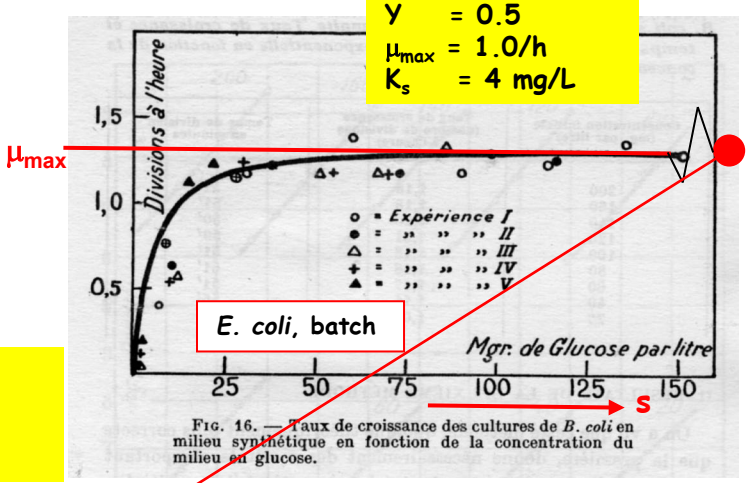


FIG. 16. — Taux de croissance des cultures de *B. coli* en milieu synthétique en fonction de la concentration du milieu en glucose.

Pump

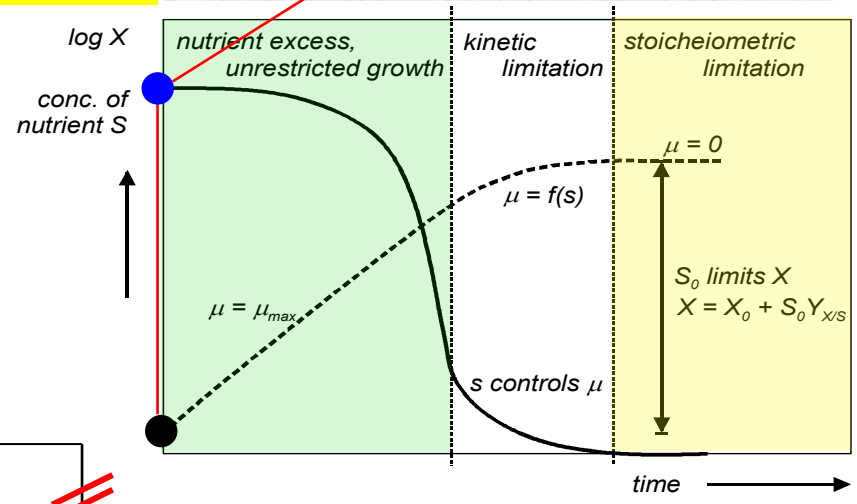
V = 1 Liter
X_{tot} = 0.05 g

Limiting C-source
Glucose: 1 g/L

Fresh medium

Bioreactor with culture

Overflow



Starting a continuous culture

Phase 1:
Batch mode

T = 1 h
μ = 1/h
s = 0.9 g/L
x = 0.05 + 0.05 g/L

Y = 0.5
μ_{max} = 1.0/h
K_s = 4 mg/L

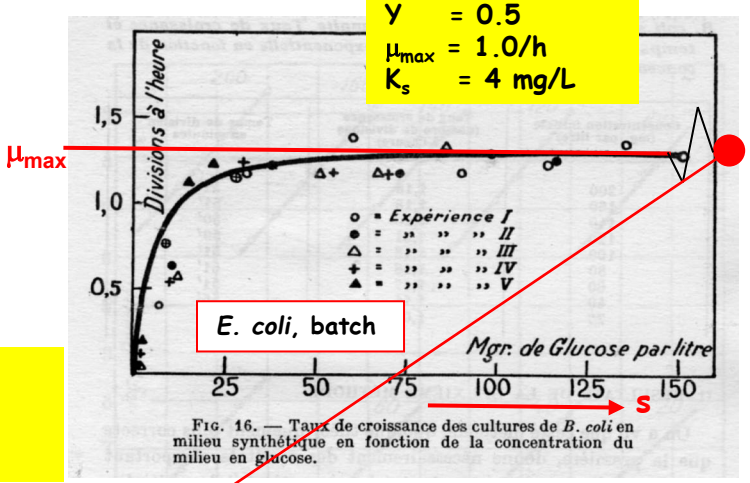


Fig. 16. — Taux de croissance des cultures de *B. coli* en milieu synthétique en fonction de la concentration du milieu en glucose.

Pump

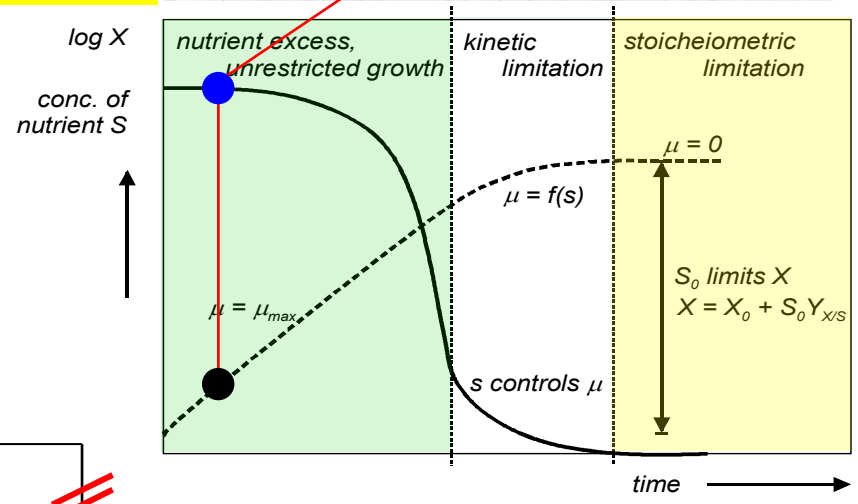
V = 1 Liter
X_{tot} = 0.10 g

Limiting C-source
Glucose: 1 g/L

Fresh medium

Bioreactor with culture

Overflow



Starting a continuous culture

Phase 1:
Batch mode

T = 2 h
μ = 1/h
s = 0.7 g/L
x = 0.05 + 0.15 g/L

Y = 0.5
μ_{max} = 1.0/h
K_s = 4 mg/L

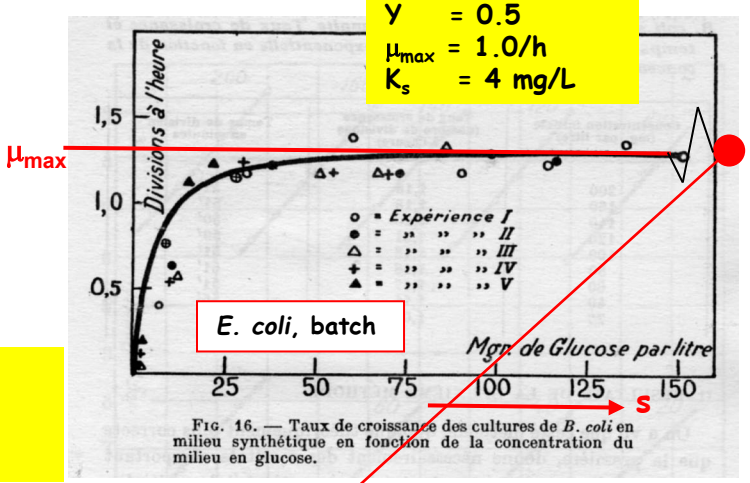
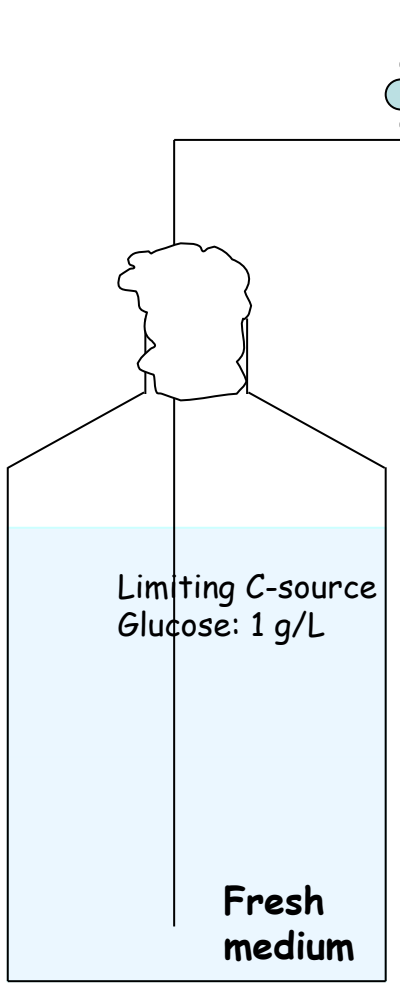
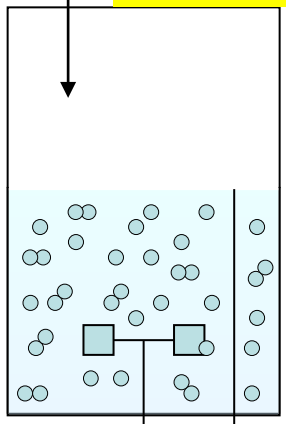


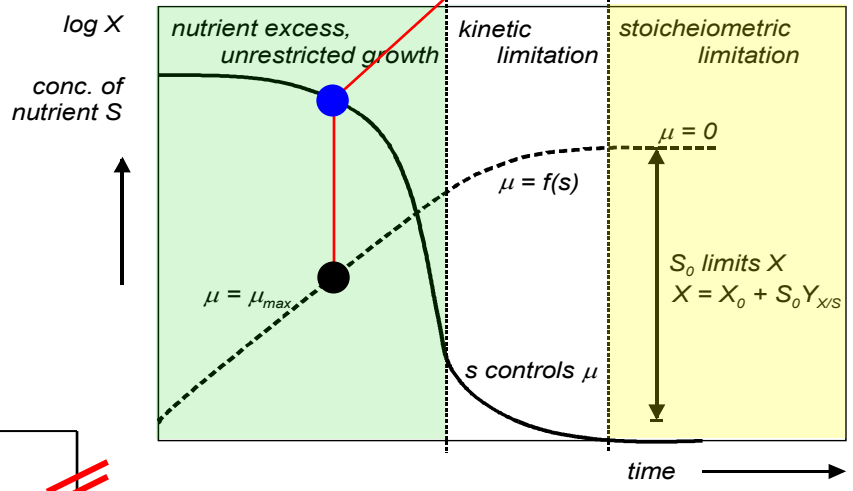
FIG. 16. — Taux de croissance des cultures de *B. coli* en milieu synthétique en fonction de la concentration du milieu en glucose.



V = 1 Liter
X_{tot} = 0.20 g



Overflow



Starting a continuous culture

Phase 1:
Batch mode

T = 3 h
μ = 1/h
s = 0.3 g/L
x = 0.05 + 0.35 g/L

Y = 0.5
μ_{max} = 1.0/h
K_s = 4 mg/L

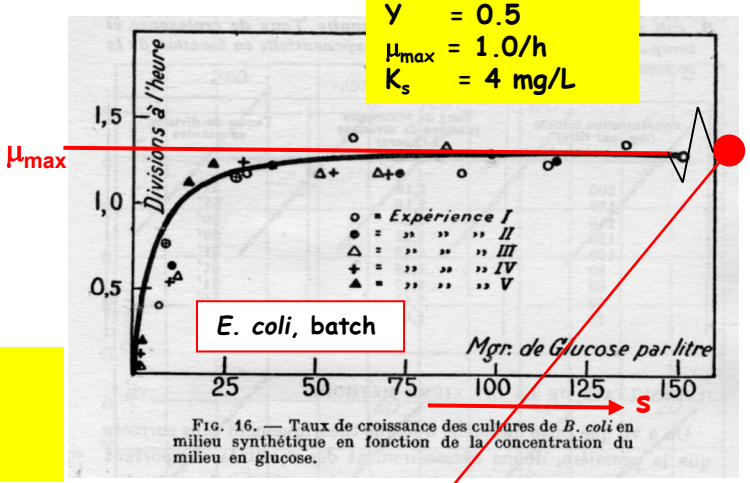


Fig. 16. — Taux de croissance des cultures de *B. coli* en milieu synthétique en fonction de la concentration du milieu en glucose.

Pump

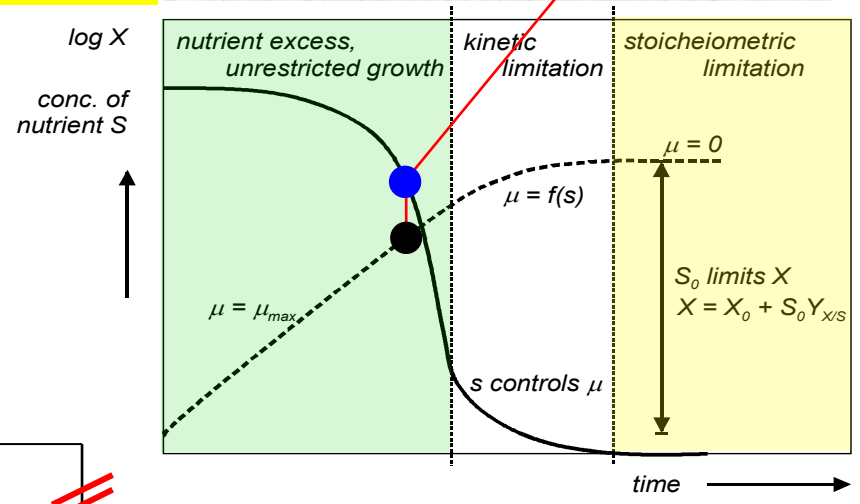
V = 1 Liter
X_{tot} = 0.40 g

Limiting C-source
Glucose: 1 g/L

Fresh medium

Bioreactor with culture

Overflow



Starting a continuous culture

Phase 1:
Batch mode

T = 3.5 h
μ ~ 1/h
s = 0.1 g/L
x = 0.05 + 0.45 g/L

Y = 0.5
μ_{max} = 1.0/h
K_s = 4 mg/L

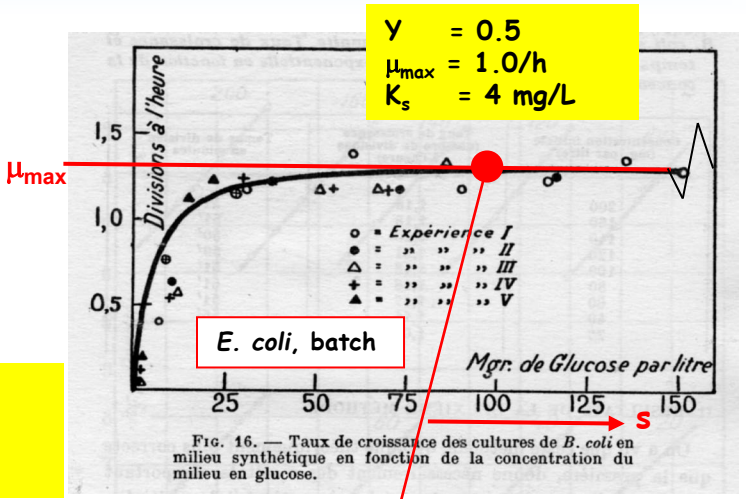
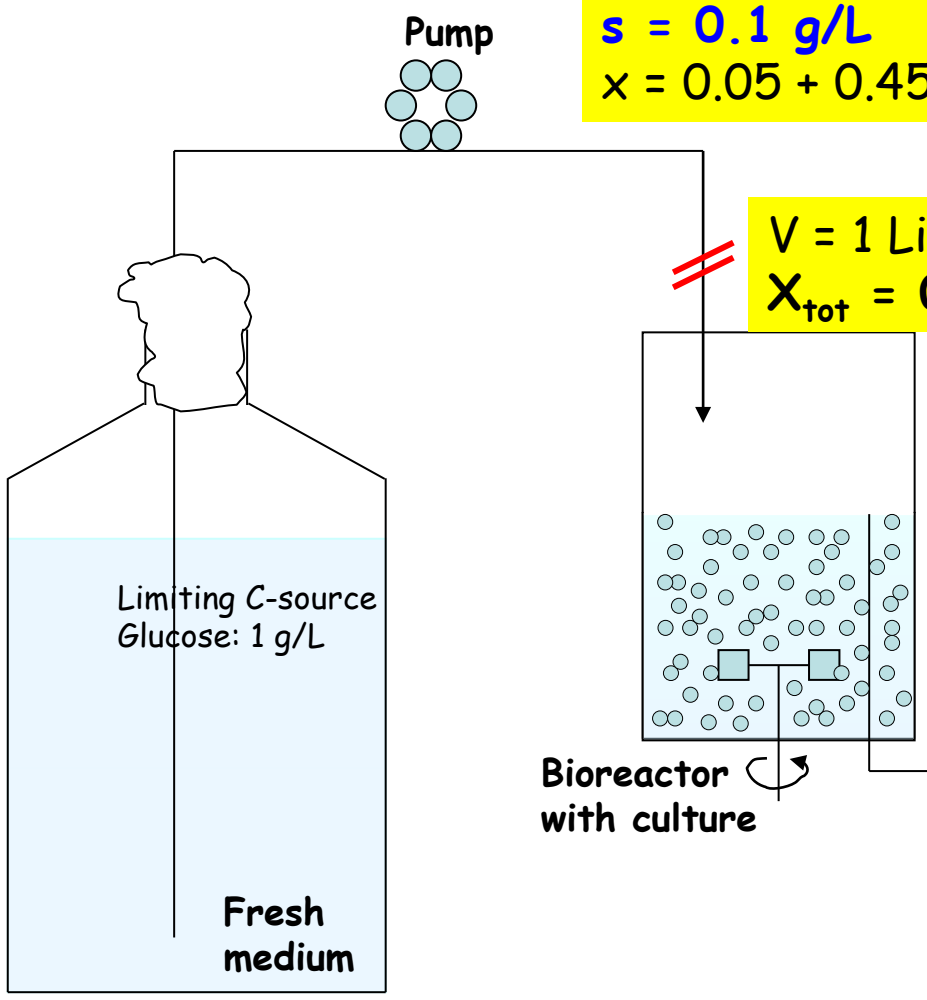
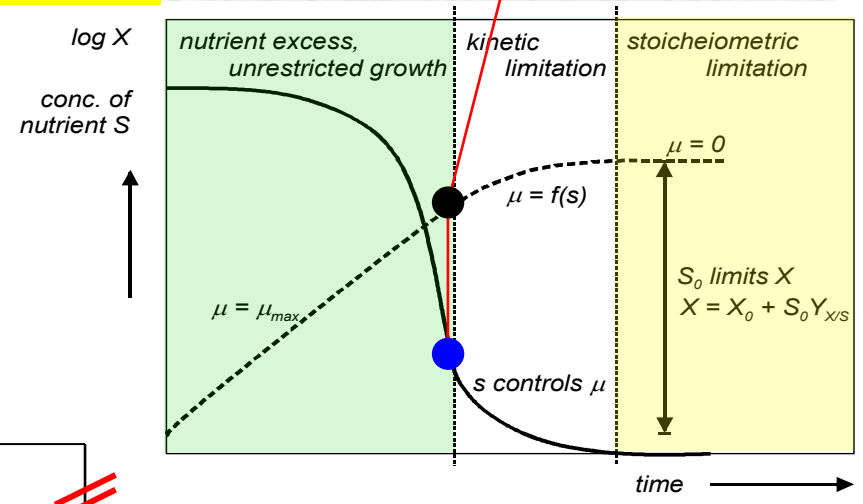


FIG. 16. — Taux de croissance des cultures de *B. coli* en milieu synthétique en fonction de la concentration du milieu en glucose.



V = 1 Liter
X_{tot} = 0.50 g



Starting a continuous culture

Phase 1:
Batch mode

T = 3.8 h
μ ~ 0.86/h
s = 0.025 g/L
x = 0.05 + 0.49 g/L

Y = 0.5
μ_{max} = 1.0/h
K_s = 4 mg/L

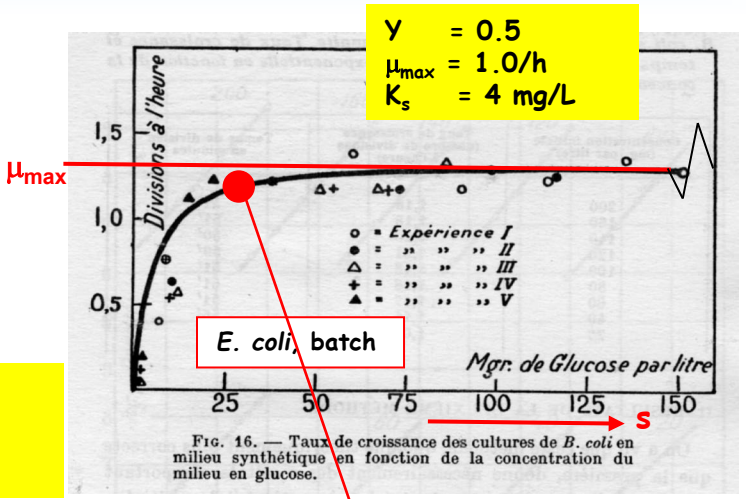


FIG. 16. — Taux de croissance des cultures de *B. coli* en milieu synthétique en fonction de la concentration du milieu en glucose.

Pump

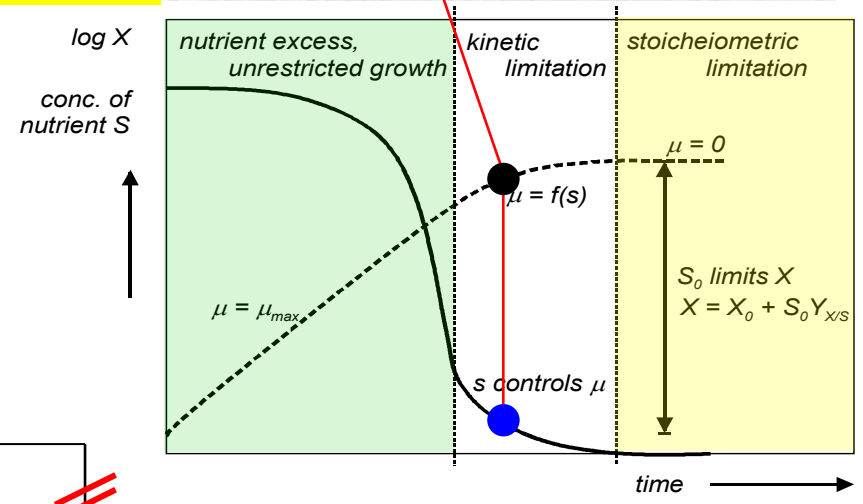
V = 1 Liter
X_{tot} = 0.54 g

Limiting C-source
Glucose: 1 g/L

Fresh medium

Bioreactor with culture

Overflow



Starting a continuous culture

Phase 1:
Batch mode

T = 4.0 h
μ ~ 0.5/h
s = 0.004 g/L
x = 0.05 + 0.50 g/L

Y = 0.5
μ_{max} = 1.0/h
K_s = 4 mg/L

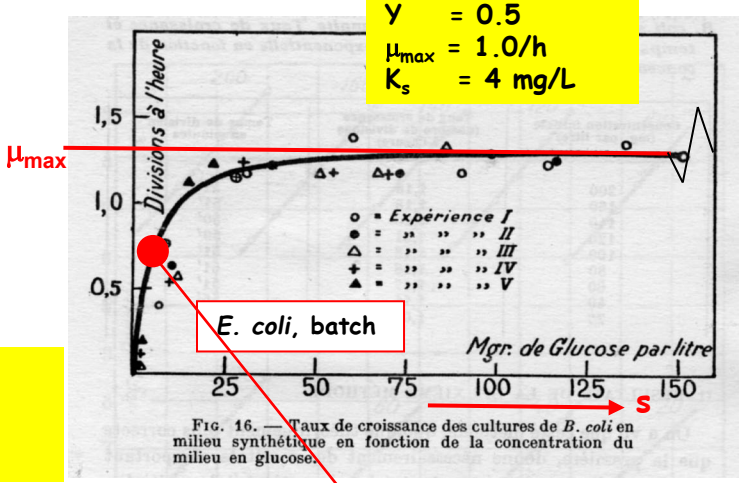
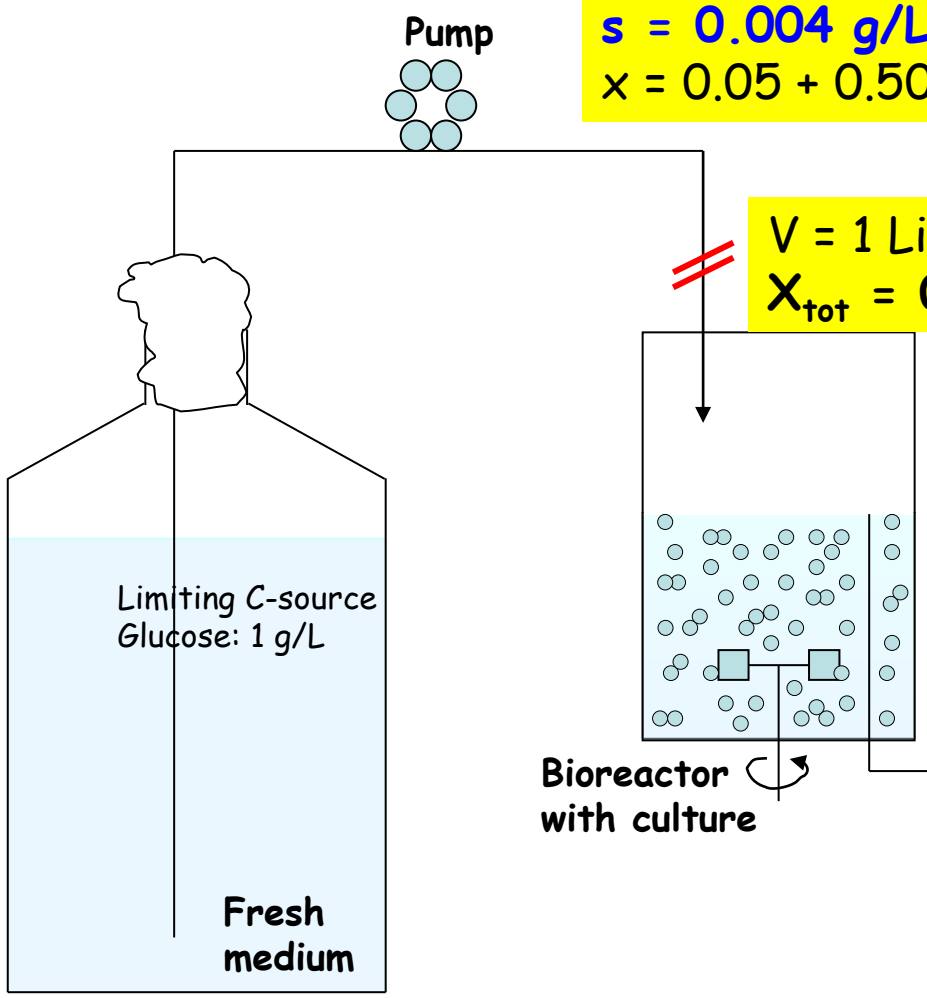
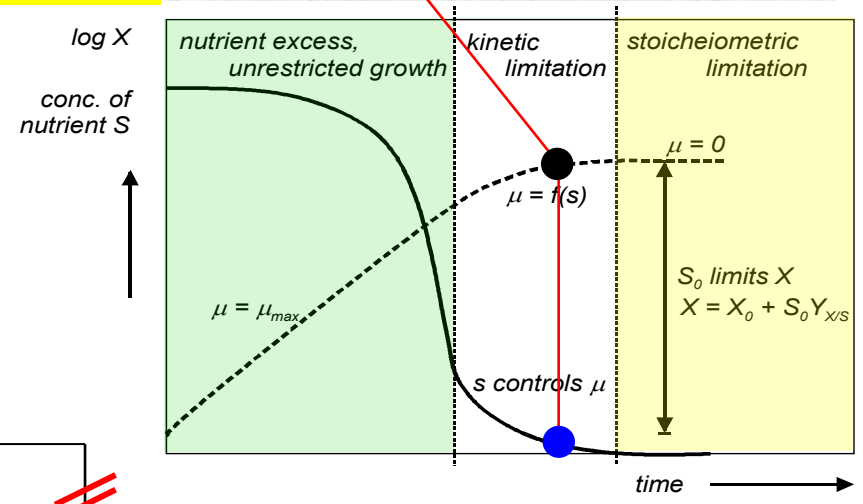


FIG. 16. — Taux de croissance des cultures de *B. coli* en milieu synthétique en fonction de la concentration du milieu en glucose.



V = 1 Liter
X_{tot} = 0.55 g



Starting a continuous culture

Phase 1:
Batch mode

T = 4.5 h
μ ~ 0.27/h
s = 0.0015 g/L
x = 0.05 + 0.50 g/L

Y = 0.5
μ_{max} = 1.0/h
K_s = 4 mg/L

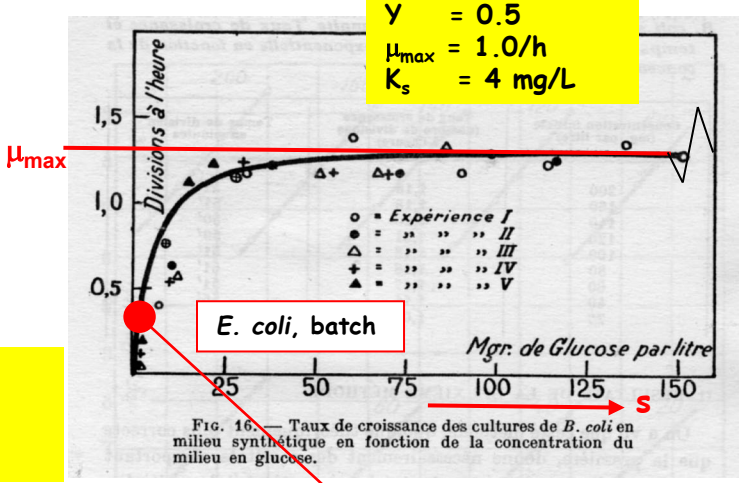
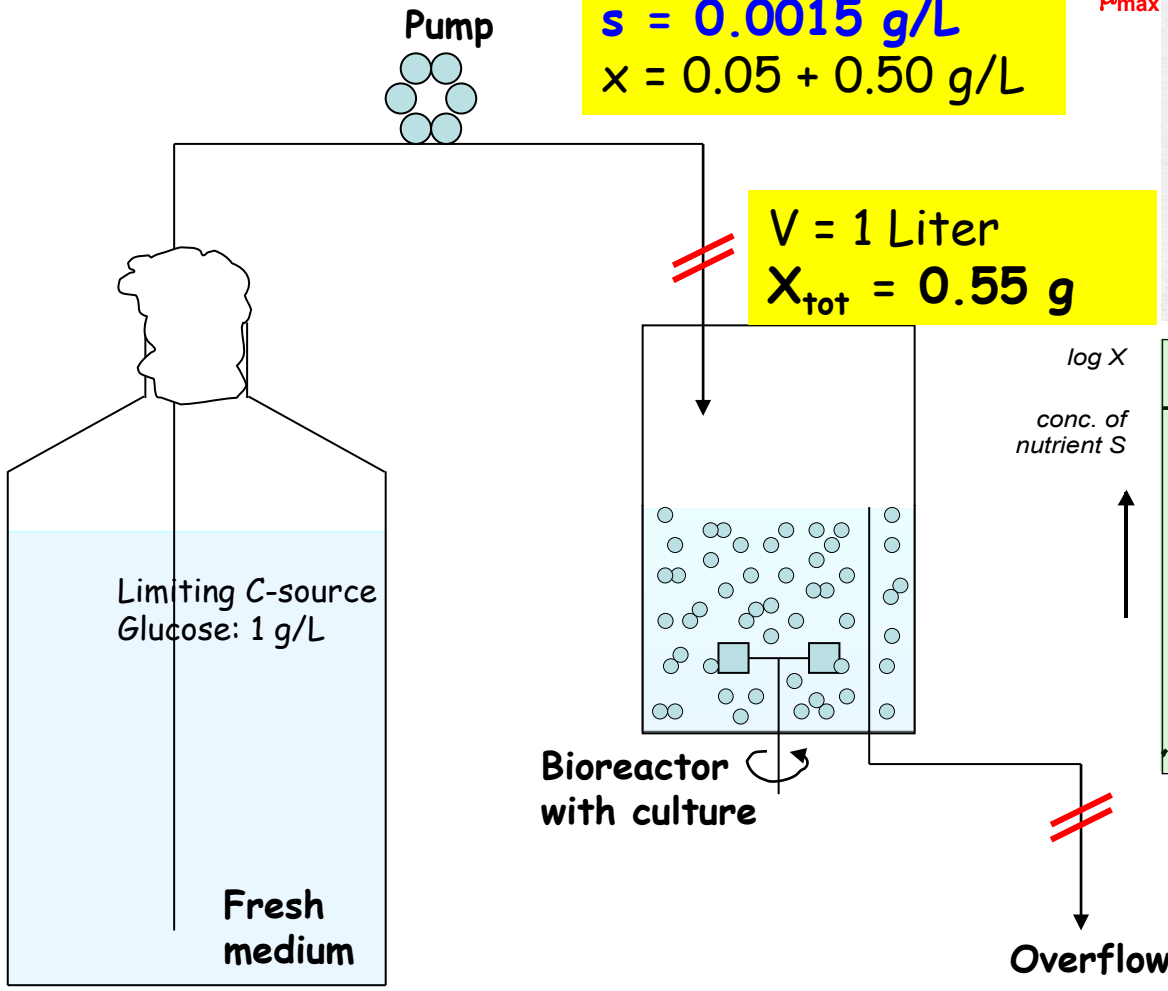
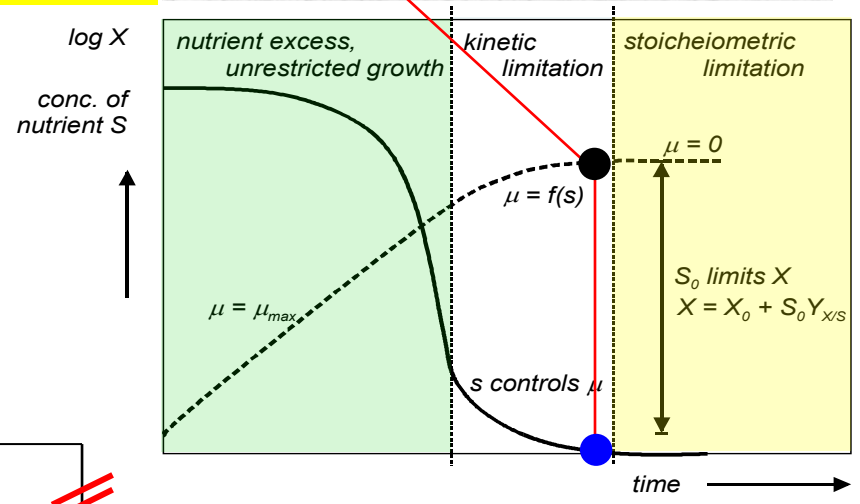


FIG. 16. — Taux de croissance des cultures de *B. coli* en milieu synthétique en fonction de la concentration du milieu en glucose.





Starting a continuous culture

Phase 1:
Batch mode

T = 6.0 h
μ ~ 0.0/h (stat.)
s = 0.000 g/L
x = 0.05 + 0.50 g/L

Y = 0.5
μ_{max} = 1.0/h
K_s = 4 mg/L

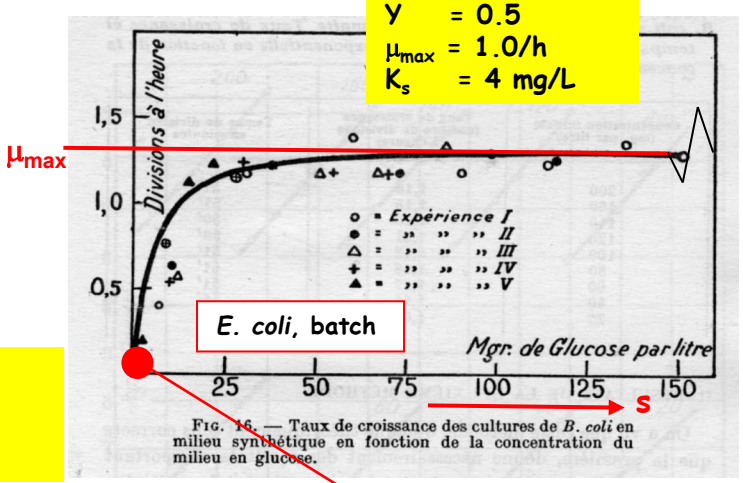


Fig. 16. — Taux de croissance des cultures de *B. coli* en milieu synthétique en fonction de la concentration du milieu en glucose.

Pump

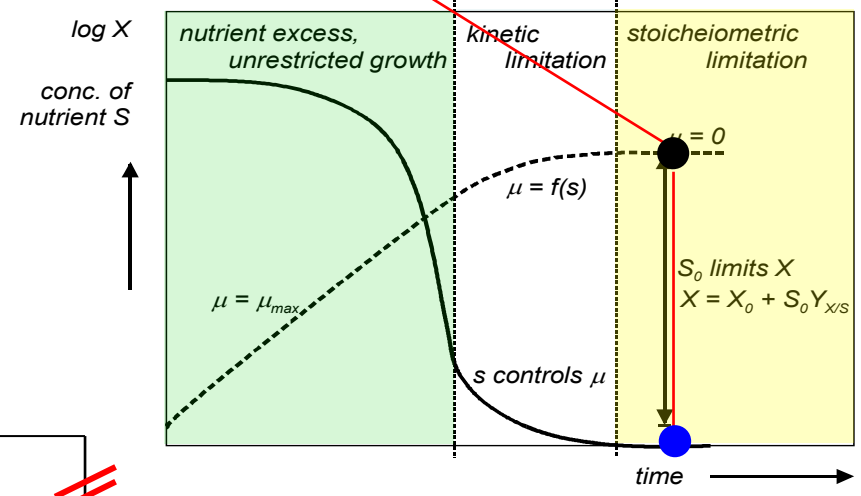
V = 1 Liter
X_{tot} = 0.55 g

Limiting C-source
Glucose: 1 g/L

Fresh medium

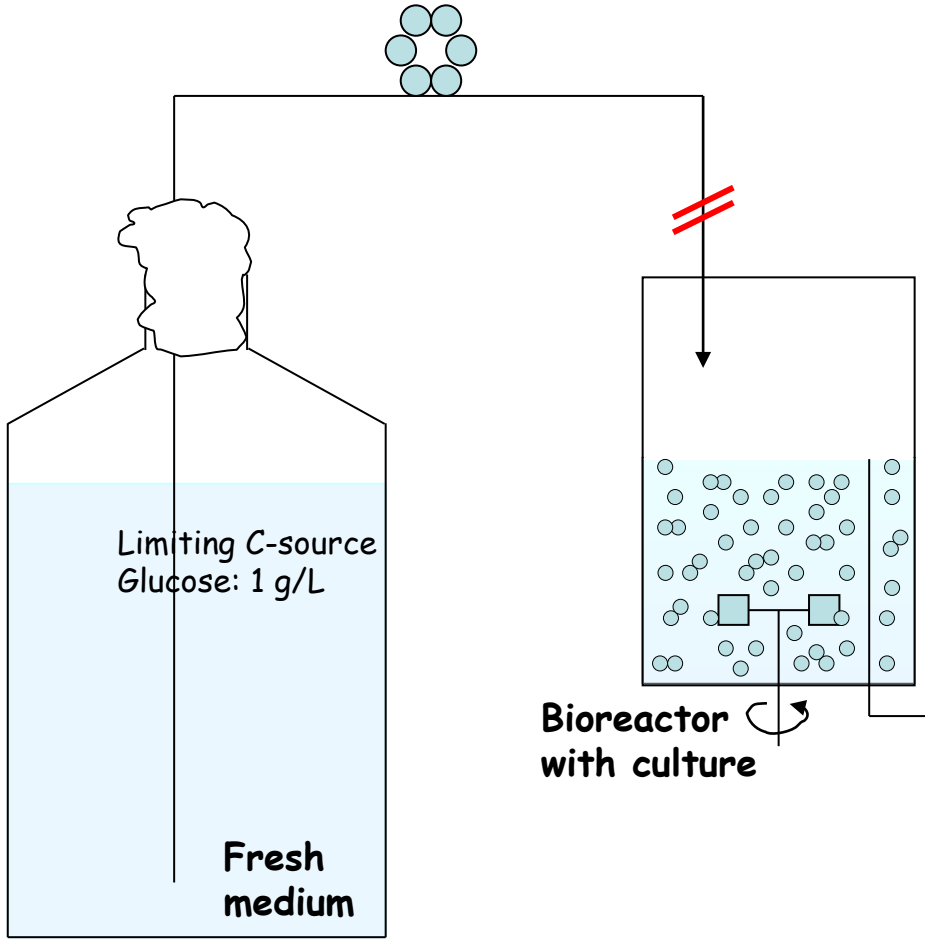
Bioreactor with culture

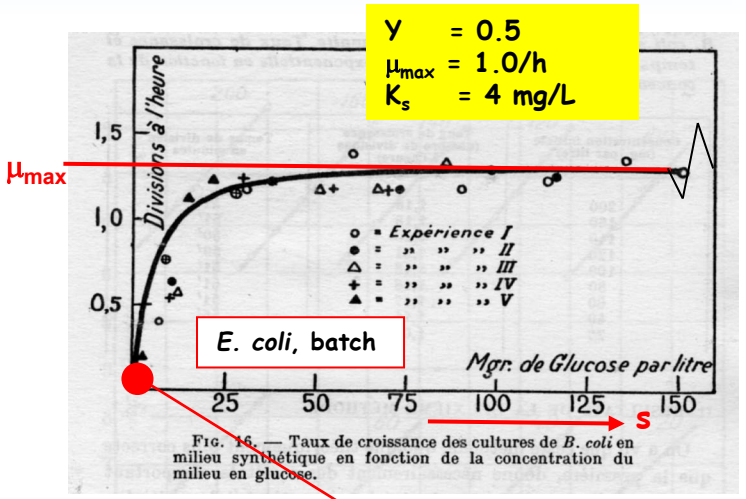
Overflow

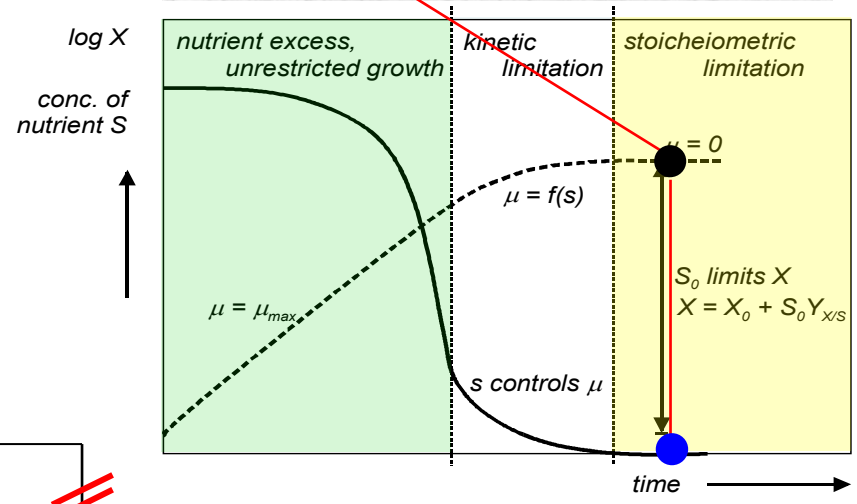


Starting a continuous culture

Can we keep the cells growing?

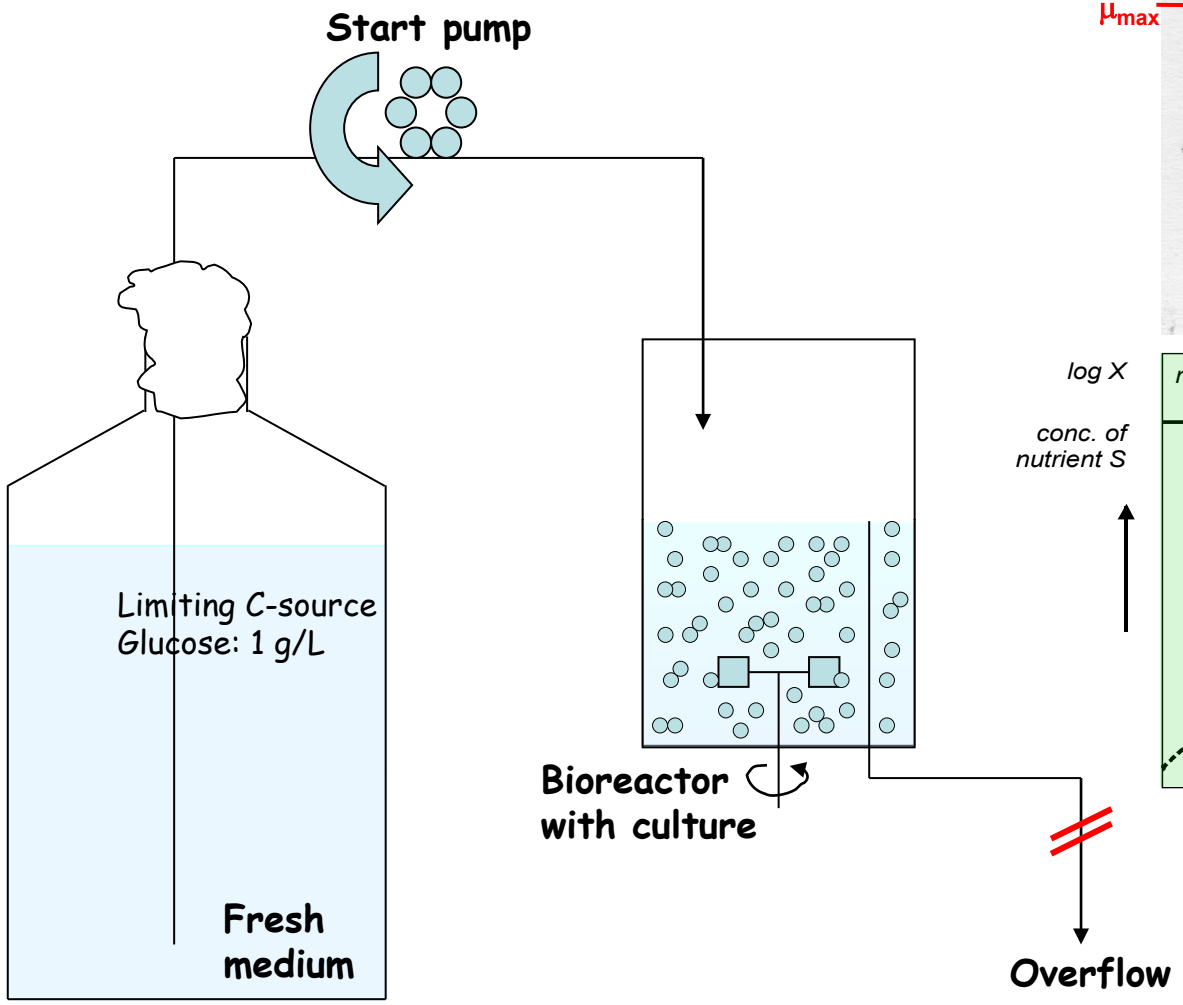


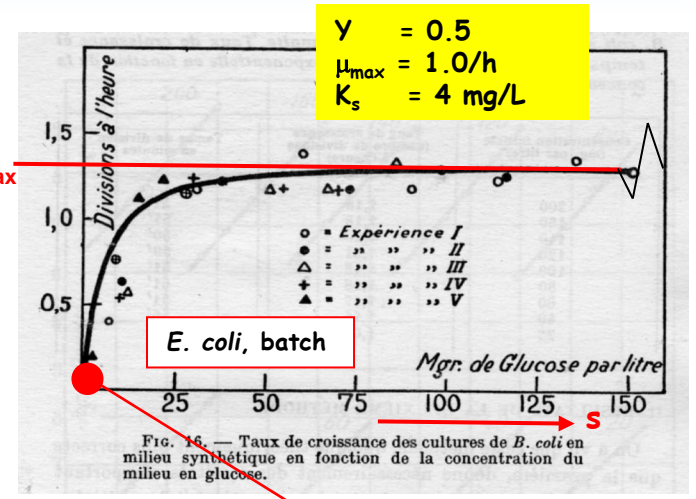


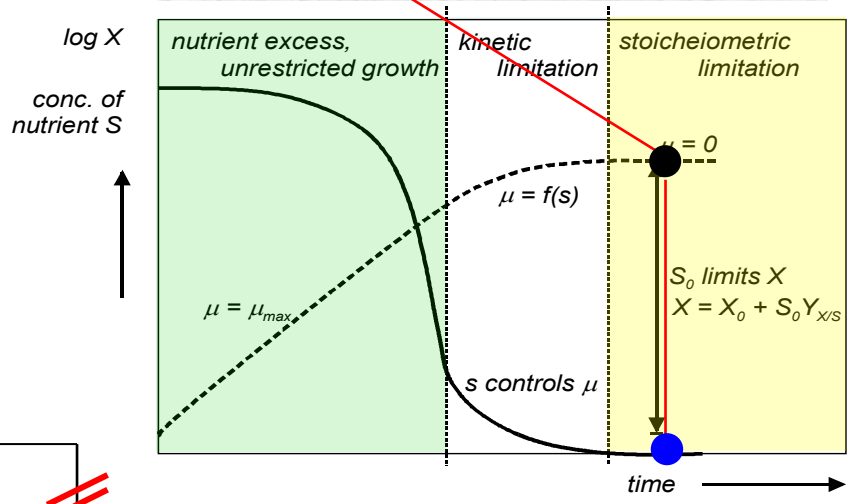


Starting a continuous culture

Of course: add fresh medium !







Starting a continuous culture

Phase 2:
Fed batch mode

T = 6.01 h
μ ~ 0.195/h (~D)
s ~ 0.0010 g/L
x = 0.55 g/L

Start pump

Feed: 0.2 L/h
(200 mg glucose / h)
D = F/V=0.2/h

V = 1 Liter
X_{tot} = 0.55 g

Limiting C-source
Glucose: 1 g/L

Fresh medium

Bioreactor with culture

Overflow

Y = 0.5
μ_{max} = 1.0/h
K_s = 4 mg/L

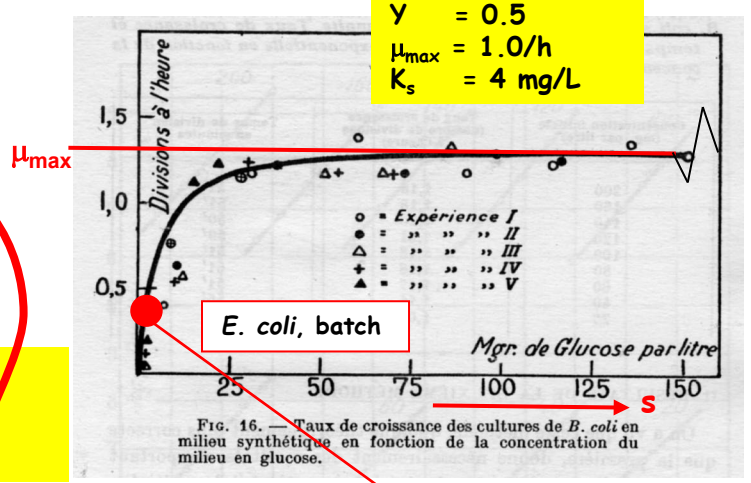
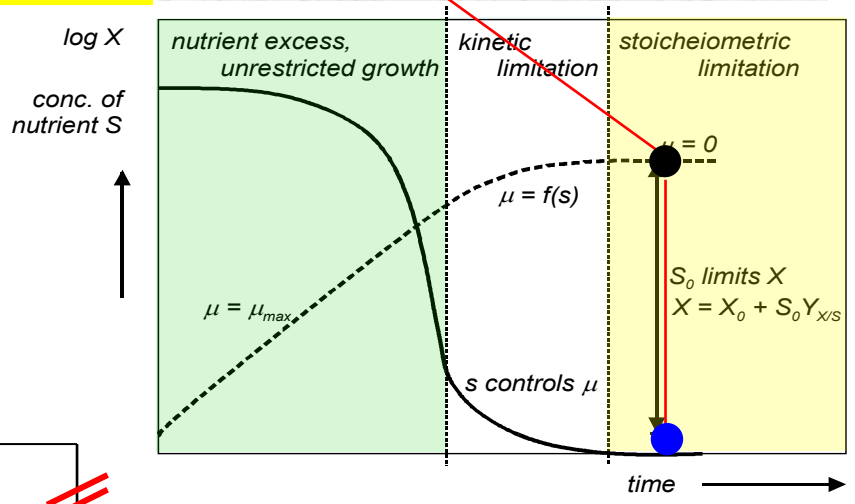


FIG. 16. — Taux de croissance des cultures de *B. coli* en milieu synthétique en fonction de la concentration du milieu en glucose.



Starting a continuous culture

Phase 2:
Fed batch mode

T = 7.0 h
μ ~ 0.17/h (~D)
s ~ 0.0008 g/L
x = 0.54 g/L

Start pump

Feed: 0.2 L/h
(200 mg glucose/h)

V = 1.2 Liter
X_{tot} = 0.65 g

Limiting C-source
Glucose: 1 g/L

Fresh medium

Bioreactor with culture

Overflow

Y = 0.5
μ_{max} = 1.0/h
K_s = 4 mg/L

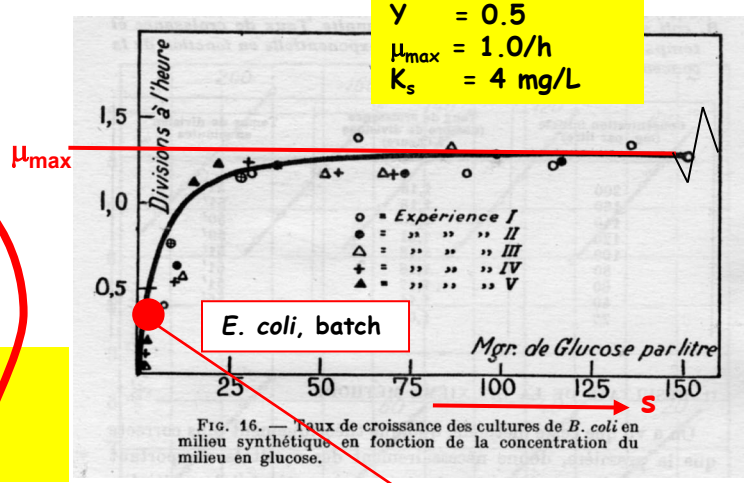
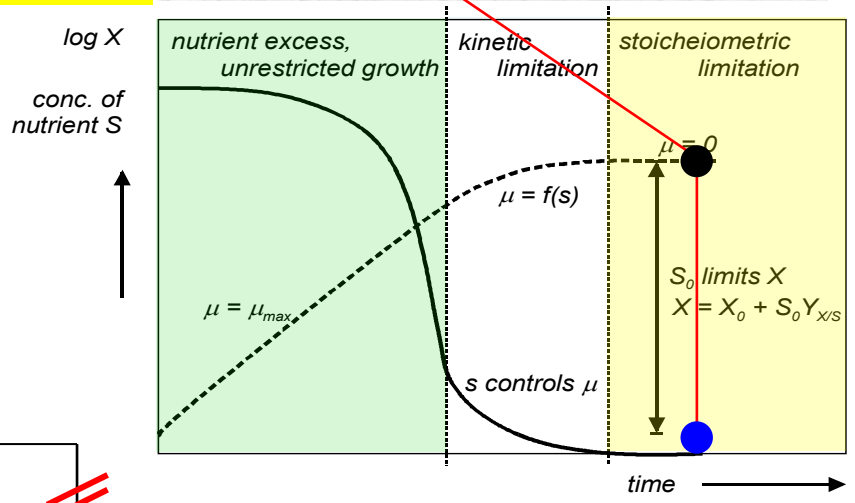


FIG. 16. — Taux de croissance des cultures de *B. coli* en milieu synthétique en fonction de la concentration du milieu en glucose.



Starting a continuous culture

Phase 2:
Fed batch mode

T = 7 > 8.0 h
μ ~ 0.17 > 0.14/h
s ~ 0.0007 g/L
x = 0.535 g/L

Start pump

Feed: 0.2 L/h
(200 mg glucose/h)

V = 1.4 Liter
X_{tot} = 0.75 g

Limiting C-source
Glucose: 1 g/L

Fresh medium

Bioreactor with culture

Overflow

Y = 0.5
μ_{max} = 1.0/h
K_s = 4 mg/L

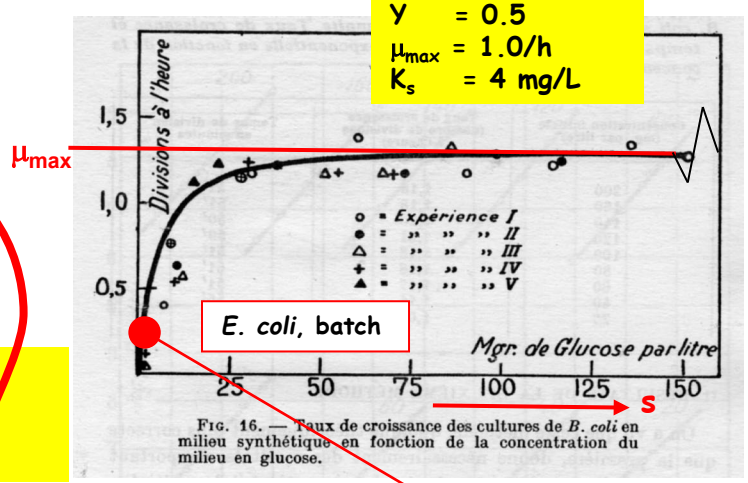
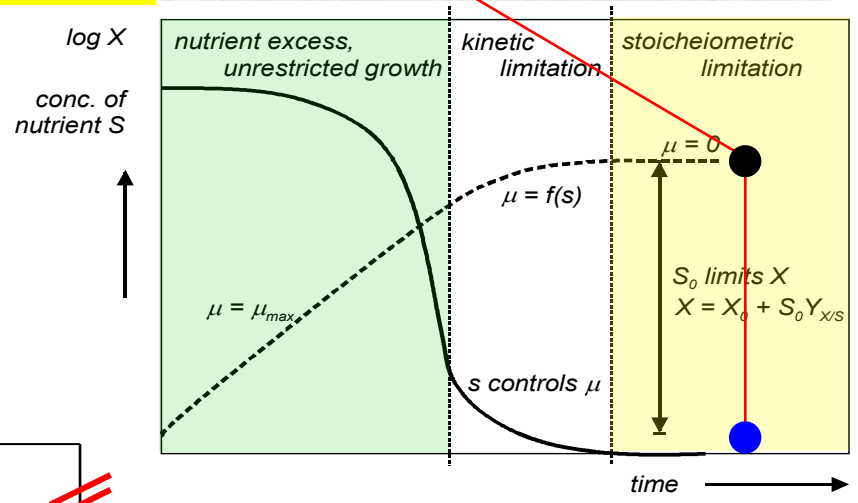


FIG. 16. — Taux de croissance des cultures de *B. coli* en milieu synthétique en fonction de la concentration du milieu en glucose.



Starting a continuous culture

Phase 2:
Fed batch mode

T = 8 > 9.0 h
μ ~ 0.14 > 0.12/h
s ~ 0.0005 g/L
x = 0.53 g/L

Start pump

Feed: 0.2 L/h
(200 mg glucose/h)

V = 1.6 Liter
X_{tot} = 0.85 g

Limiting C-source
Glucose: 1 g/L

Fresh medium

Bioreactor with culture

Overflow

Y = 0.5
μ_{max} = 1.0/h
K_s = 4 mg/L

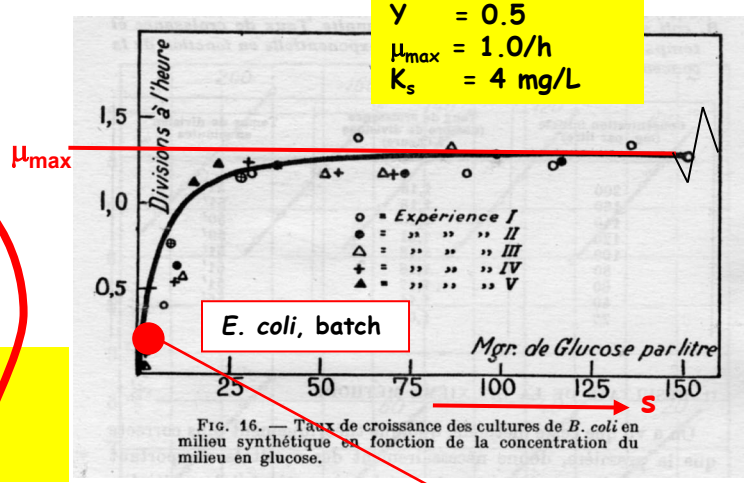
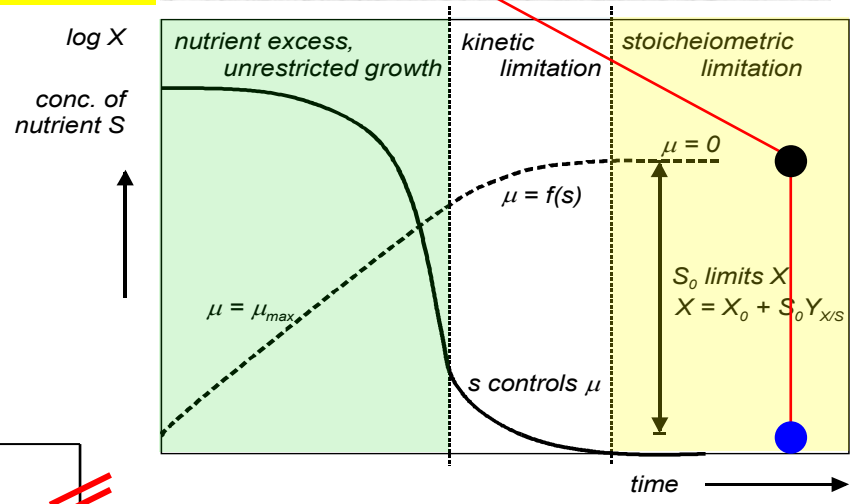


FIG. 16. — Taux de croissance des cultures de *B. coli* en milieu synthétique en fonction de la concentration du milieu en glucose.



Starting a continuous culture

Phase 2:
Fed batch mode

T = 9 > 10.0 h
μ ~ 0.12 > 0.11/h
s = 0.0004 g/L
x = 0.52 g/L

Start pump

Feed: 0.2 L/h
(200 mg glucose/h)

V = 1.8 Liter
X_{tot} = 0.95 g

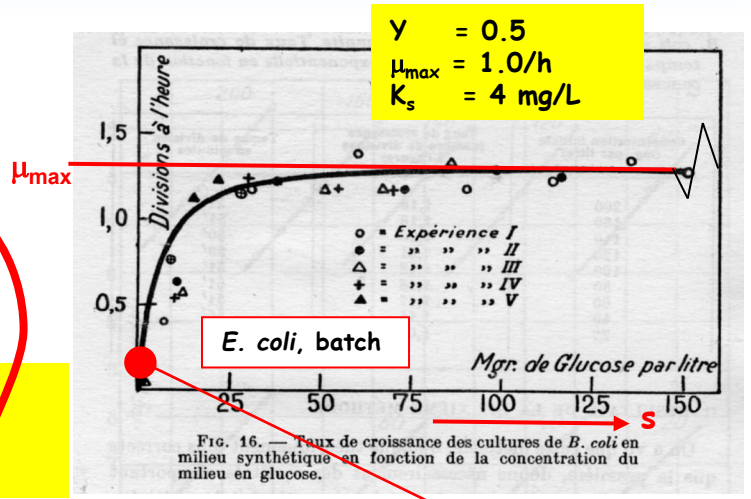
Limiting C-source
Glucose: 1 g/L

Fresh medium

Bioreactor with culture

Overflow

Y = 0.5
μ_{max} = 1.0/h
K_s = 4 mg/L



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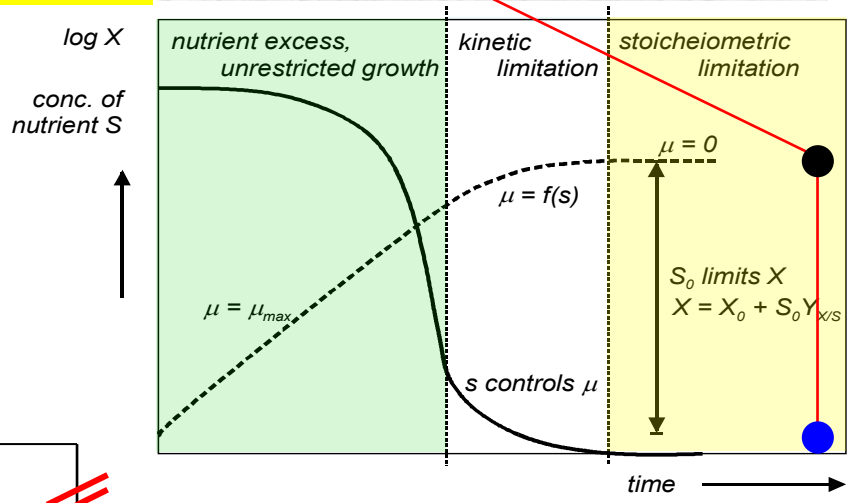
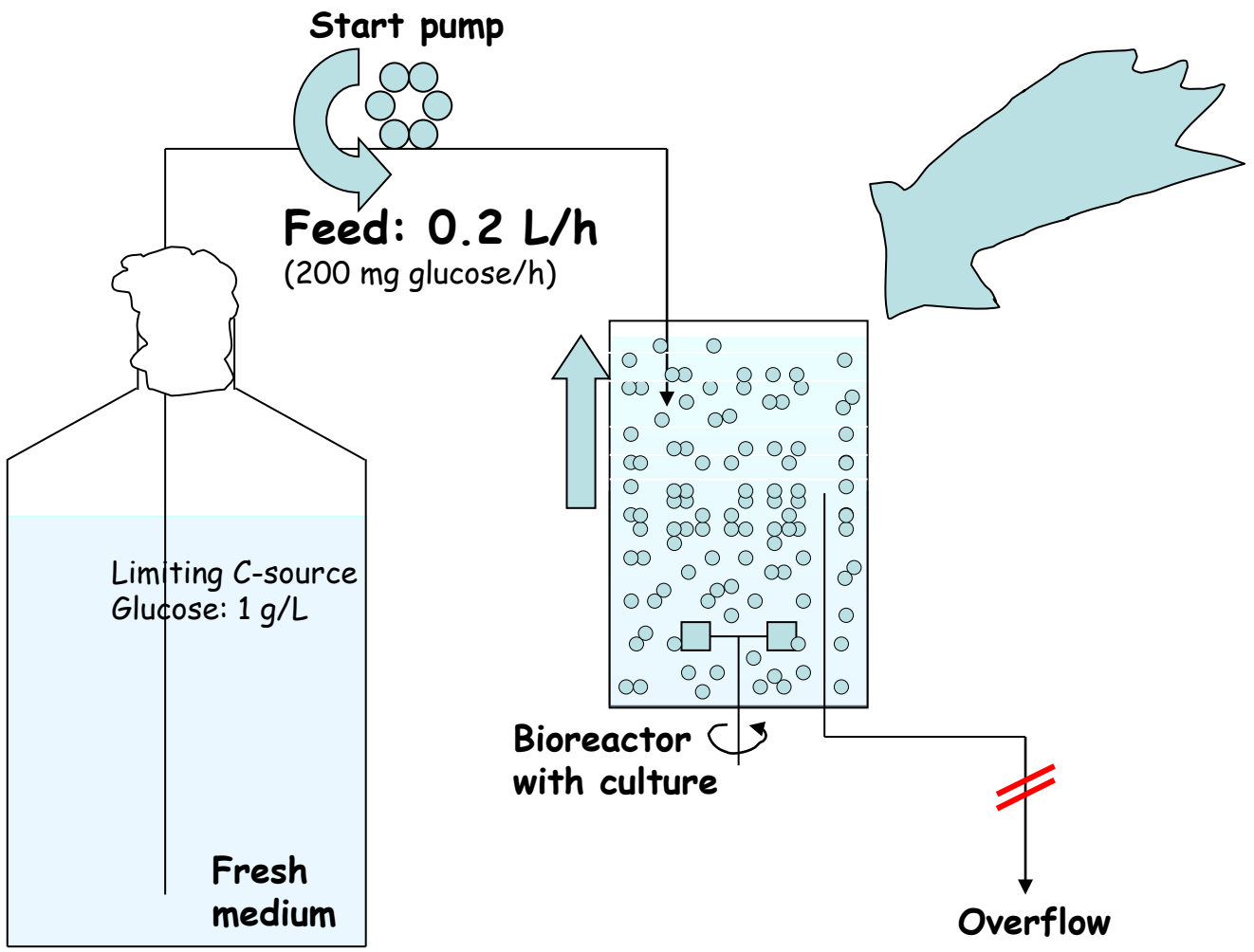


Starting a continuous culture

Phase 2:
Fed batch mode

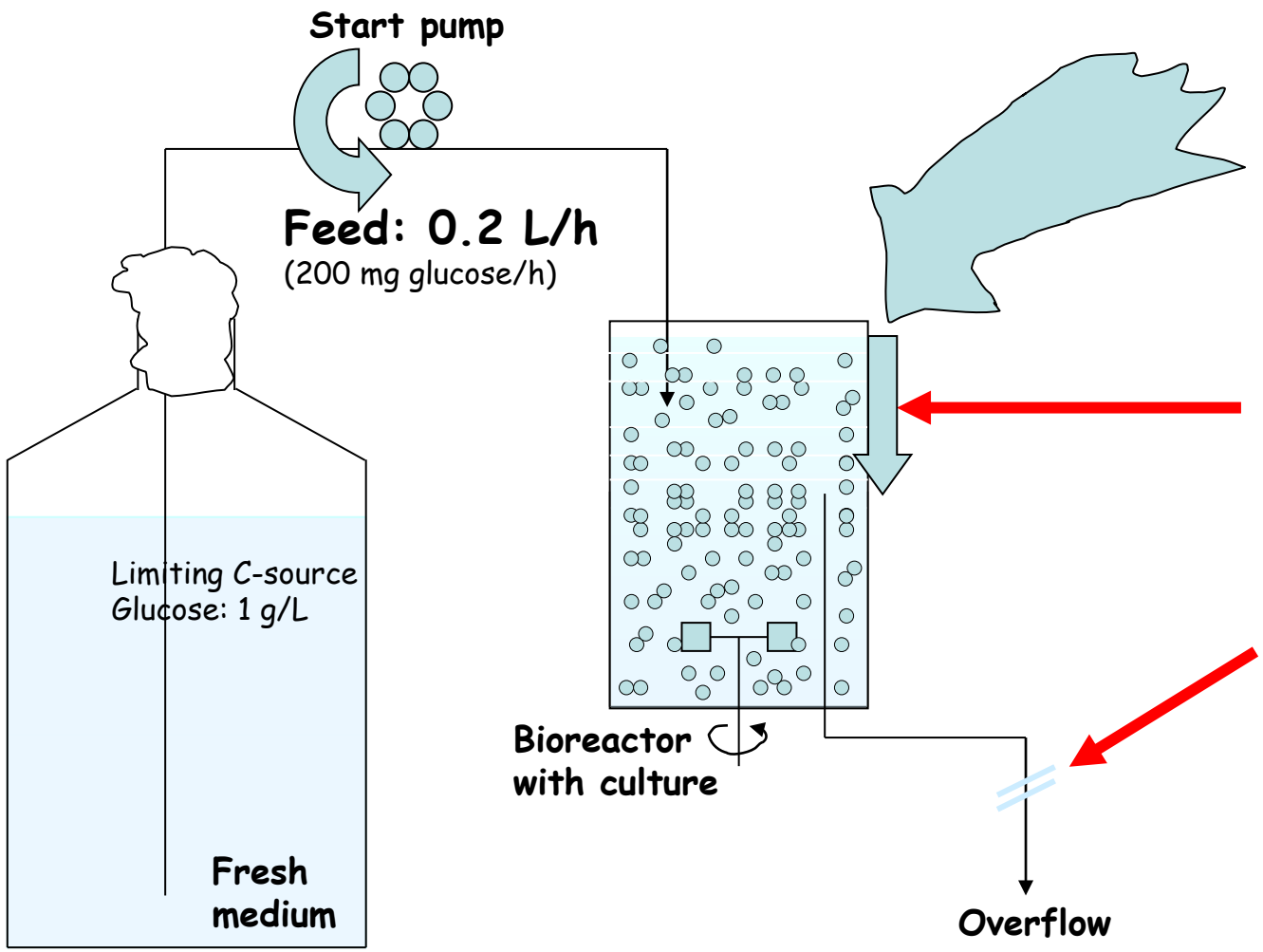


The same amount of glucose per hour is fed to more and more cells!

Result: μ is continuously decreasing

Starting a continuous culture

Phase 3:
Continuous mode



The same amount of glucose per hour is fed to more and more cells!

Result: μ is continuously decreasing

Reduce the total biomass in the reactor to the initial amount

Open overflow !!!

Starting a continuous culture

Phase 3:
Continuous mode

T = 11 h
μ ~ 0.195/h (~D)
s = 0.0010 g/L
x = 0.52 g/L

Start pump

Feed: 0.2 L/h
(200 mg glucose/h)
D = F/V=0.2/h

V = 1 Liter
X_{tot} = 0.52 g

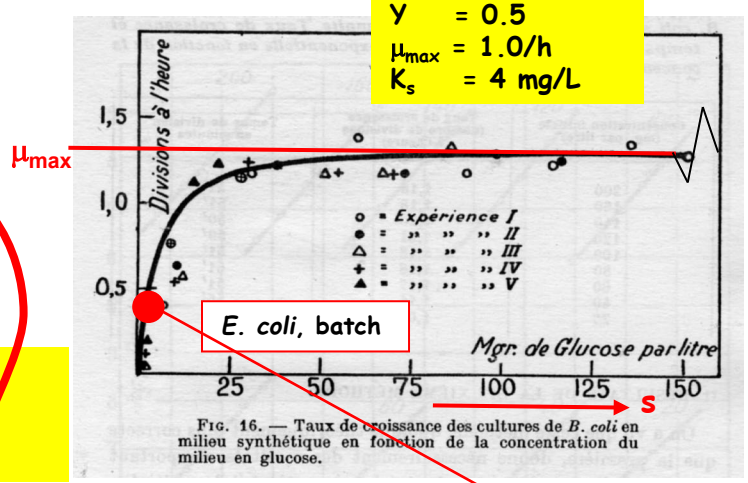
Limiting C-source
Glucose: 1 g/L

Fresh medium

Bioreactor with culture

Overflow

Y = 0.5
μ_{max} = 1.0/h
K_s = 4 mg/L



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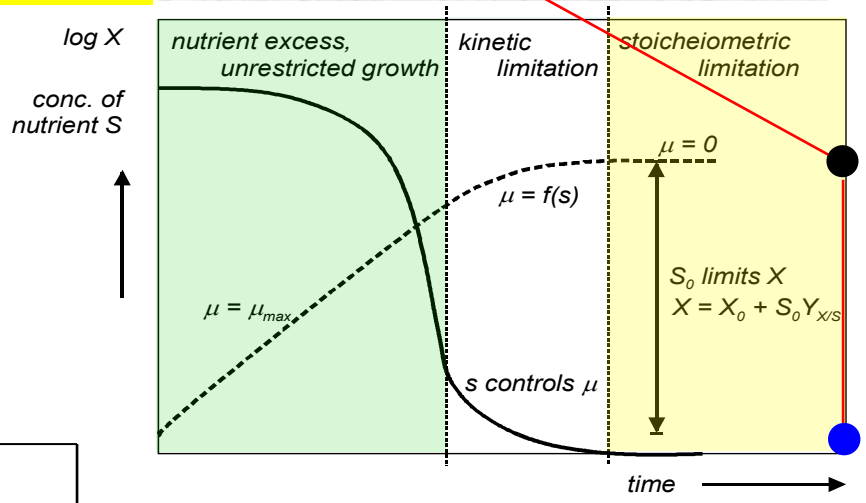


The continuous culture becomes a chemostat culture: a state of continuous growth establishes

Phase 3:
Continuous mode

T = 11 h
μ ~ 0.199/h (~D)
s = 0.0011 g/L
x = 0.51 g/L

Start pump

Feed: 0.2 L/h
(200 mg glucose/h)
D = F/V=0.2/h

V = 1 Liter
X_{tot} = 0.51 g

Limiting C-source
Glucose: 1 g/L

Fresh medium

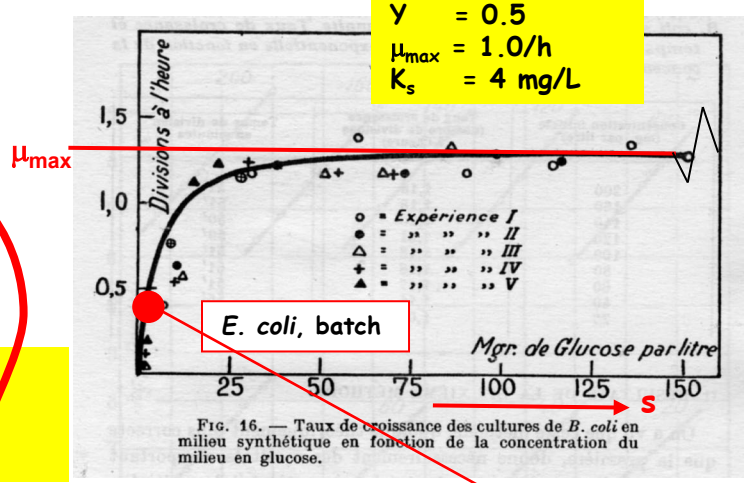
Bioreactor with culture

Cells newly formed / h

= Cells removed / h

Overflow

Y = 0.5
μ_{max} = 1.0/h
K_s = 4 mg/L



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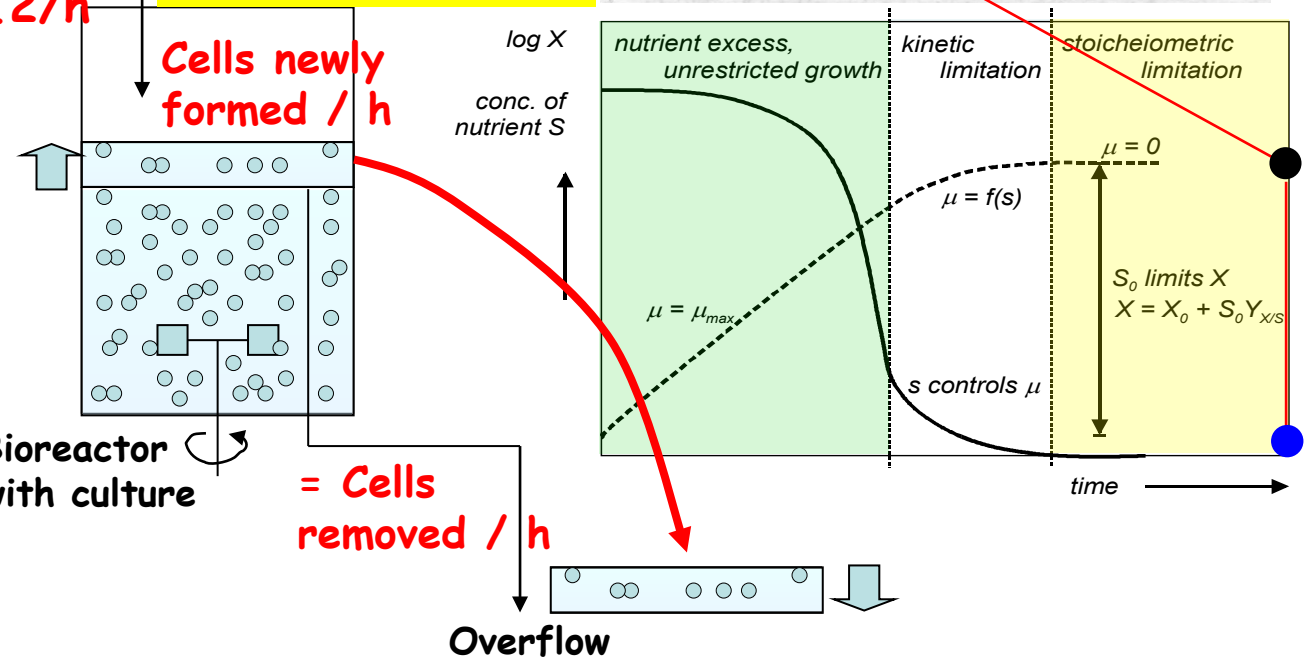


The continuous culture IS a chemostat culture: steady-state conditions have established

Phase 3:
Continuous mode

T = 11 h
μ ~ 0.20/h (=D)
s = 0.0011 g/L
x = 0.499 g/L

Start pump

Feed: 0.2 L/h
(200 mg glucose/h)
D = F/V=0.2/h

V = 1 Liter
X_{tot} = 0.499 g

V,x,s,μ = const.

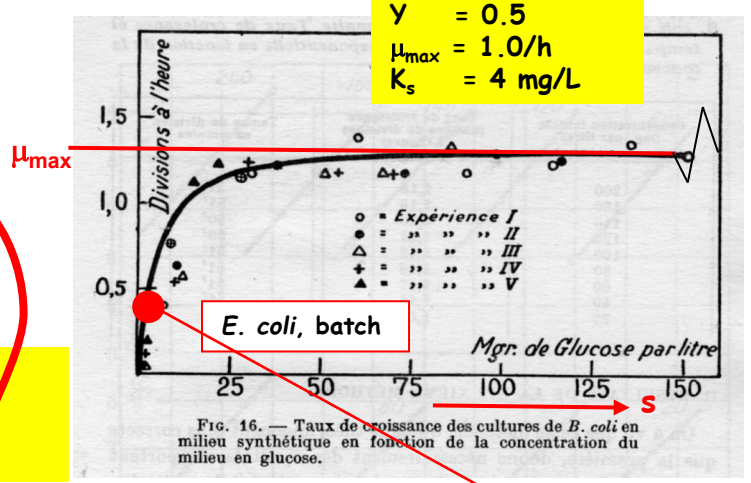
Limiting C-source
Glucose: 1 g/L

Fresh medium

Bioreactor with culture

Overflow

Y = 0.5
μ_{max} = 1.0/h
K_s = 4 mg/L



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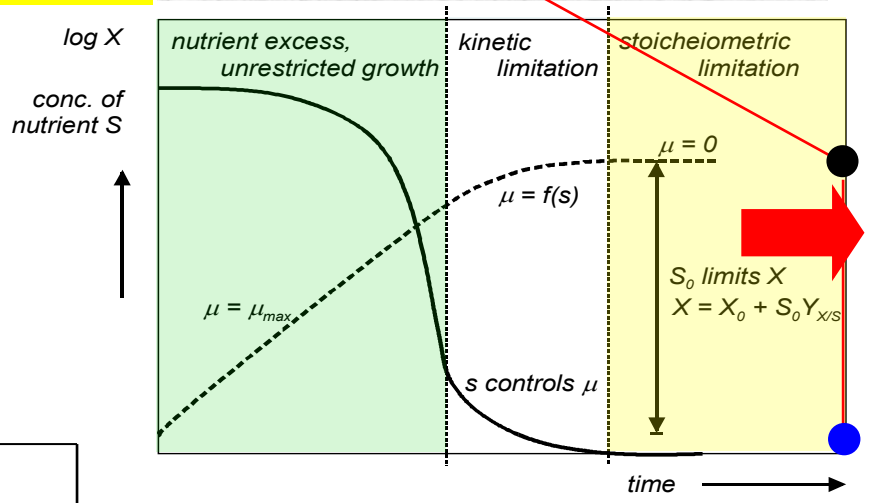


Determination of yield coefficients

$Y_{X/S}$, $Y_{P/S}$ etc.

Growth equation:

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

In chemostat plot of 1/Y versus 1/D gives straight line which **does not pass through origin**
(if $Y_{X/E}$ were independent of μ then it would!)

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

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

Determination of maintenance energy

Pirt equation:

$$\frac{1}{Y_{X/S}} = \frac{1}{Y^G} + \frac{m_s}{\mu}$$

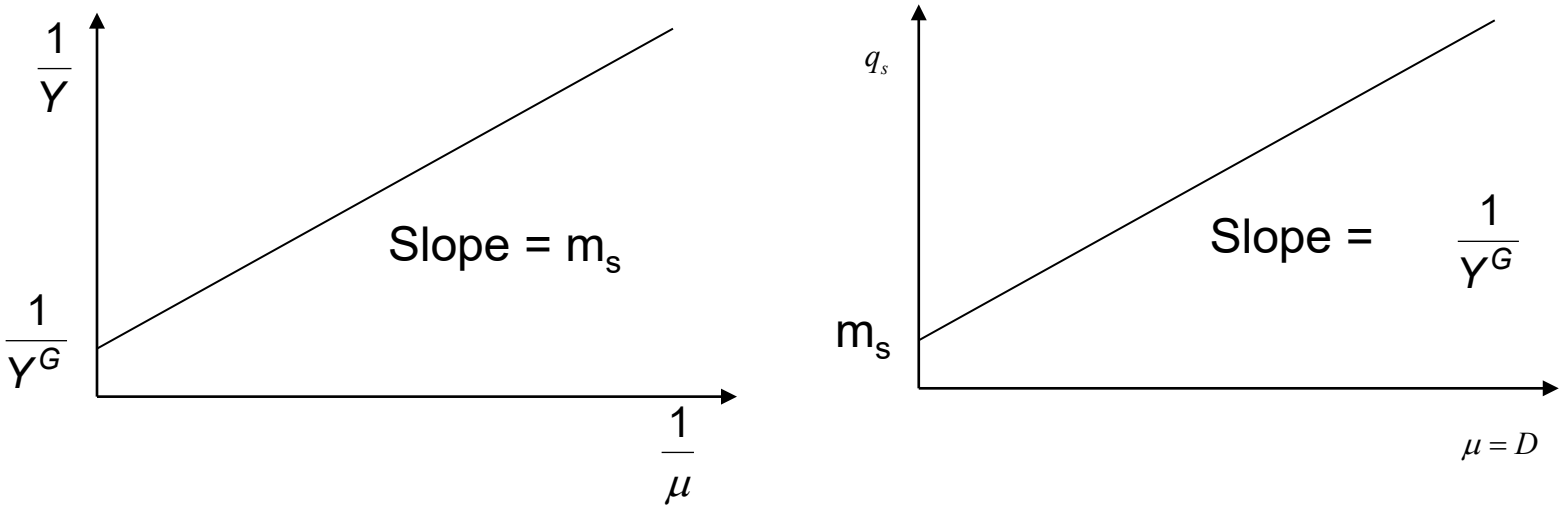
Y^G: True growth yield on substrate s [g g^{-1}]
m_s: maintenance energy [g g^{-1} h^{-1}]

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_s and Y^G

Presentation of the data in a graph

$$\frac{1}{Y_{X/S}} = \frac{1}{Y^G} + \frac{m_s}{\mu} \quad \longrightarrow \quad q_s = \frac{\mu}{Y} = \frac{\mu}{Y^G} + m_s$$

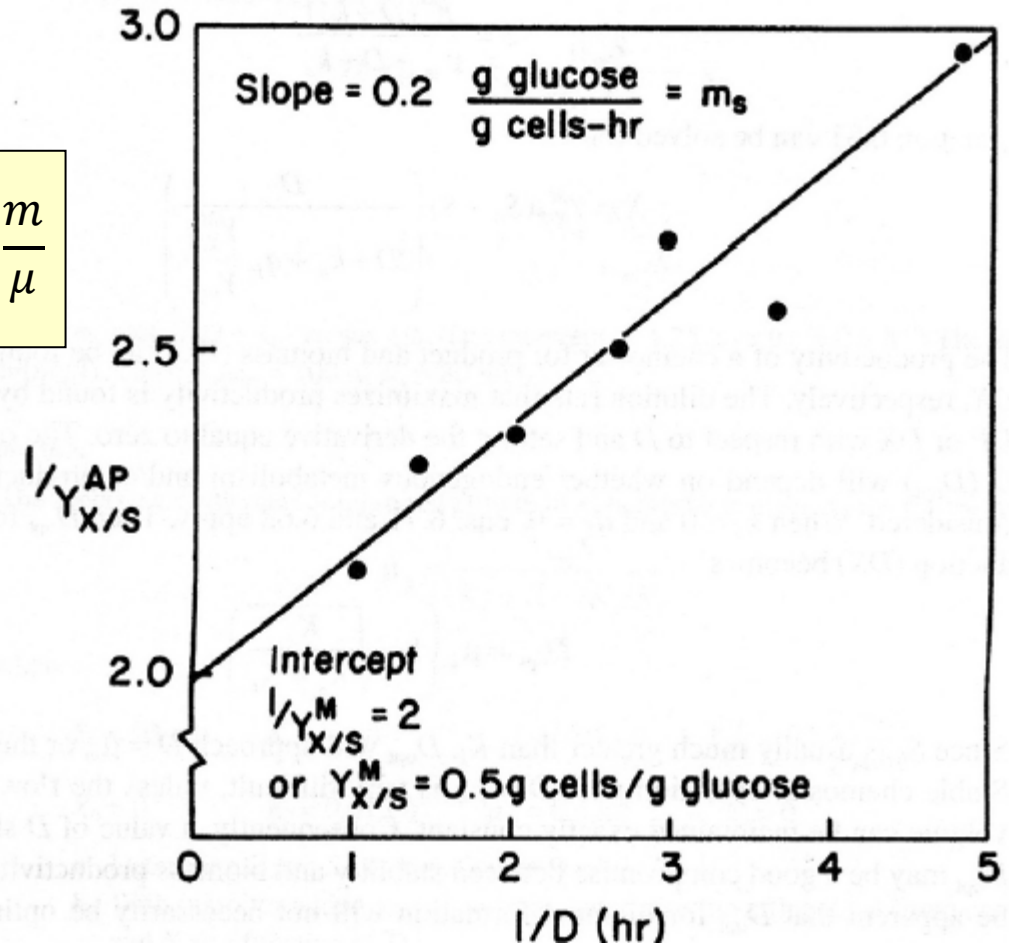


Range of values m_s: in *E. coli*: 0.072 – 0.090 g glucose g^{-1} cdw h^{-1}

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

Experimental determination of m and Y

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

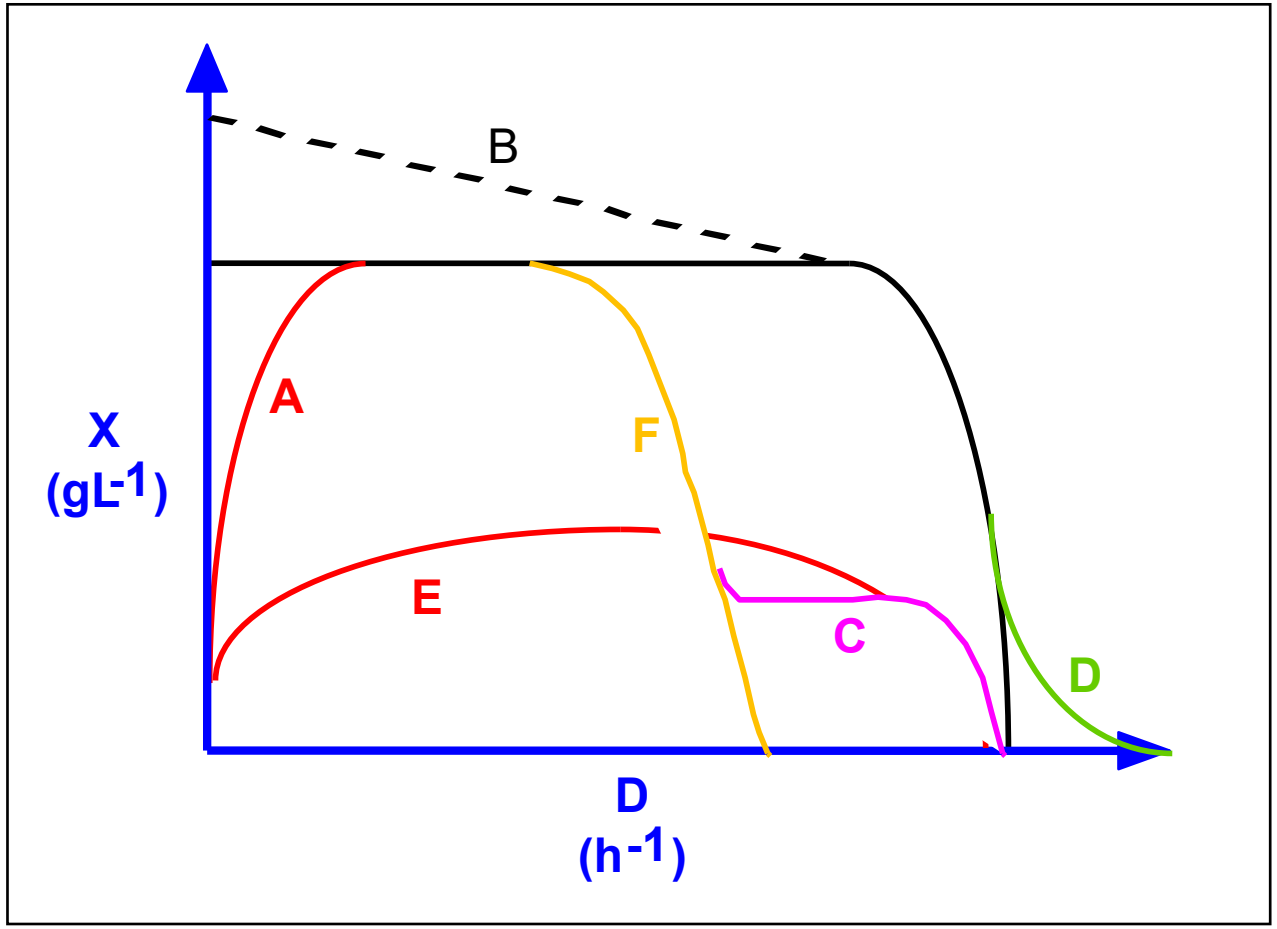


Question

- 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 $\boldsymbol{D_{crit}}$ **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.

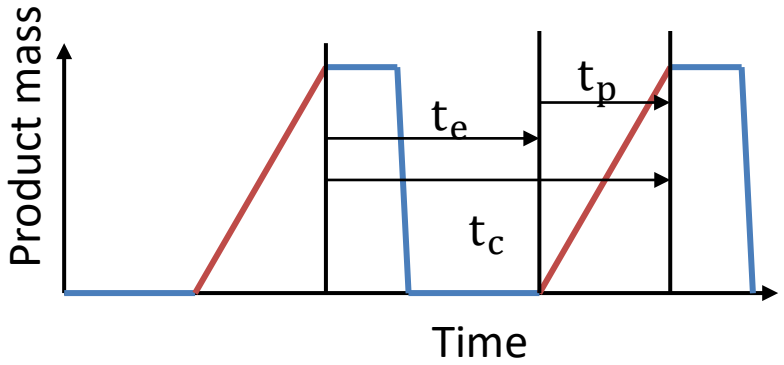
Definition of productivity

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

ΔM_{out}: Product mass generated by process

Δt: Time frame of consideration

Batch (idealized)



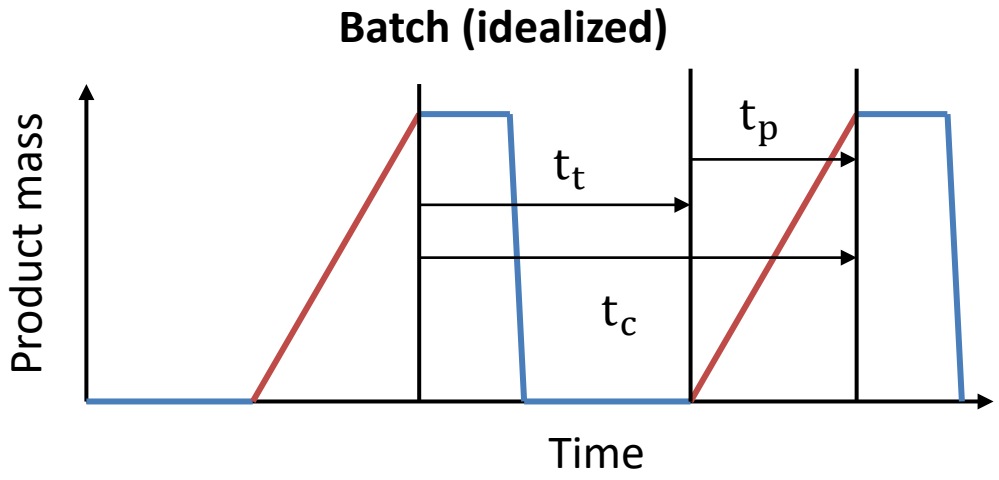
The relevant time frame needs to be defined (see next slide).

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 through-out t_p.

The total batch time, t_c is given by:

$$t_c = t_p + t_t = \frac{\ln \frac{X}{X_0}}{\mu_{max}} + t_t$$

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

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

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

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:

$$P^V_{conti} = \tilde{m}^I \cdot D \quad \left[\frac{\mathrm{g}}{\mathrm{Lh}}\right]$$

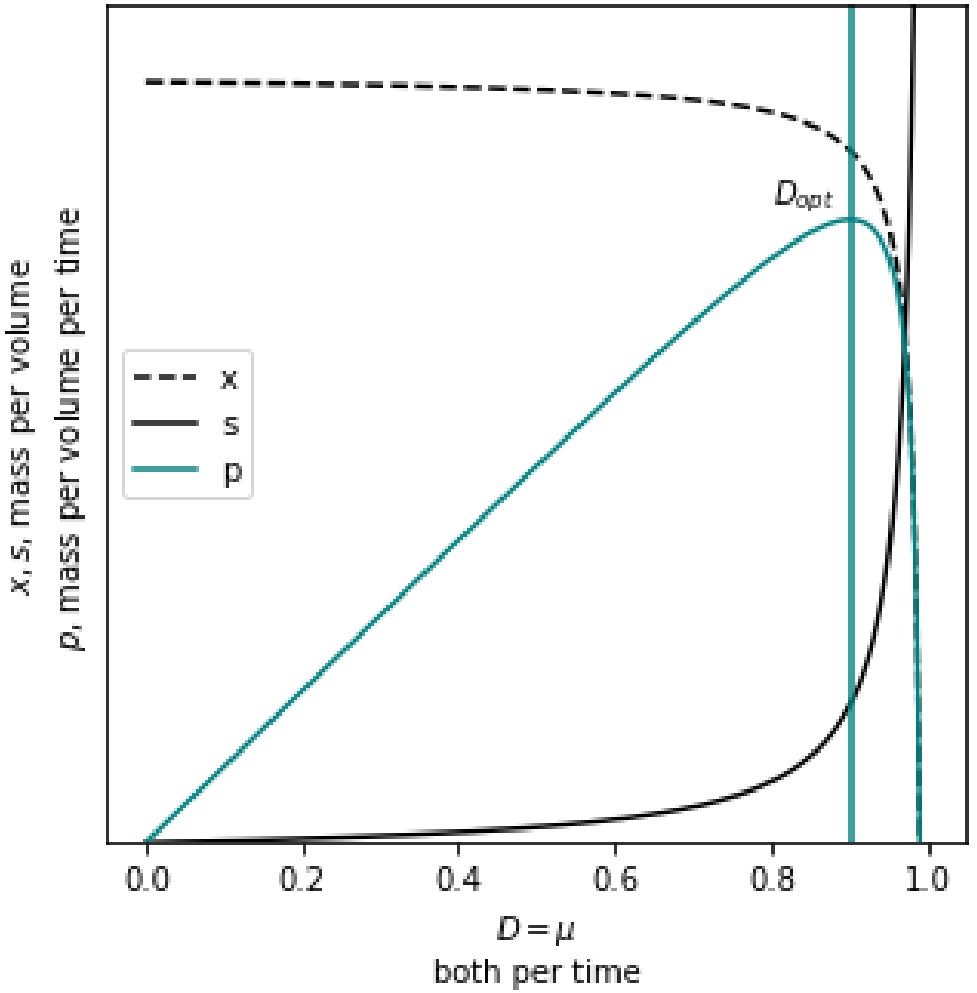
For a chemostat in steady-state:

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

Inserting:

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

Chemostat productivity of a primary product



P^V_{conti} has an optimum in the x-D diagram:

- 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.

Question: How do you calculate D_{opt}?

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

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.

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

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

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

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

Remember:
$P^G = P^V \cdot V$

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.).

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

Note:

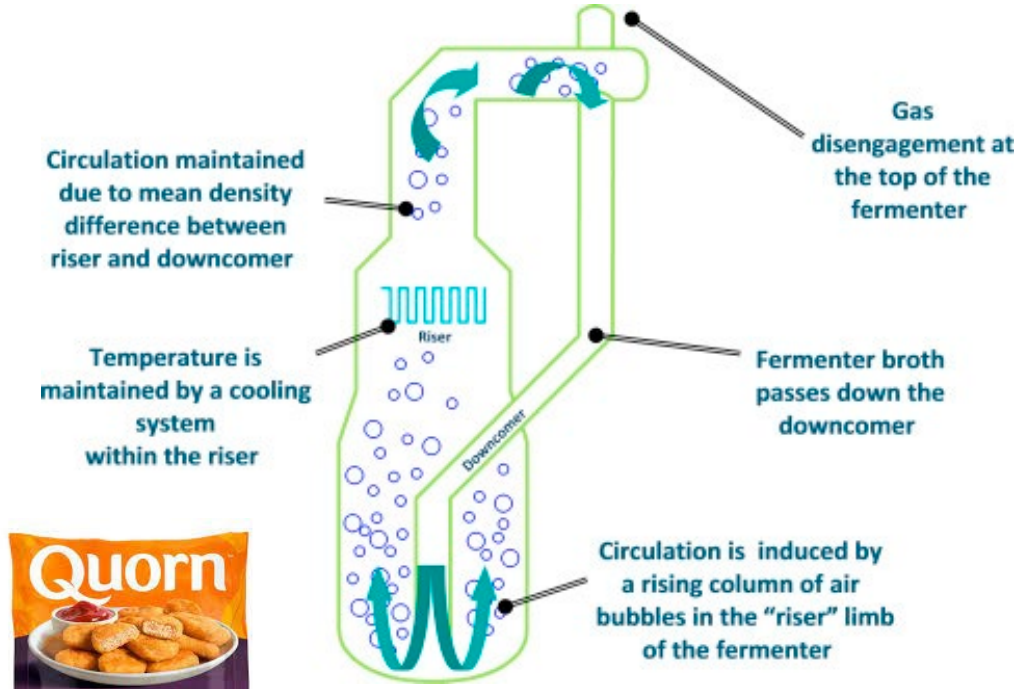
- This formula only gives a **rough estimate** in the increase of the productivity in a chemostat compared to batch culture.
- It neglects the **startup, shutdown and turnover time of the chemostat**
- It assumes production in the chemostat at the optimal dilution rate, which leaves a notable fraction 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

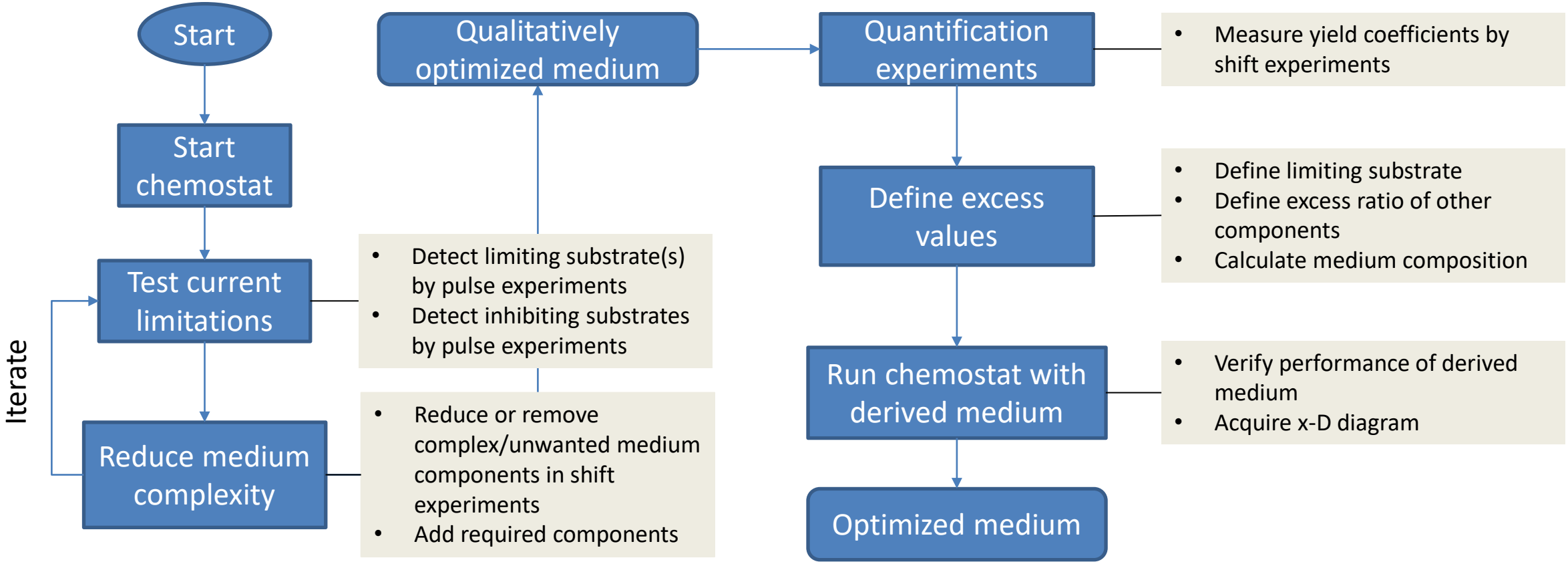
Questions

- 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}.

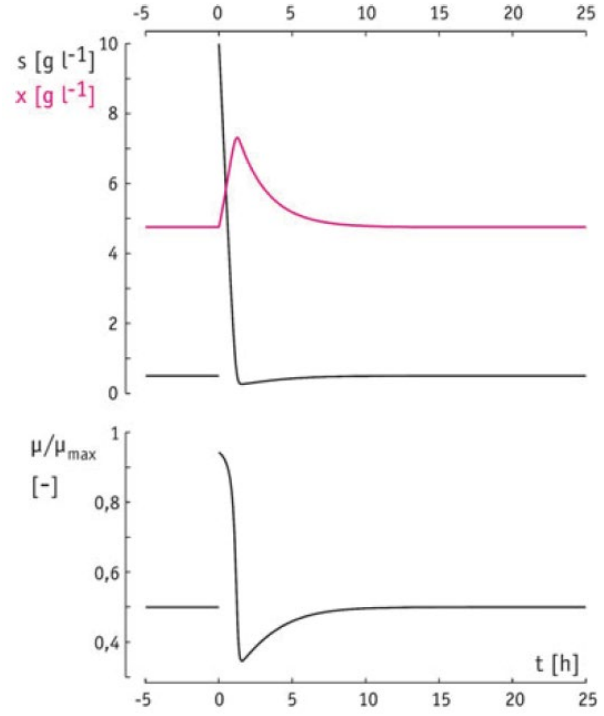
Medium optimization in a chemostat

- 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

Medium design: Pulse experiments



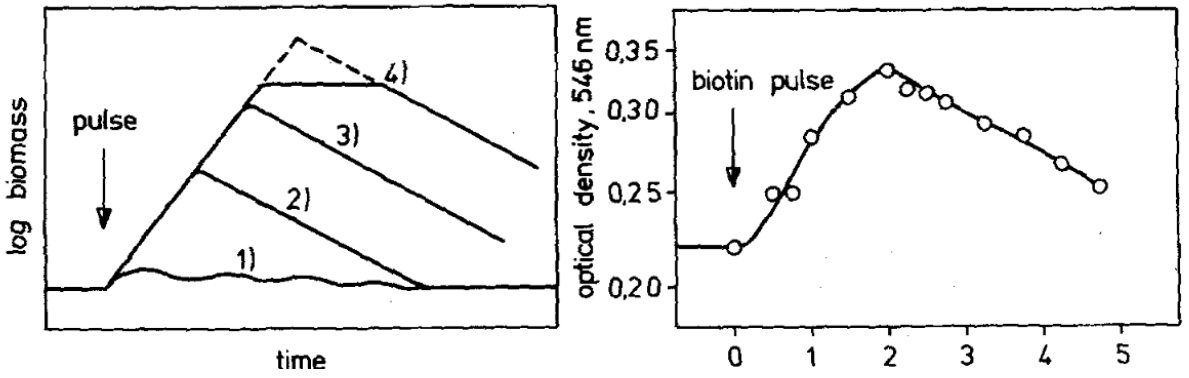
H. Chieml, 2011

- 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:

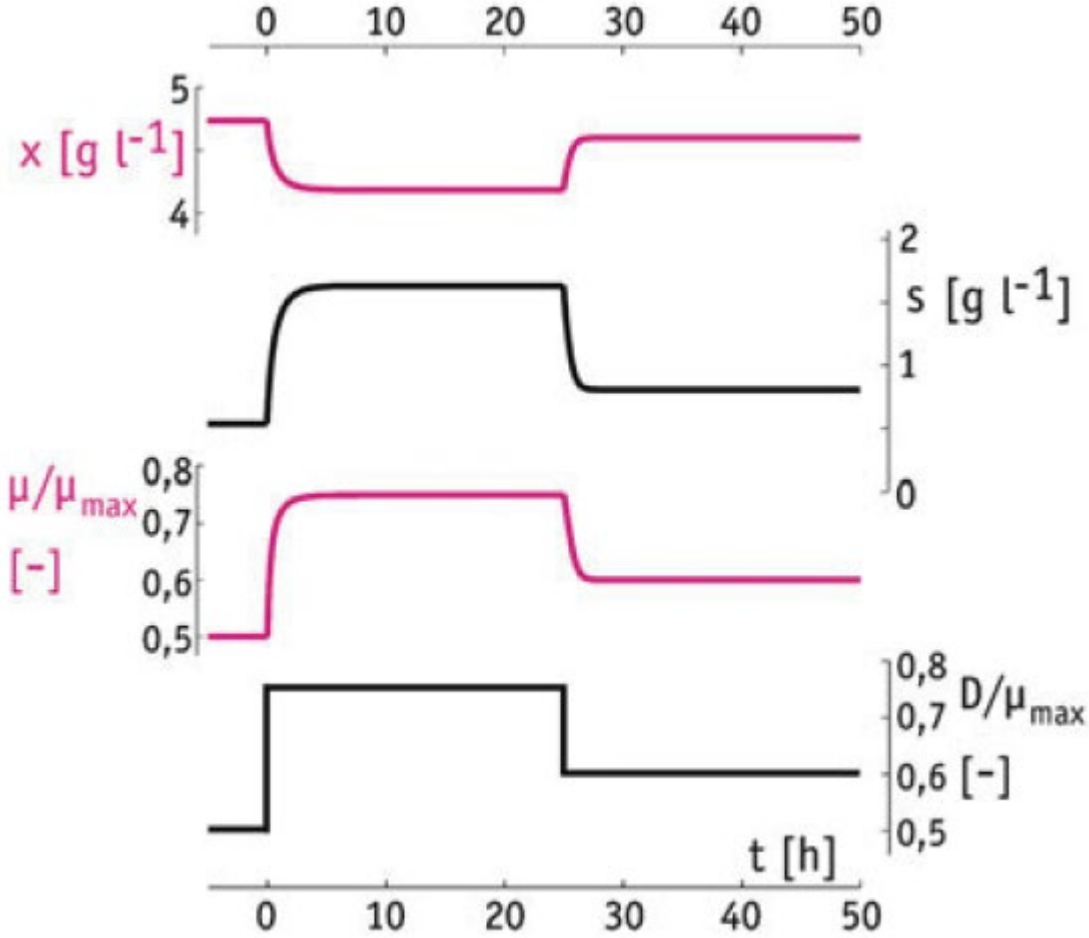
1) Not limiting substrate
2) Limiting substrate
3) Limiting substrate
4) Initially the limiting substrate, with second substrate becoming limiting during pulse.



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

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).

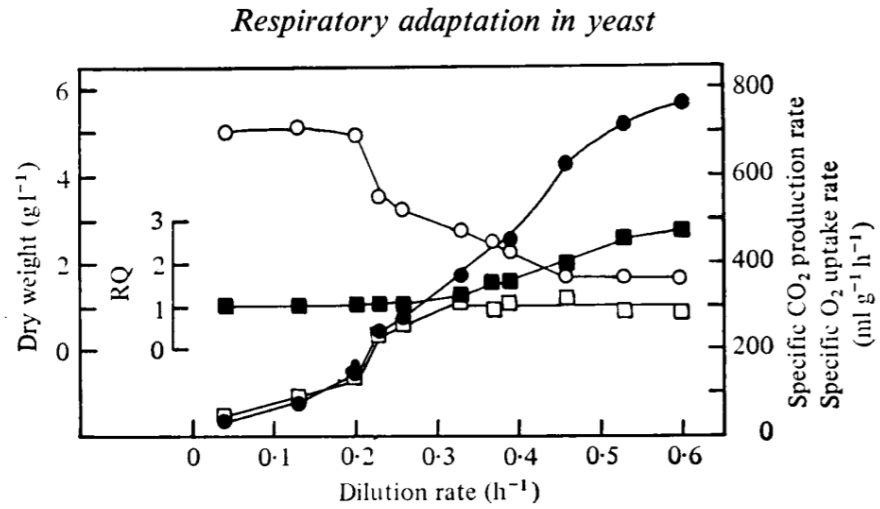


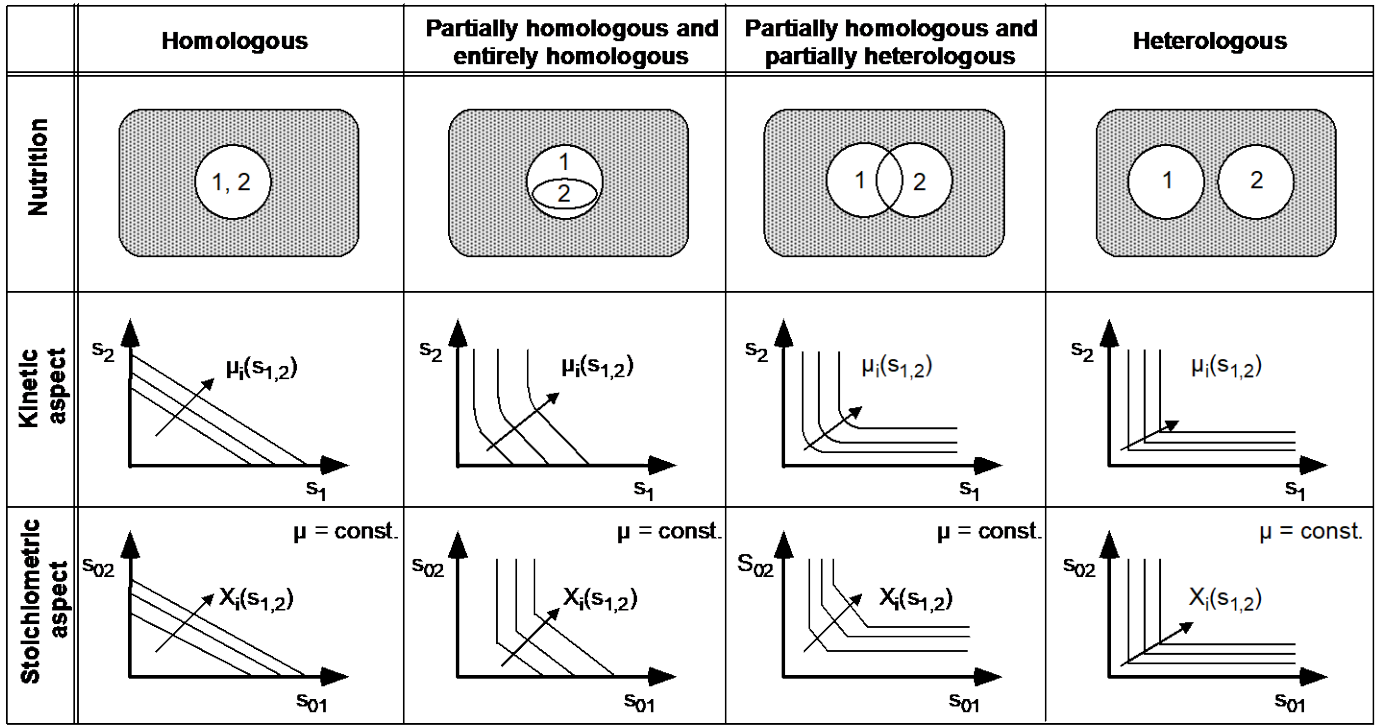
Fig. 1. Continuous culture of *S. cerevisiae* with glucose as the limiting substrate. Estimations were dry weight (○), specific carbon dioxide production rate (●), specific oxygen uptake rate (□) and respiratory quotient, RQ (■). The glucose feed concentration was 9·93 g l⁻¹.

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

- The chemostat is a great instrument to study the cell physiology of microorganisms.
- Pulse-shift experiments trigger non-steady state situations that reveal particular nutritional needs (nutrient limitations), whereas a decrease of activity can indicate toxic interactions.
- Continuous cultivation is becoming more important in future because of the good productivity and new DSP options.

10. Advanced continuous cultivation

Functionality of two nutrients

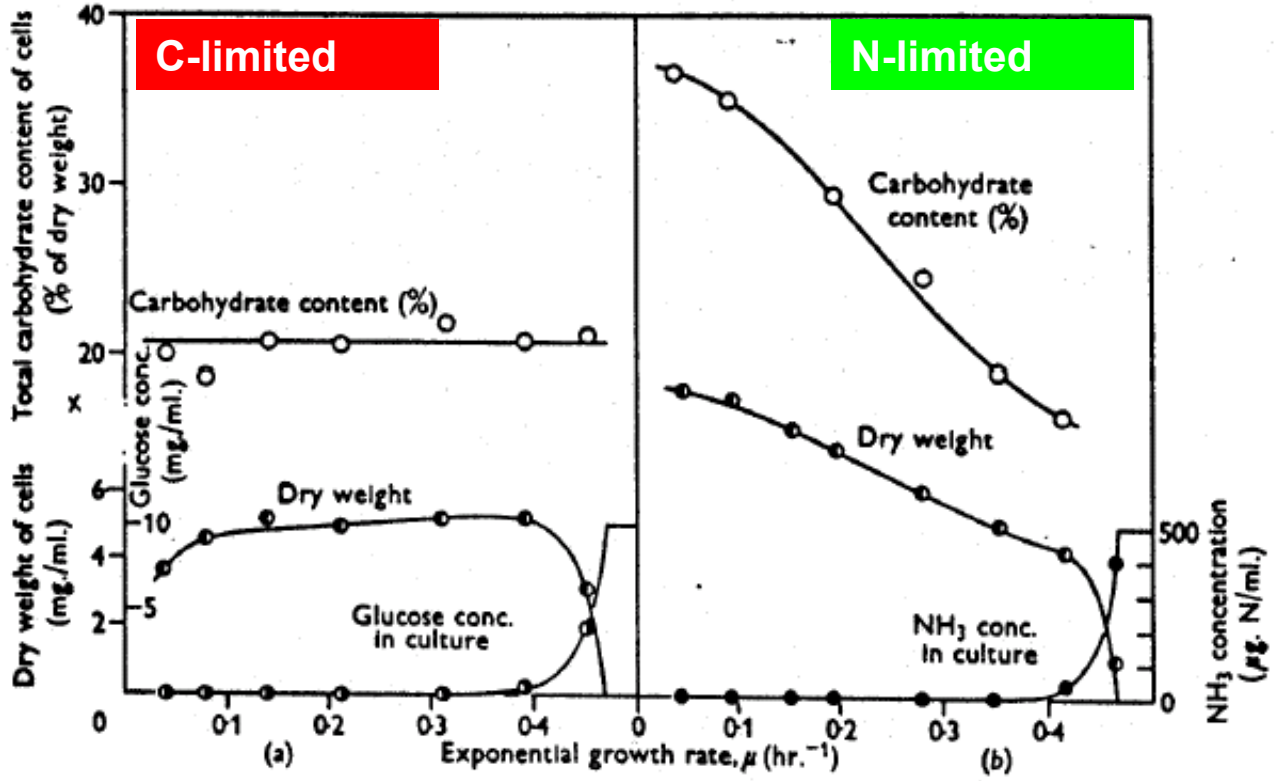


Glucose	Glutamine	Serine	Glucose
Pyruvat	Glucose	Cystein	Ammonium

M. Zinn, B. Witholt, T. Egli, *J. Biotechnol.* **113**, 263-279 (2004).

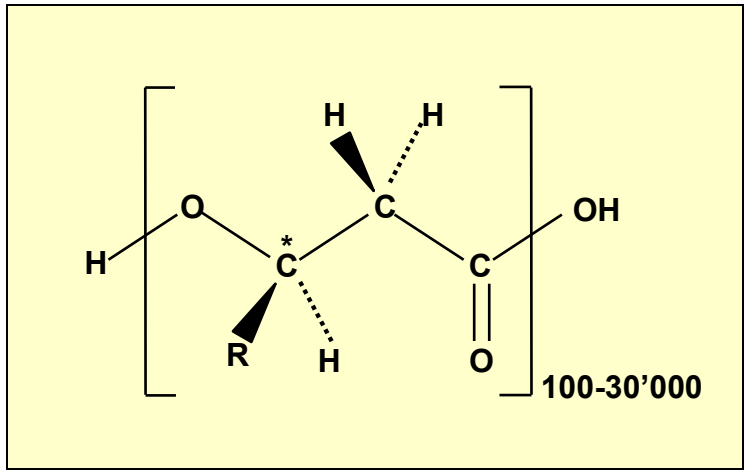
Influence of μ (=dilution rate) on biomass concentration for glucose and ammonium as limiting nutrients

Real data for a fodder yeast (*Candida utilis*)



From: Herbert & Tempest (unpublished data)

Poly([*R*]-3-hydroxyalkanoate) (PHA)



R = C_1-C_2 Short chain length PHA (sclPHA)

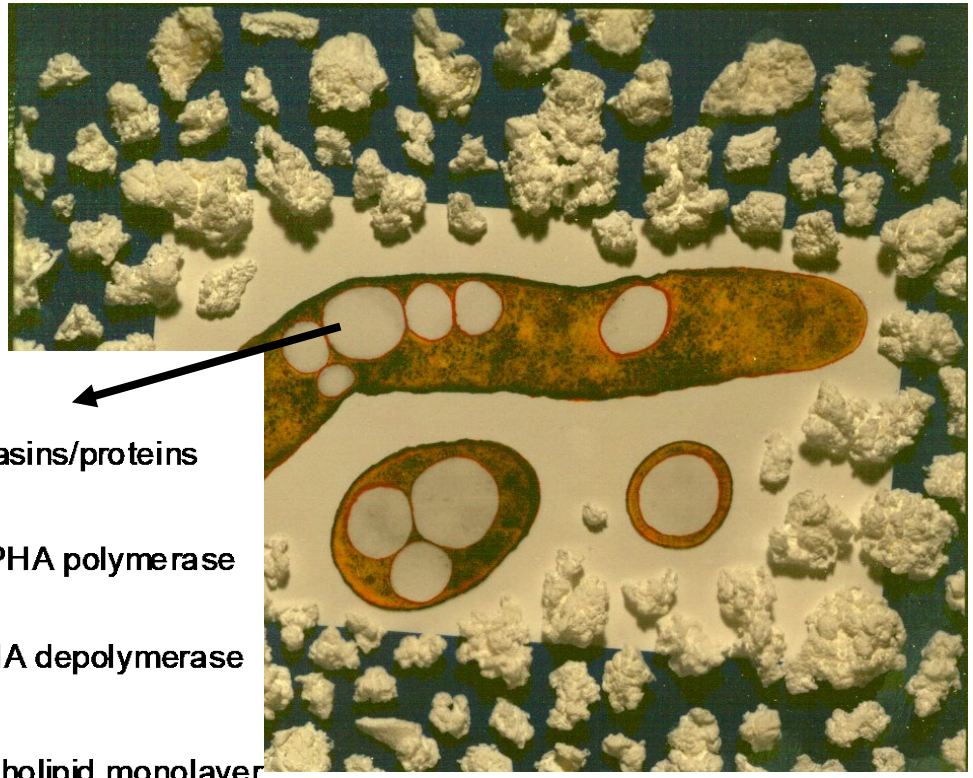
R = C_3-C_9 Medium chain length PHA (mclPHA)

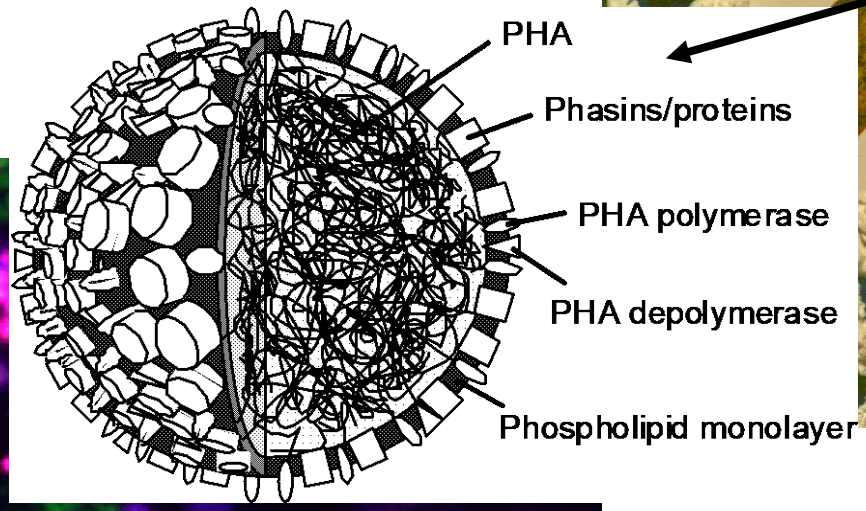
R = C_{10}-? Long chain length PHA (lclPHA)

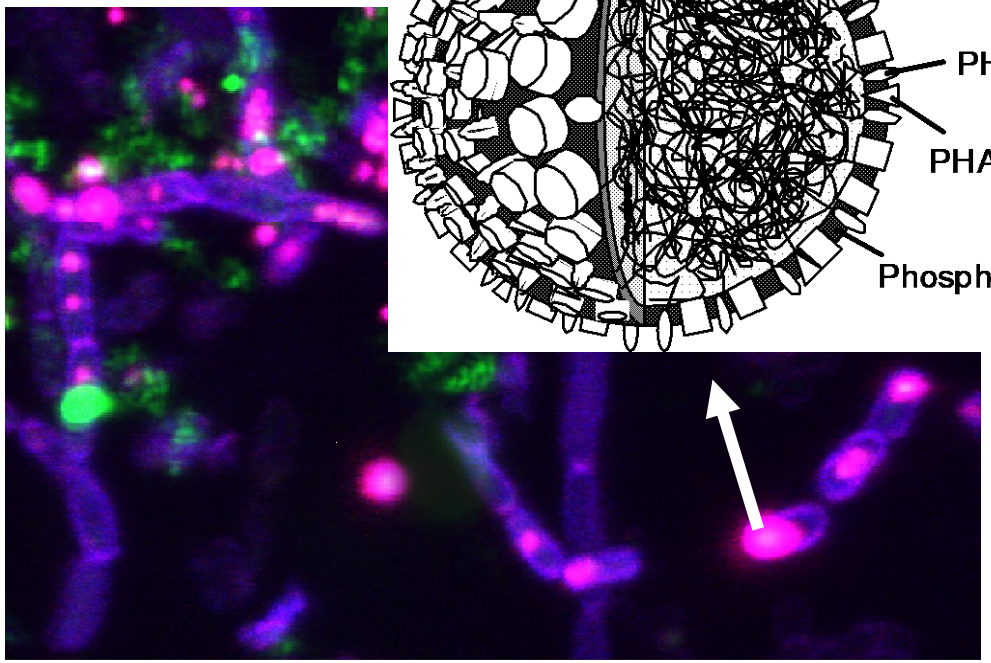
Typical PHA production strains

Cupriavidus necator

scl-PHA





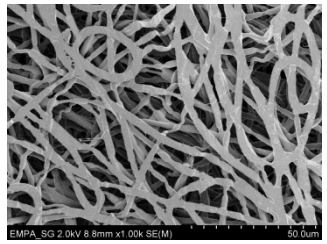


Pseudomonas putida GPo1

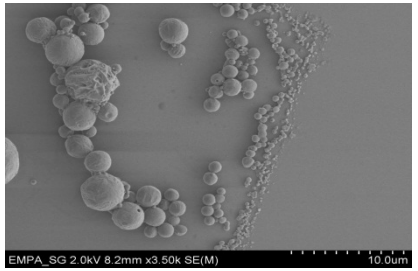
mcl-PHA

Processing of PHA

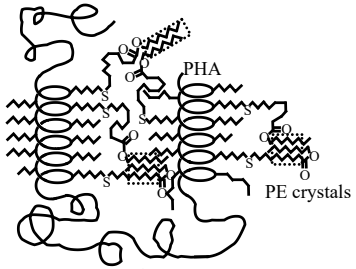
Melt spinning



Electro-spinning

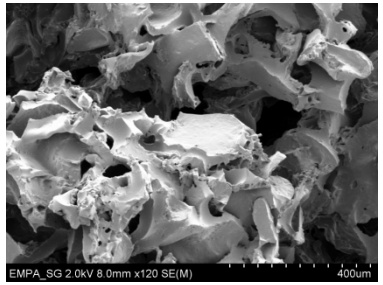


Beads



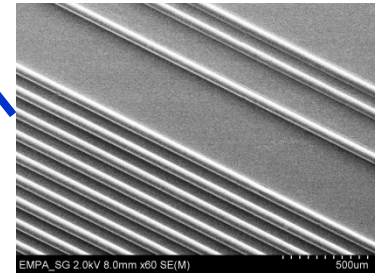
Chemical modificiation

scl-PHA mcl-PHA



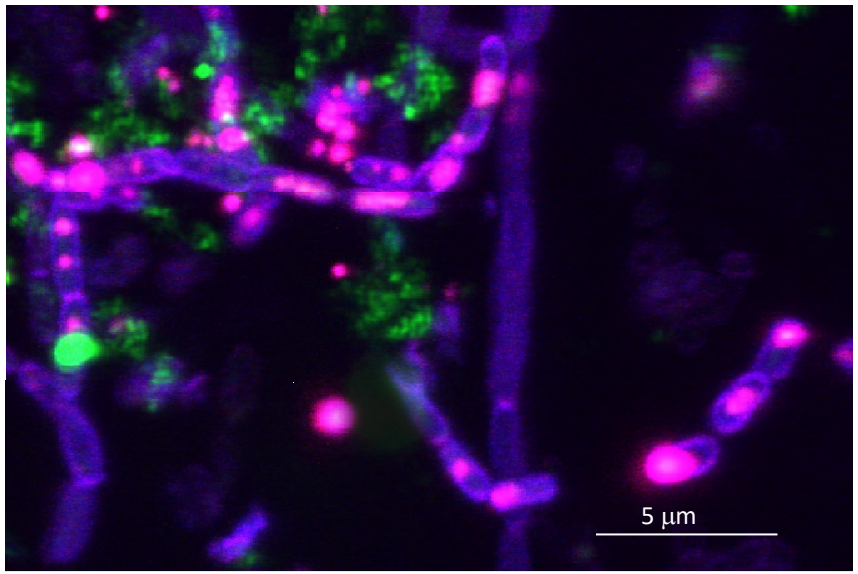
Porous structures

Surface coatings



Defined structures

Biosynthesis of mcl-PHA



Reason for accumulation:

Starvation by a nutrient

Intracellular limitation

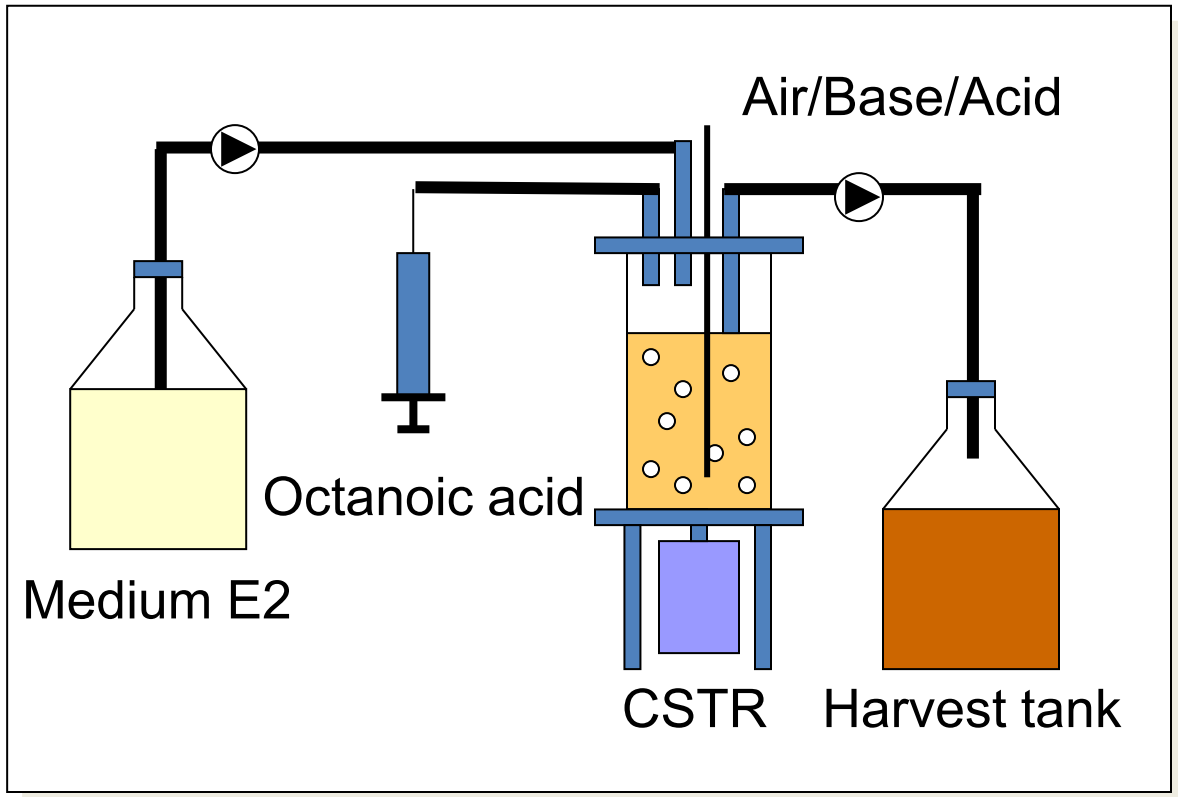
Limitation by a nutrient

Type of PHA accumulation:

Non-growth associated
(Fed-)Batch cultivation

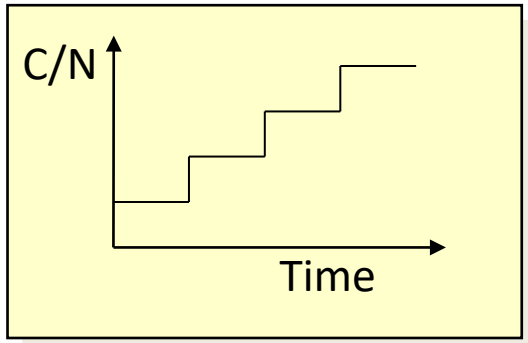
Growth associated
Continuous cultivation

Pseudomonas putida GPo1 in the chemostat



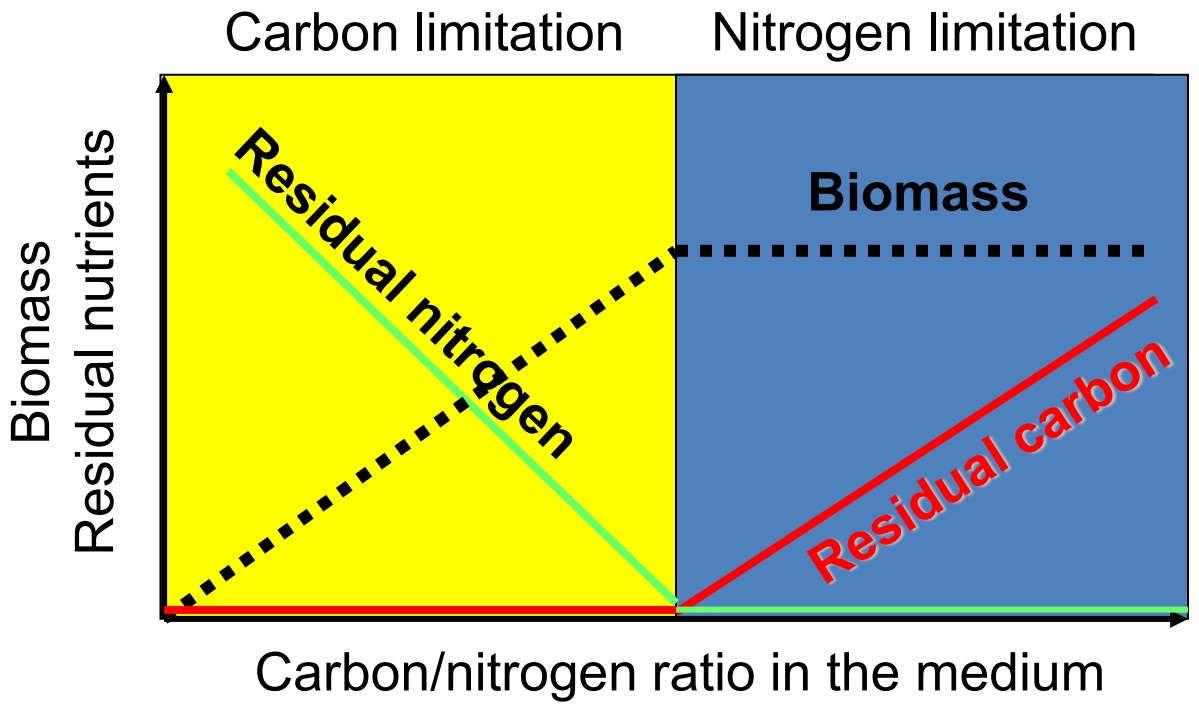
Adjustable parameters:

- pH
- temperature
- aeration
- specific growth rate (D)
- nutrition



You remember the Liebig's law…

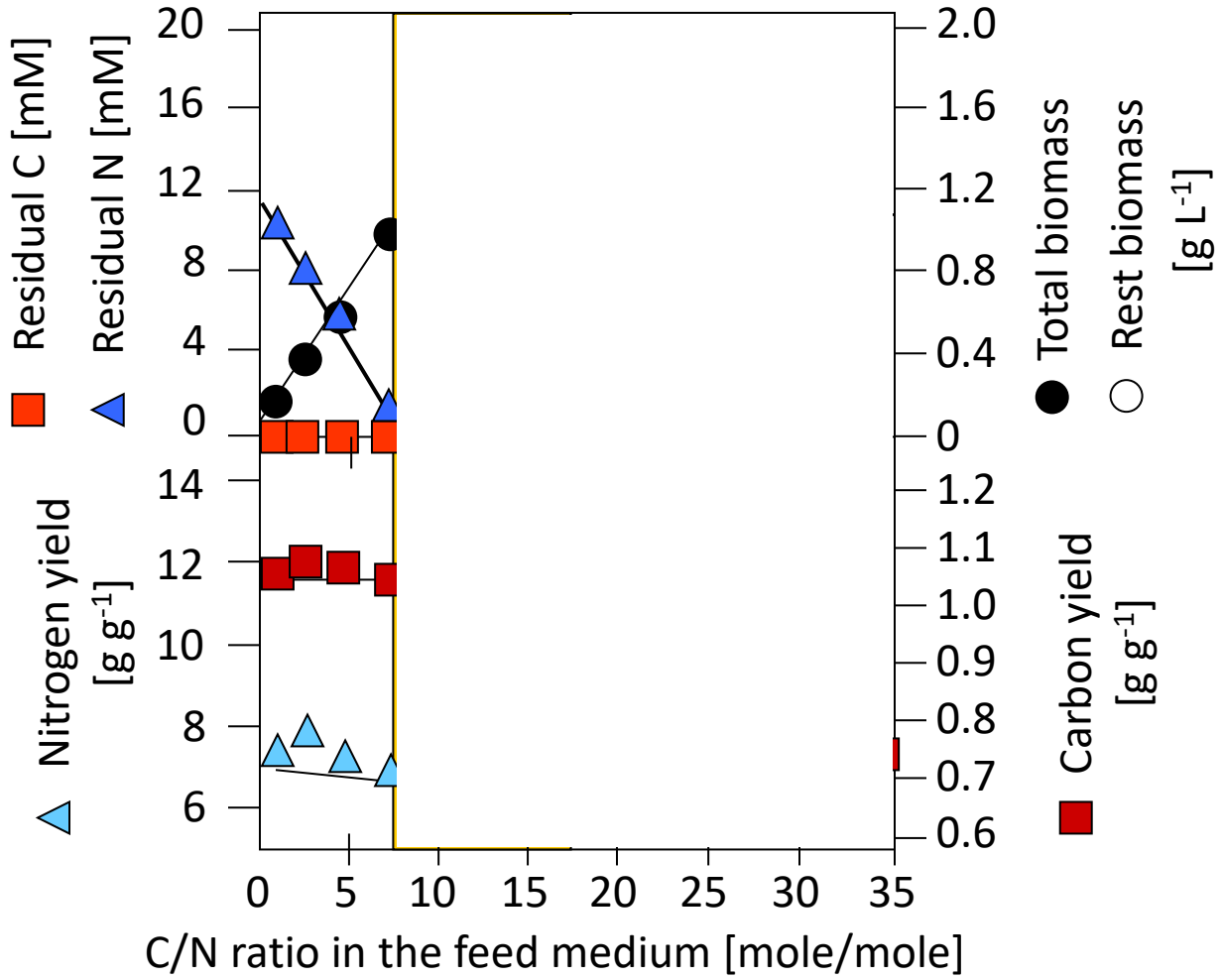
$X = Y_{X/C} * C$ $X = Y_{X/N} * N$



(nitrogen source is kept constant)

Growth in a chemostat at different C/N ratios

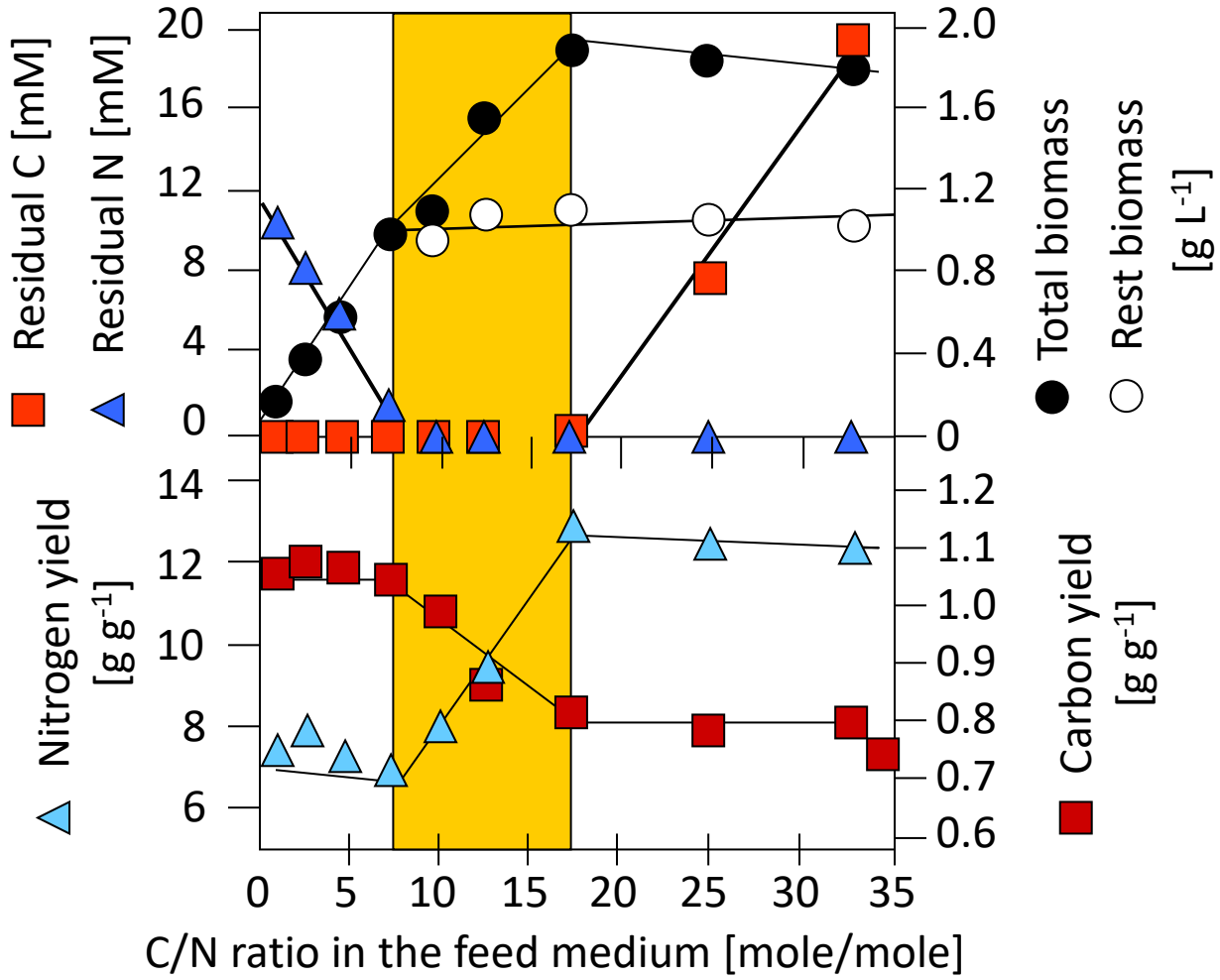
- *Pseudomonas putida* GPo1, D = 0.2 h^{-1}
- Octanoic acid in feed increased, ammonium sulfate constant



Durner *et al.* (2001) *Biotechnol. Bioeng.*72:278-288

Dual (C,N) limited growth in chemostat

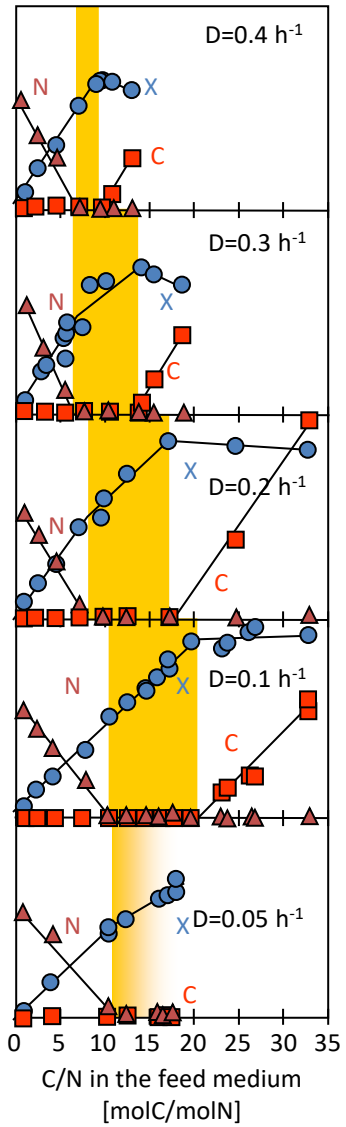
- *Pseudomonas putida* GPo1, D = 0.2 h^{-1}
- Octanoic acid in feed increased, ammonium sulfate constant



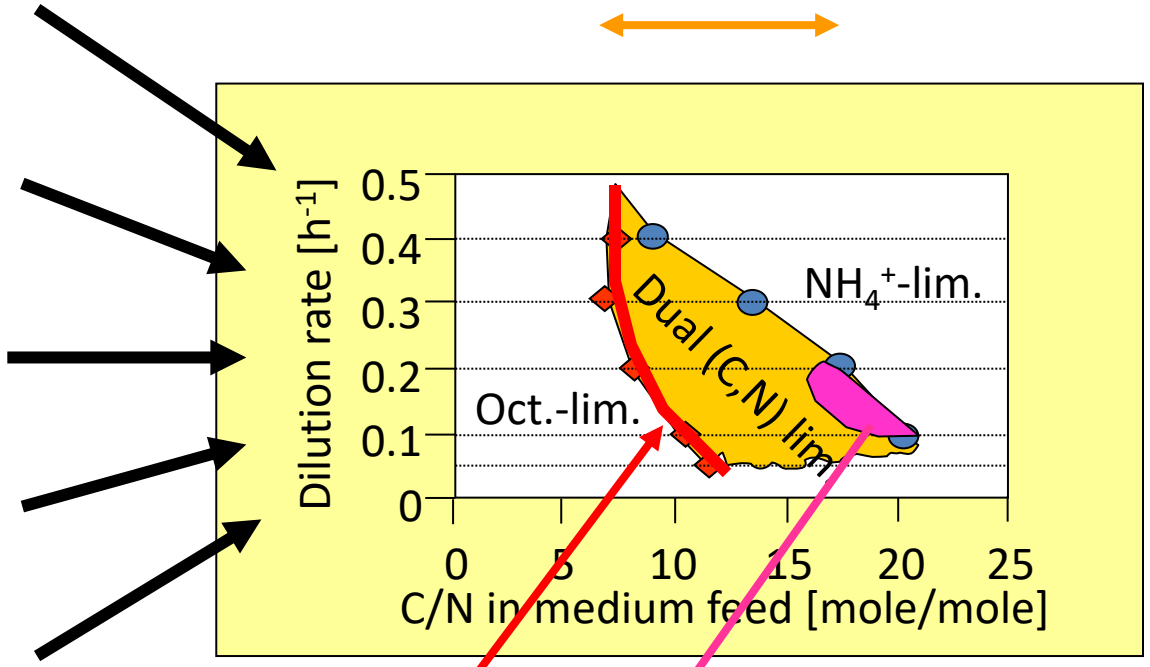
Durner *et al.* (2001) *Biotechnol. Bioeng.*72:278-288

The “Banana” curve of *P. putida* GPo1

C source: Octanoic acid, medium CCMT



Capacity of storing C (PHA)

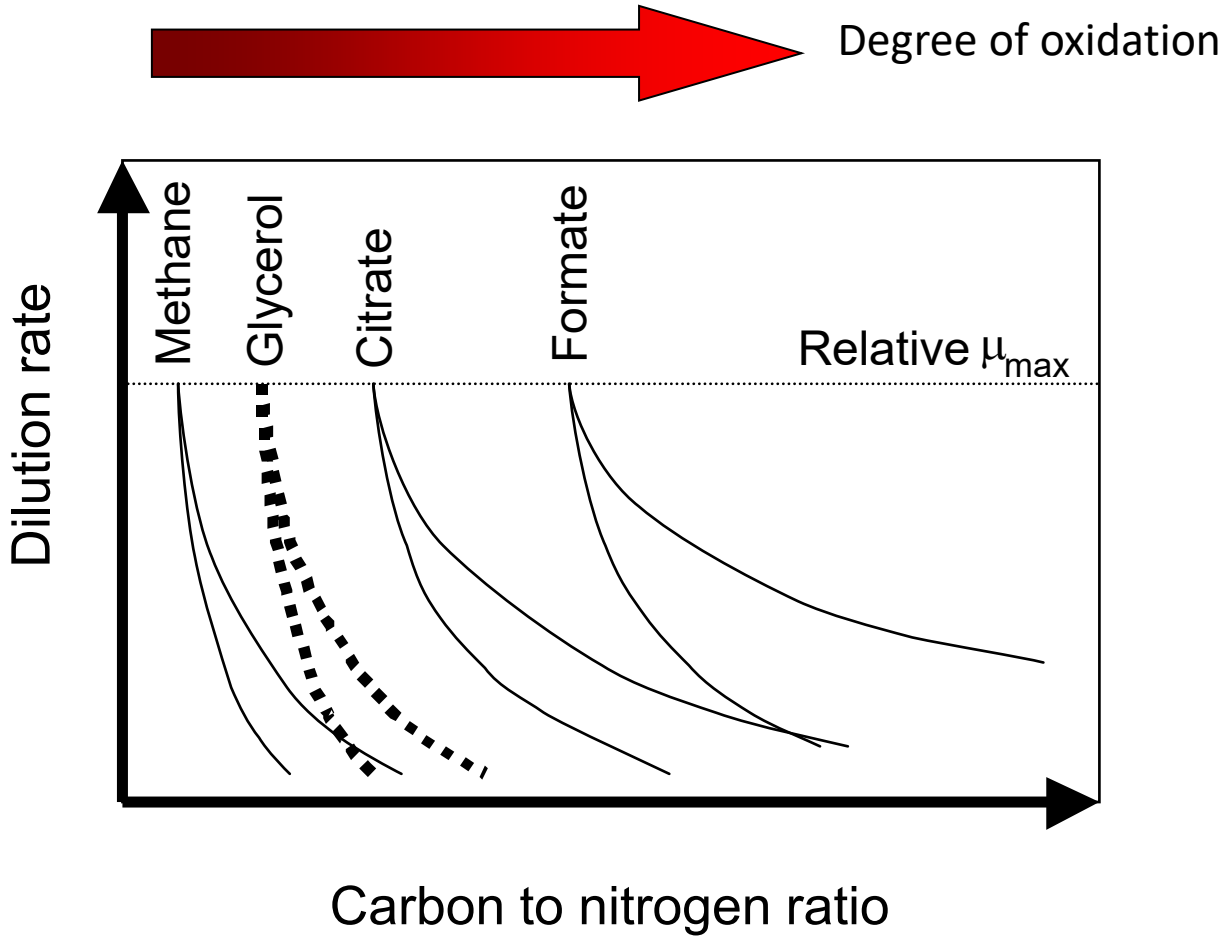


Influence of cell maintenance

Optimal production conditions

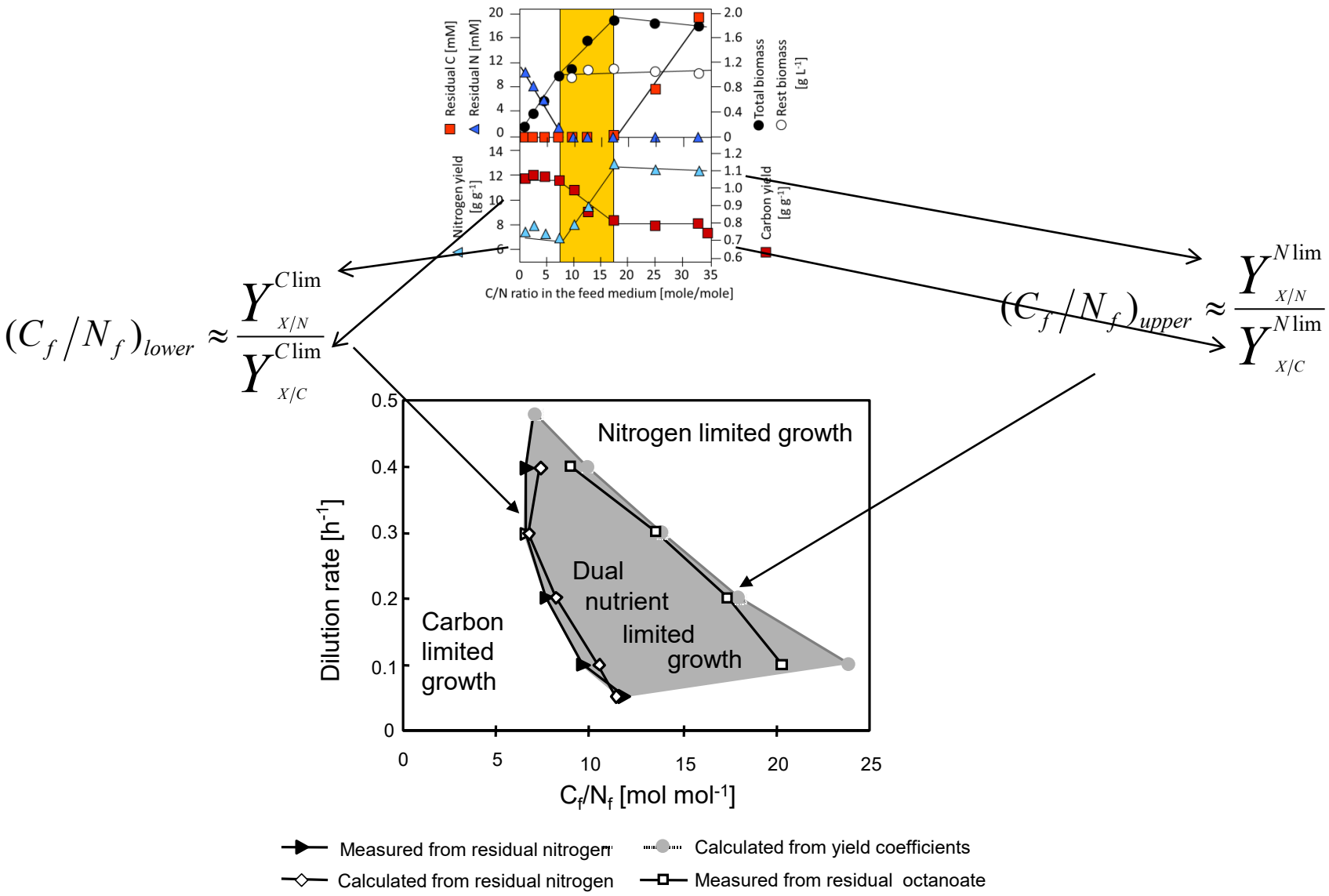
R. Durner *et al.* (2000) *Appl. Environ. Microbiol.* 66:3408-3414

Influence of substrates on the banana curve

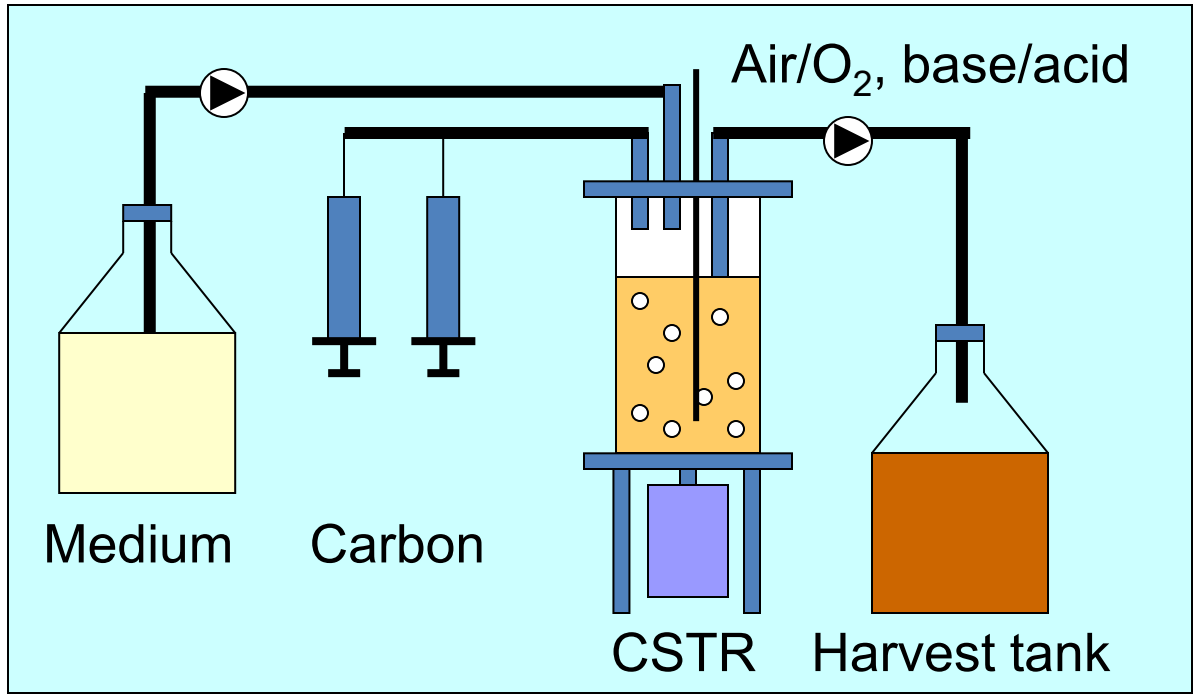


Zinn, M., B. Witholt, and T. Egli (2004) *J. Biotechnol.* 113:263-279.

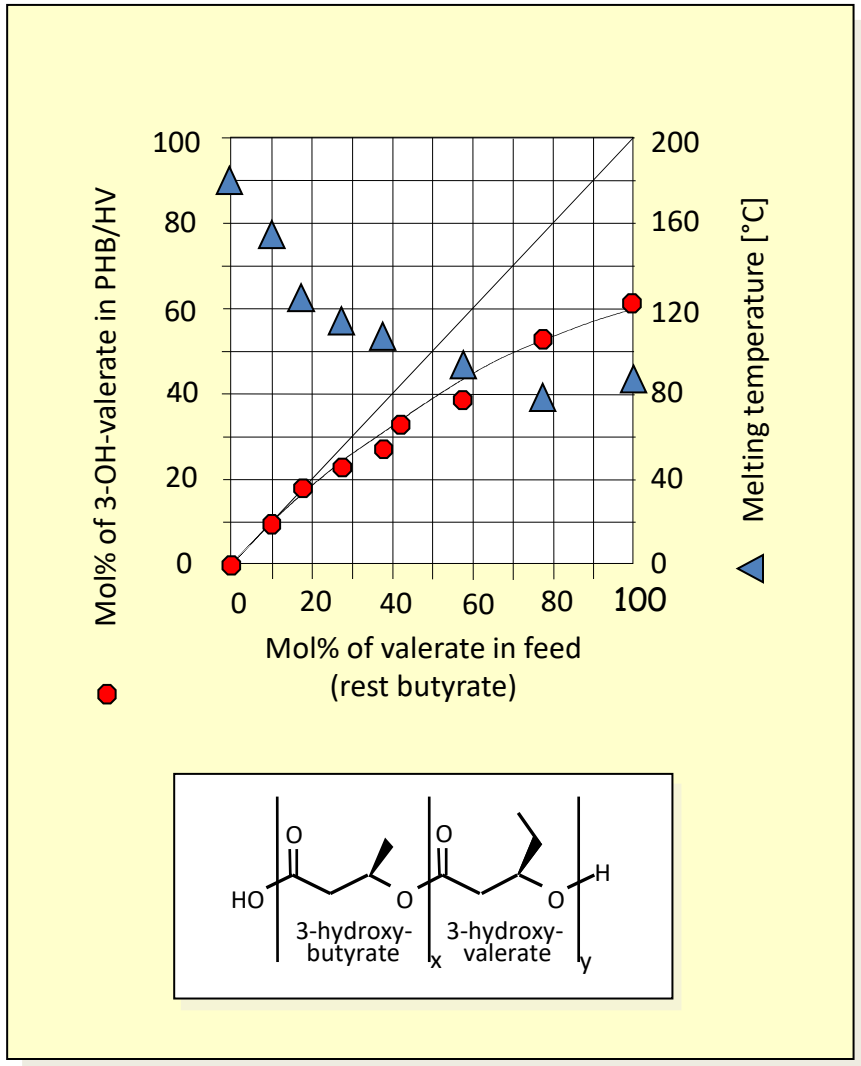
Prediction of the banana curve



Production of tailor-made PHA



Tailored melting temperature



Ralstonia eutropha

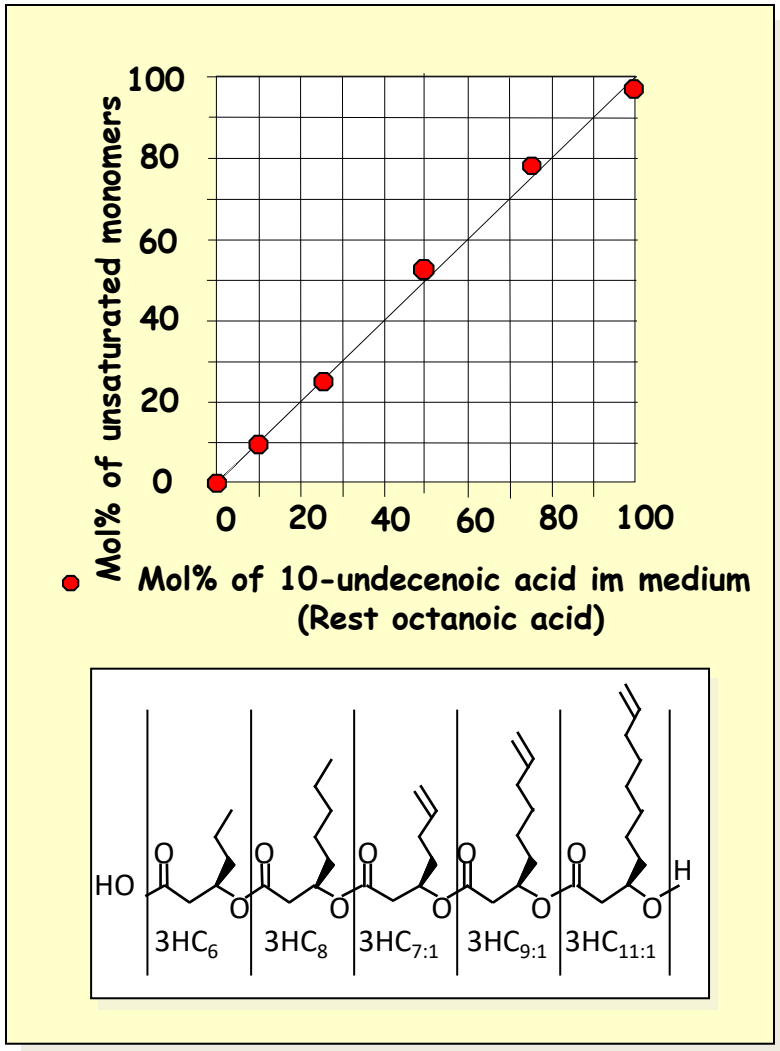
Chemostat (D = 0.1 h^{-1}, C/N = 14 g g^{-1})

C: butyric and valeric acid (C=const.)

N: ammonium sulfate (N=const.)

M. Zinn *et al.* (2003) *Acta Biotechnol.* 23:309-316

Tailored functionality of mclPHA



***Pseudomonas putida* GPo1**

Chemostat (D = 0.1 h^{-1}, C/N = 12 g/g)

C: Octanoic acid, 10-undecenoic acid (C = const.)

N: Ammonium sulfate (N = const.)

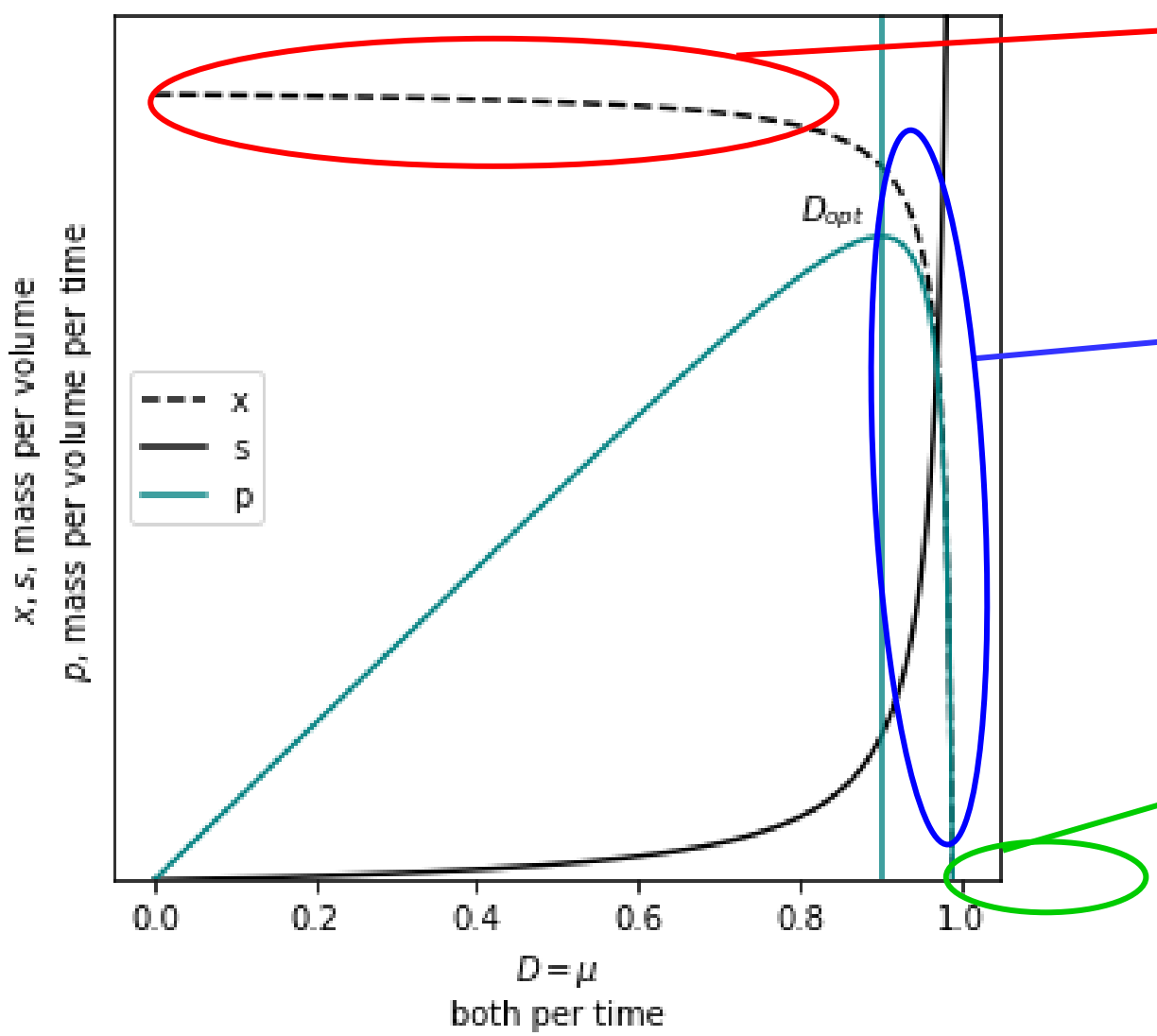
10-Undecenoate [mol%]	M_w	M_w/M_n	T_g [°C]	T_m [°C]	ΔH_m [J/g]
0	286´000	2.4	-33.1	58.1	14.5
10	251´000	1.9	-35.9	50.8	10.2
25	253´000	2.2	-39.5	44.5	7.8
50	290´000	1.9	-44.6	39.9	0.2
75	278´000	2.4	-47.4	-	-
100	290´000	2.4	-49.3	-	-

R. Hartmann, *et al.* (2006) *Biotechnol. Bioeng.* **93**:737-746

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.F_{out}
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) • E.g., cyclic shift of steady state for strain evolution

Stability of steady-states in a chemostat

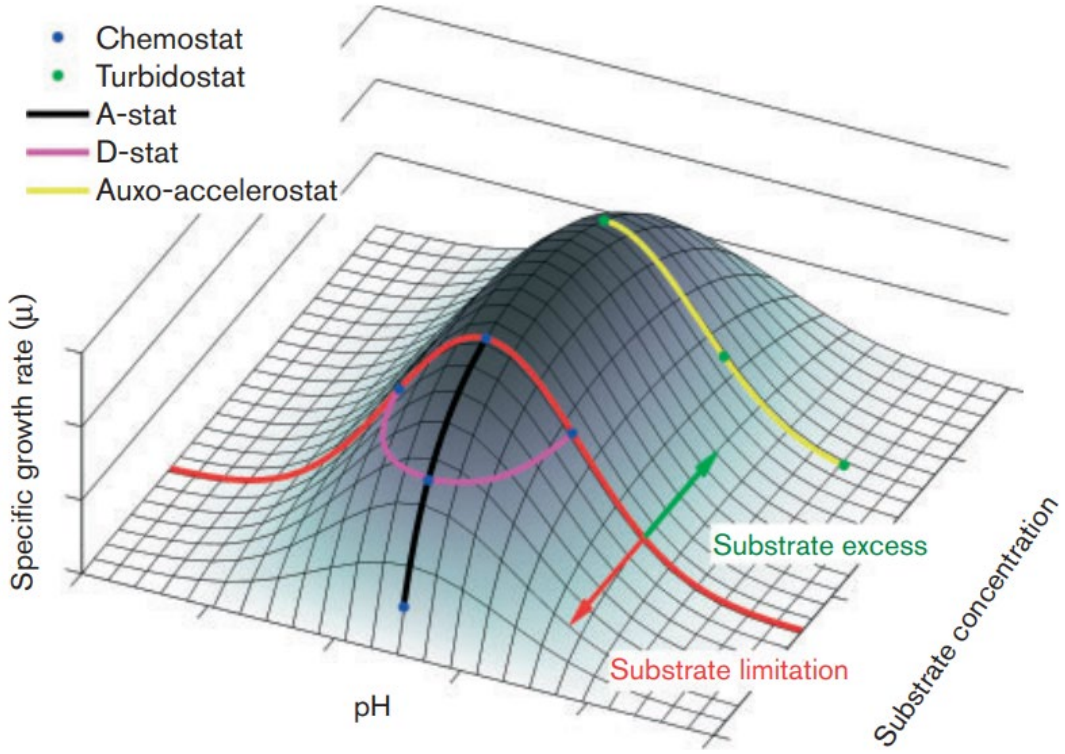


Chemostat

Auxostat

wash-out (unstable for $x > 0$)

Auxostat – Actively controlled continuous cultivations

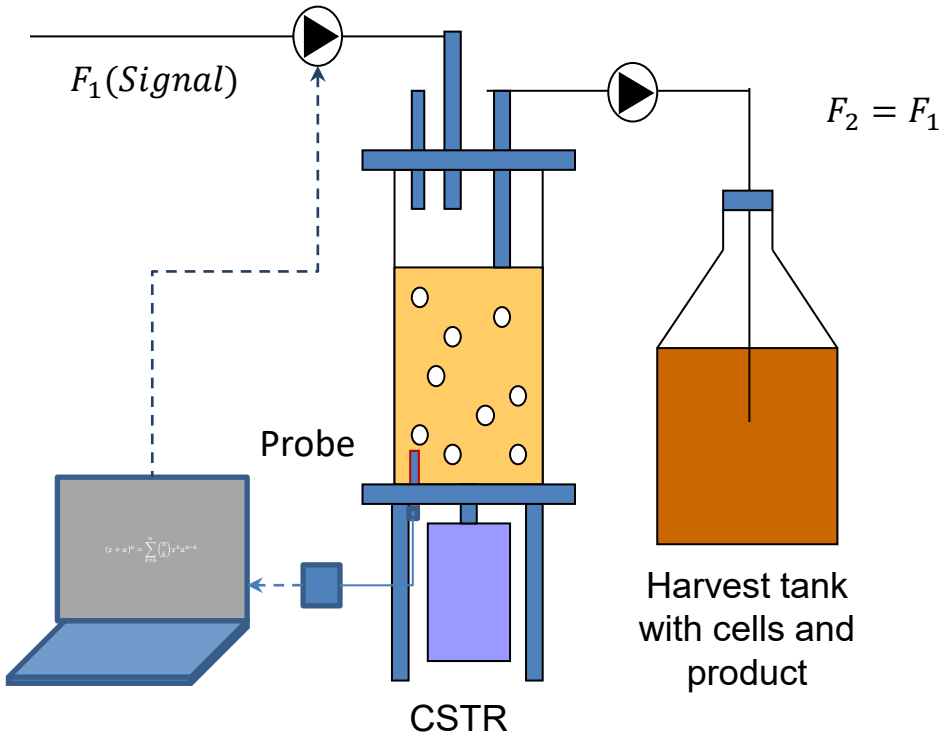


Different realizations of 'Auxo':

- 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

K. Adamberg, K. Valgepea, R. Vilu, Advanced continuous cultivation methods for systems microbiology, Microbiology 161(9) (2015) 1707-1719.

Auxostats in continuous cultures



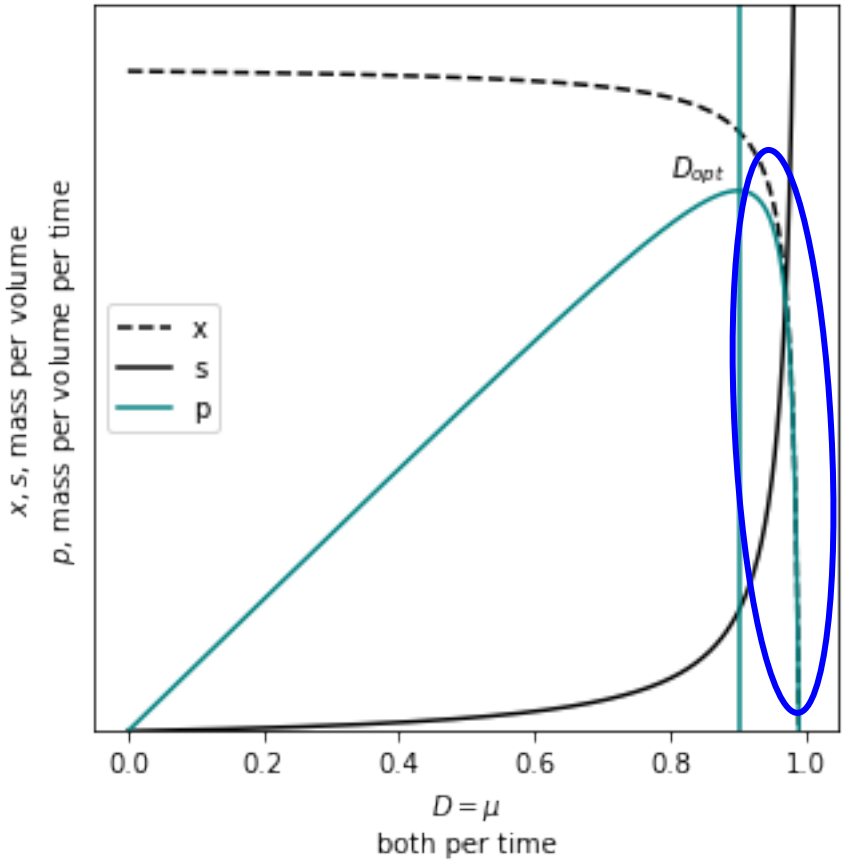
Advantages

- 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

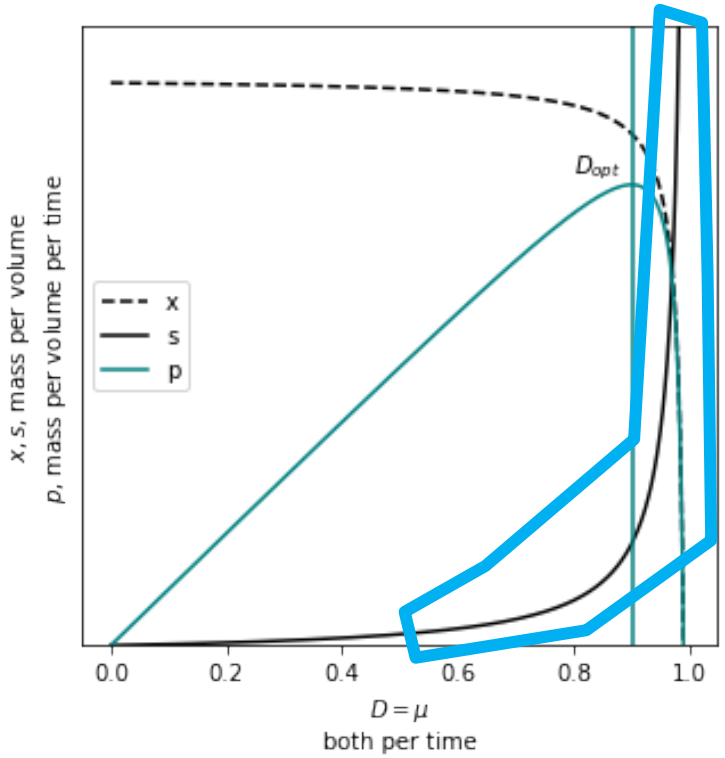


- **Turibdostat** stabilizes the turbidity:
- Assumption: $A_{665\,nm} \propto x$
- $\frac{dA_{665\,nm}}{dt} = 0 \approx \frac{dx}{dt}$

- **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.

Continuous sampling tool for at line analytics

https://www.securecell.ch/en/numera

Cobas, Roche diagnostics
At-line process analytics

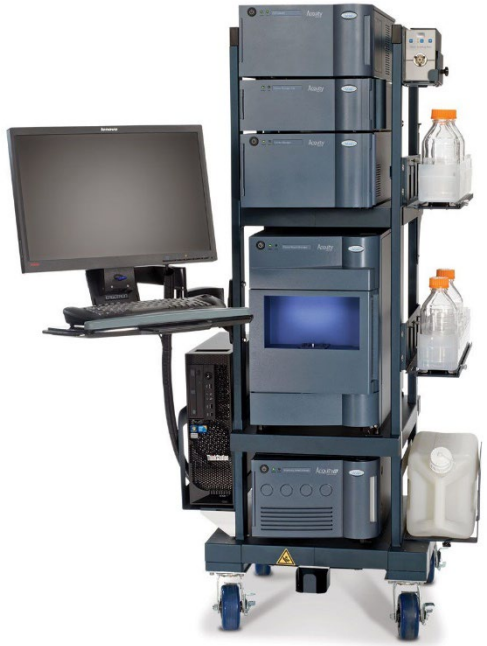
Other technologies:

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

Cobas, Roche diagnostics
At-line process analytics

Other technologies:

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

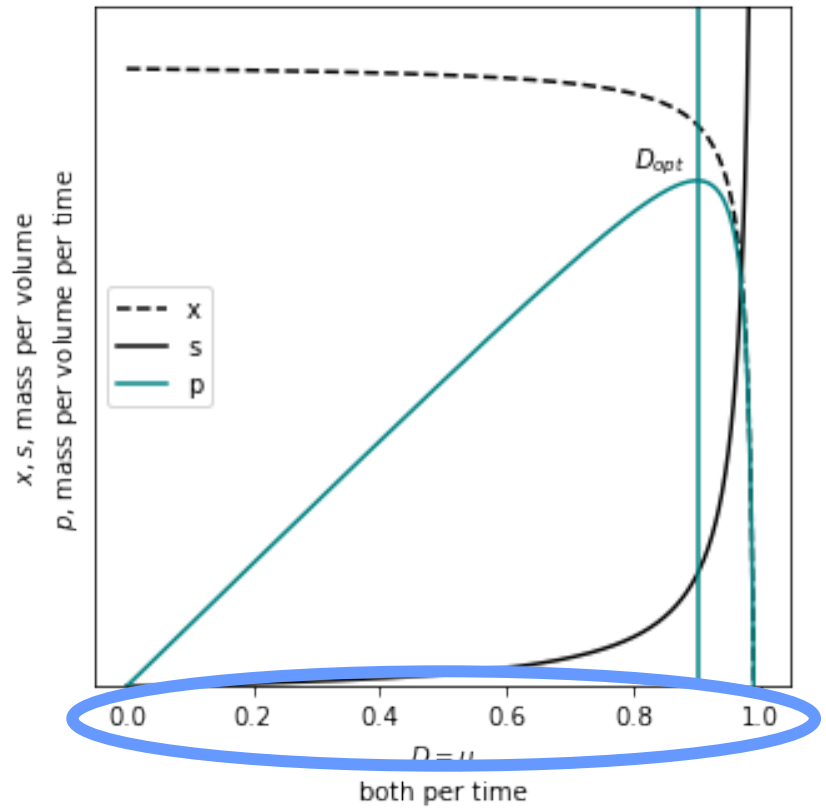


Patrol UPLC, Waters
On-line chromatography

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

pH-auxostat – the mechanism

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

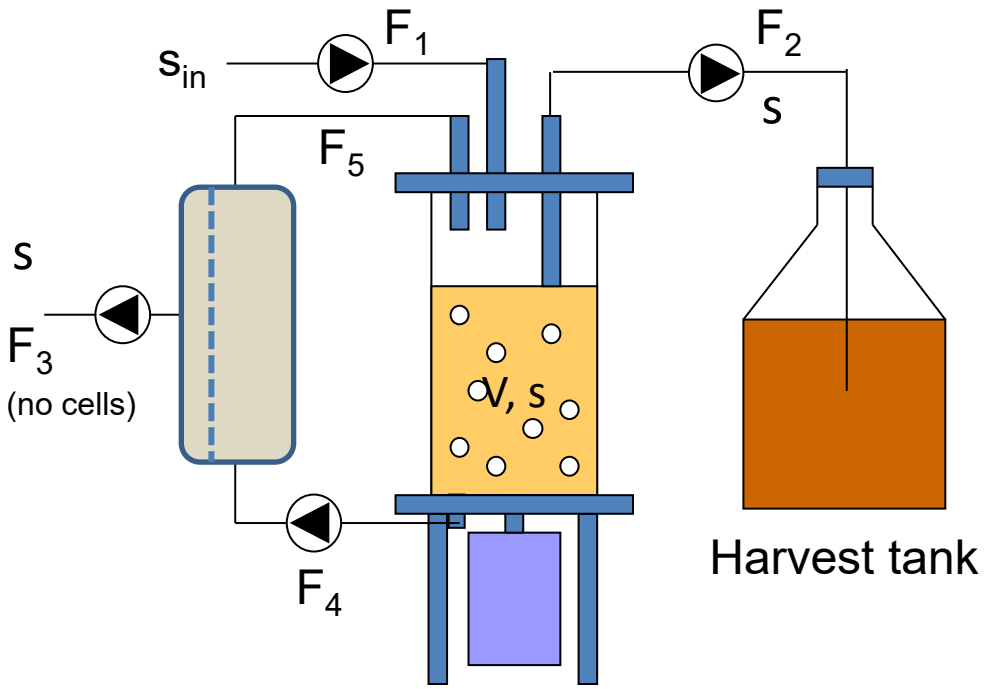


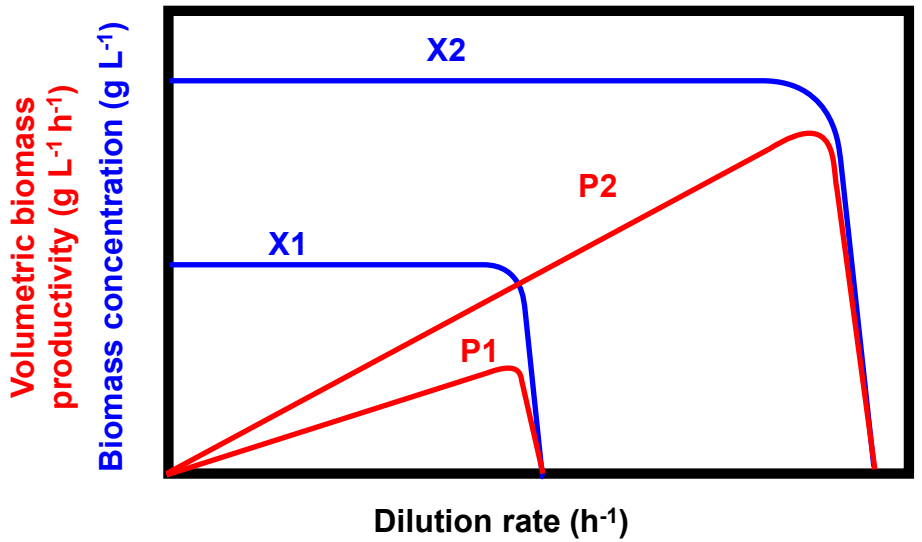
- Assumption: generation or consumption of H^+ is proportional to the growth rate
- pH is kept constant
- Substrate is fed at a rate proportional to the base/acid consumption with a proportionality constant $R > \frac{1}{Y_{H^+/S}}$
- Thus, cells are fed, when they consume substrate

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





Volumetric biomass productivity in chemostat with and without cell recycle.

$$F_1 = F_2 + F_3$$

$$0 \leq R = \frac{F_3}{F_1} \leq 1$$

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

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

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

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

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

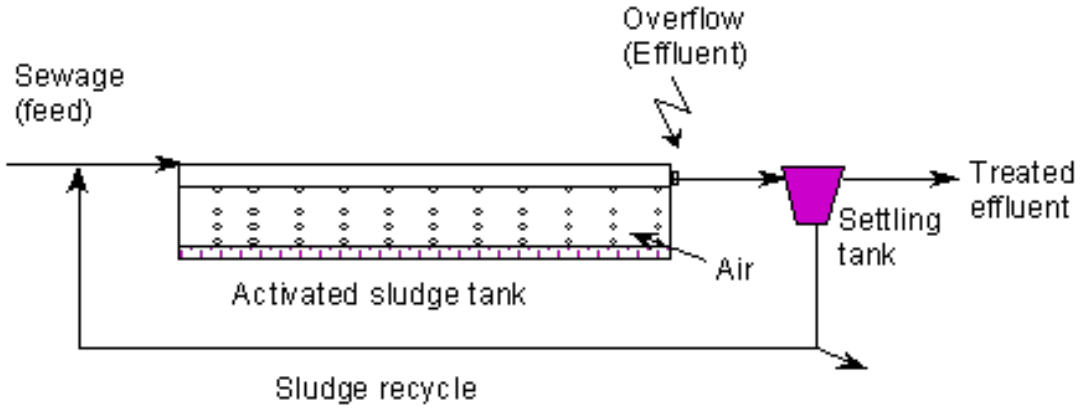
$$\tilde{p} = Y_{p/s}\,(s_{in} - \tilde{s})$$

Note: 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.

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

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 litres per hour and batch reactors simply could not cope with the load.

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

Example for enhancing the productivity with cell recycle

Continuous production of ethanol using a membrane bioreactor:

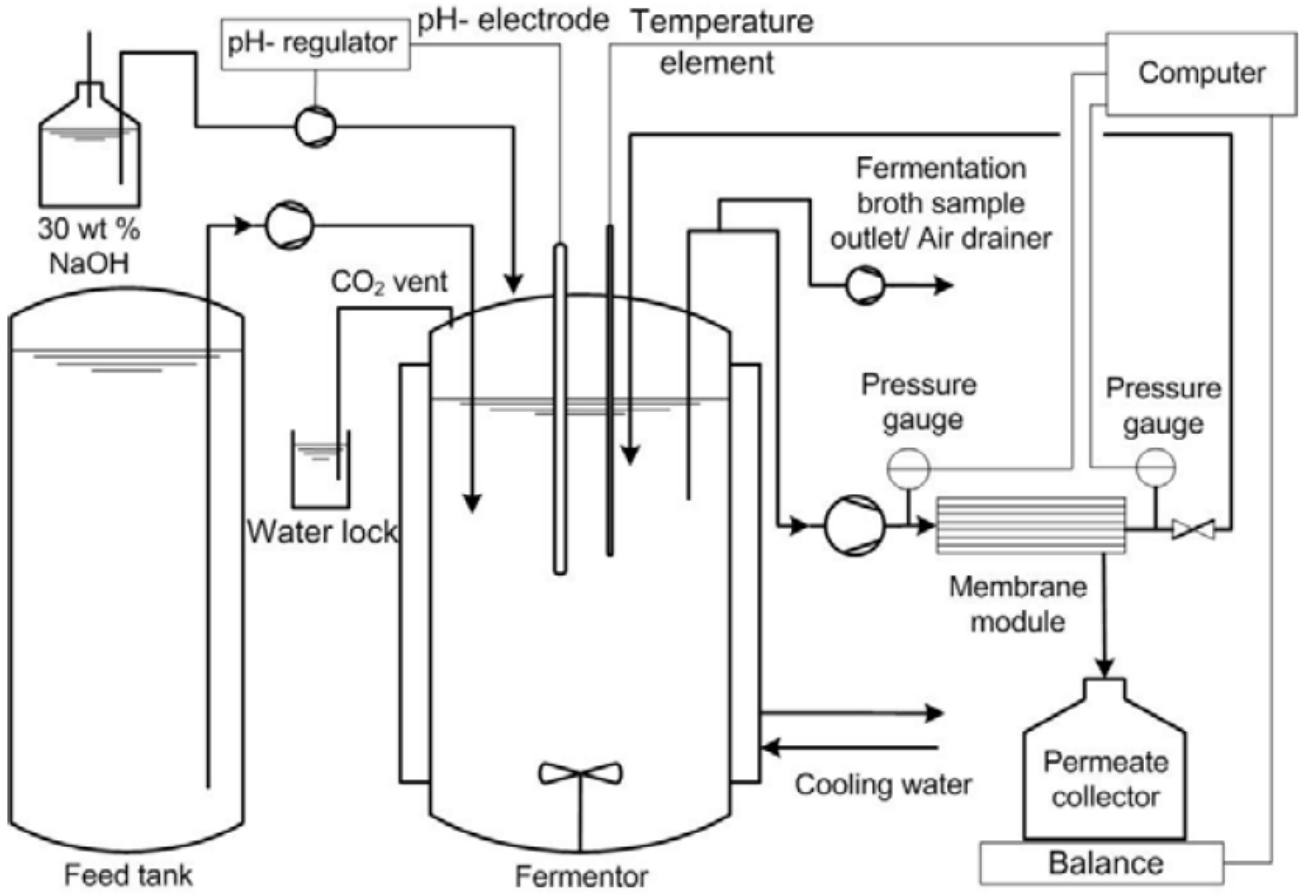
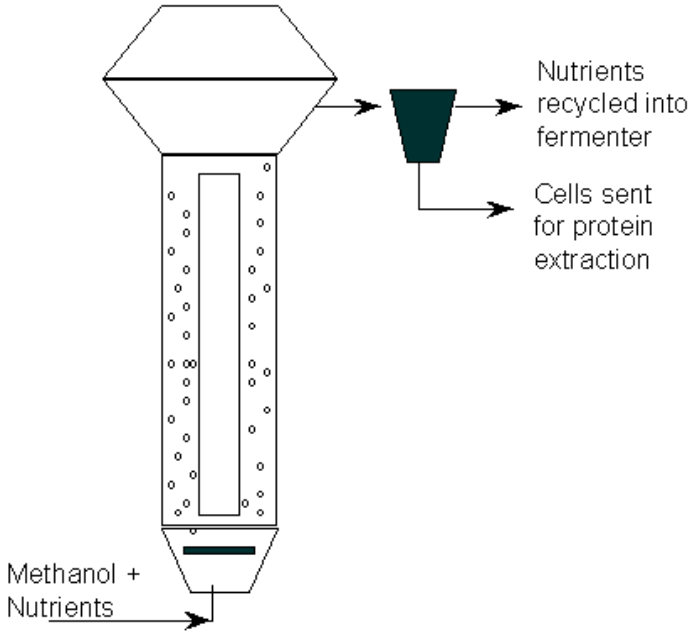


Figure 3. Equipment setup for continuous fermentation with external membranes.

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

Major applications of continuous culture reactors

Applications of continuous cultures in other industries are limited. ICI has used a 1 million-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.

Plug flow reactor

Advantages

- Reactor length corresponds to reaction time
- Works fine for fast reactions

Disadvantages

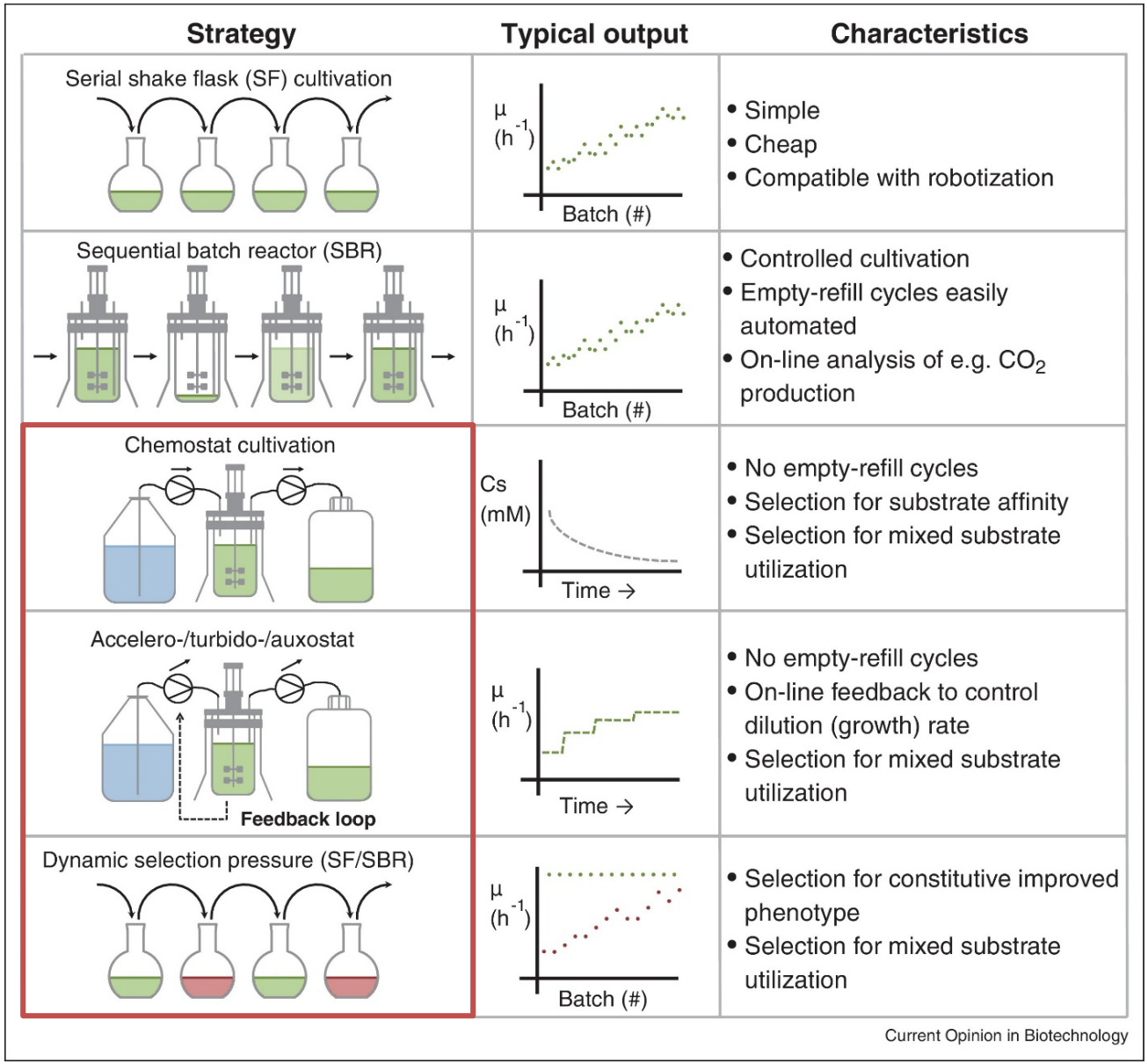
- Difficult to transfer enough oxygen (membrane tubing needed)
- pH cannot be controlled (buffered medium needed)
- Cell adhesion/biofilm formed on the tubing walls (sedimentation)
- Large resistance when small diameter of tubing is selected

https://www.indiamart.com/proddetail/pfr-plug-flow-reactor-4792522630.html

Strain evolution

- Continuous cultures can be used for strain development.
- Objectives: Selection of
  - The fastest growing strain
  - Strain with broad substrate usage
  - Improved stability in adverse conditions
  - Faster accumulation of storage material (e.g., PHAs)
- Additionally, **genetic engineering** provides powerful tools for designed optimization.

How can the different objectives be achieved?

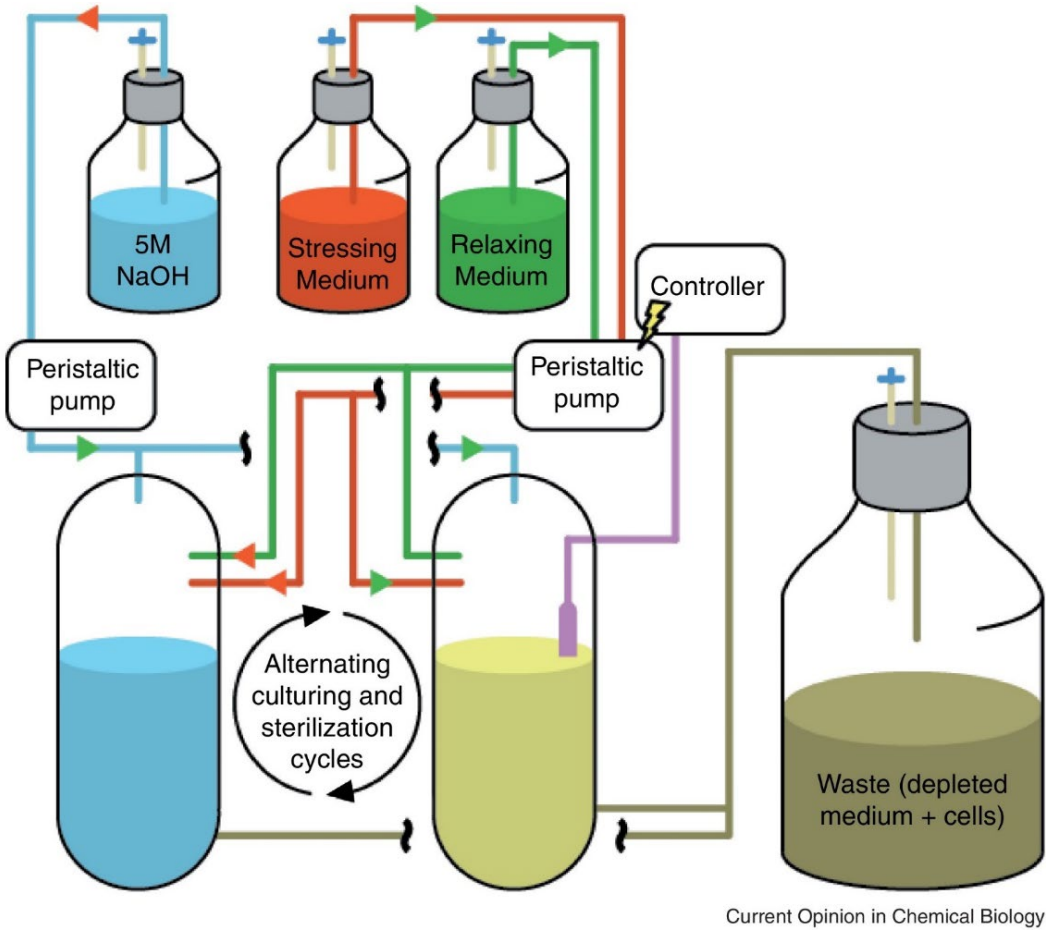


Mans, R., Daran, J. M. G., & Pronk, J. T. (2018). *Current opinion in biotechnology*, *50*, 47-56.

Strain evolution – an example

- A strain should be selected to better accept a stressing medium
- Consequently, it is alternatingly grown on a stressing and a relaxing medium

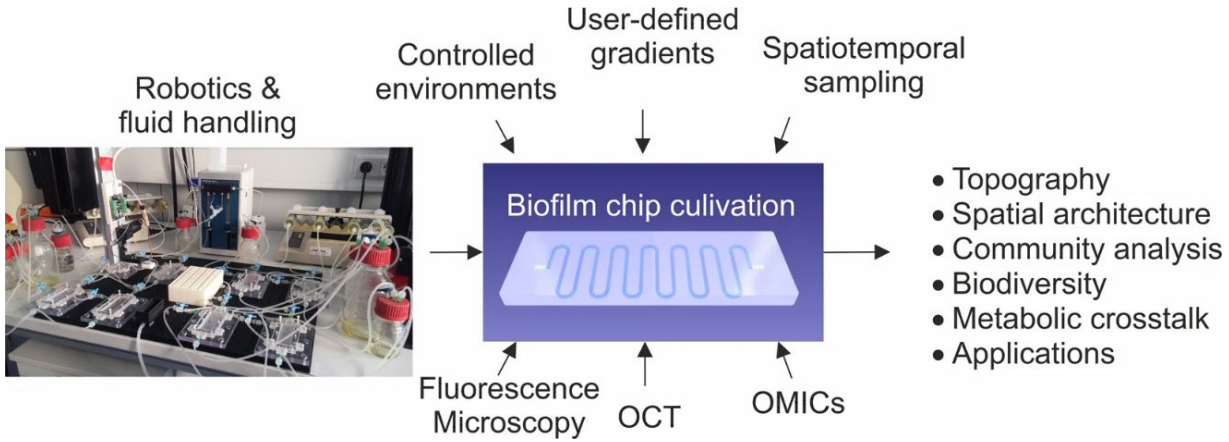
"The GM3 cultivation device. A growing culture in the first vessel is supplemented with a defined ratio of relaxing and stressing medium. This ratio is regulated by a controller connected to a turbidity meter measuring cell density. After a defined growth time, the whole culture is transferred to the second vessel to limit selection escape through biofilm formation. Following this transfer, the first vessel is sterilized with 5 M NaOH and washed extensively to limit contamination. After another defined growth period in the second vessel, the growing culture is transferred back to the first vessel, and the cycle is repeated. The selection stringency is modulated through the ratio of the relaxing and stressing media, ultimately reaching up to 100% stressing medium. The depleted medium and cells are pumped to a waste container."

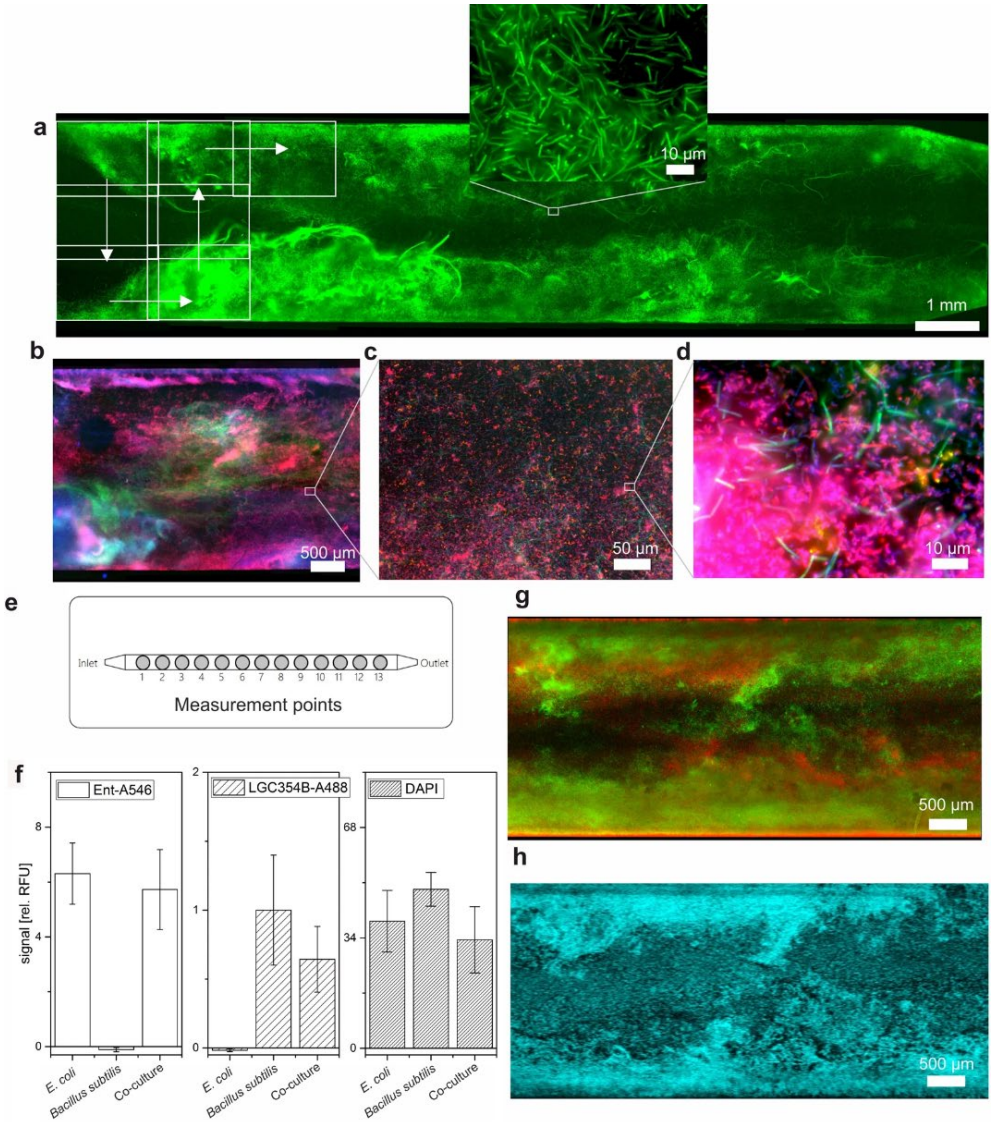


https://www.sciencedirect.com/science/article/pii/S1367593114001446

Biofilm bioreactor

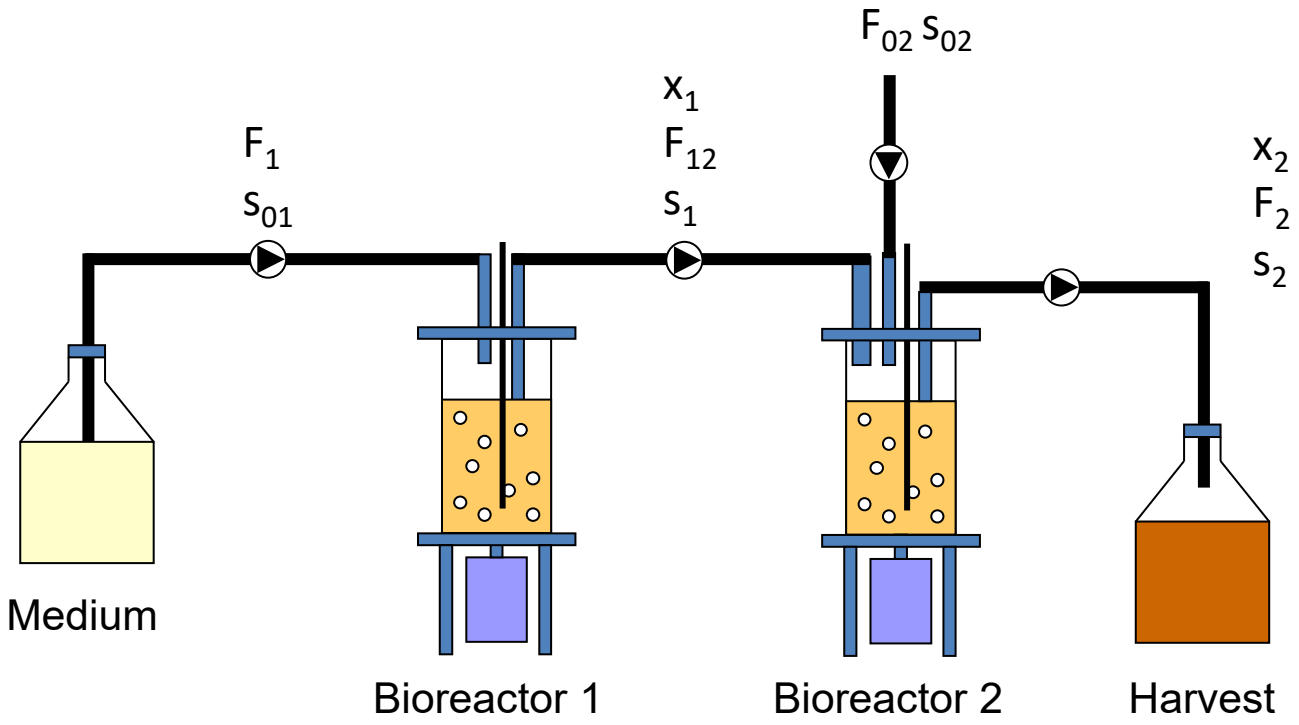
- Continuous cultivations provide unique opportunities for mimicking natural systems.
- Here: investigation of biofilms in microchannels
  - Automated platform for liquid flow, sampling and measurements
  - *E. coli* (green) and *S. subtilis* (red) were marked by fluorescent stains





Hansen, S. H., Kabbeck, T., Radtke, C. P., Krause, S., Krolitzki, E., Peschke, T., ... & Niemeyer, C. M. (2019). Machine-assisted cultivation and analysis of biofilms. *Scientific Reports*, *9*(1), 1-10.

Two-stage chemostat theory



$$D_2 = \frac{(F_{02} + F_{12})}{V_2} = \frac{F_{02}}{V_2} + \frac{F_{12}}{V_2} = \underbrace{D_{02} + D_{12}}$$

Partial dilution rates

Biomass balance in a two-stage chemostat

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

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

$$\frac{dx_2}{dt} = 0$$ During steady-state

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

$$\mu_2 = D_2 - \frac{D_{12} x_1}{x_2}$$ Consequently $\mu_2 < D_2$

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

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

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

during steady-state:

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

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

Substrate balance in a two-stage chemostat

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

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

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

With the solution:

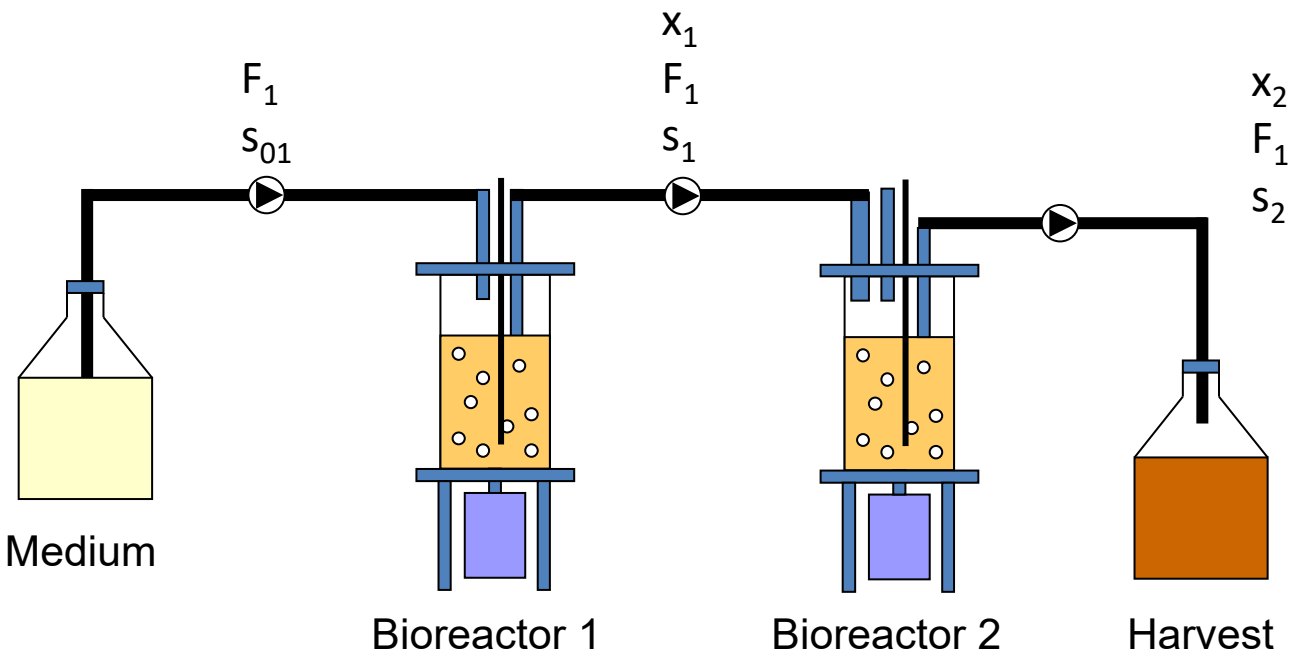
$$s_2 = \frac{-b - \sqrt{(b^2 - 4ac)}}{2a}$$

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

$$-b = \frac{\mu_{\max}D_{12}s_{01}}{D_2} + \frac{(\mu_{\max} - D_2)D_{02}s_{02}}{D_2} - D_{12}s_1 + K_sD_2$$

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

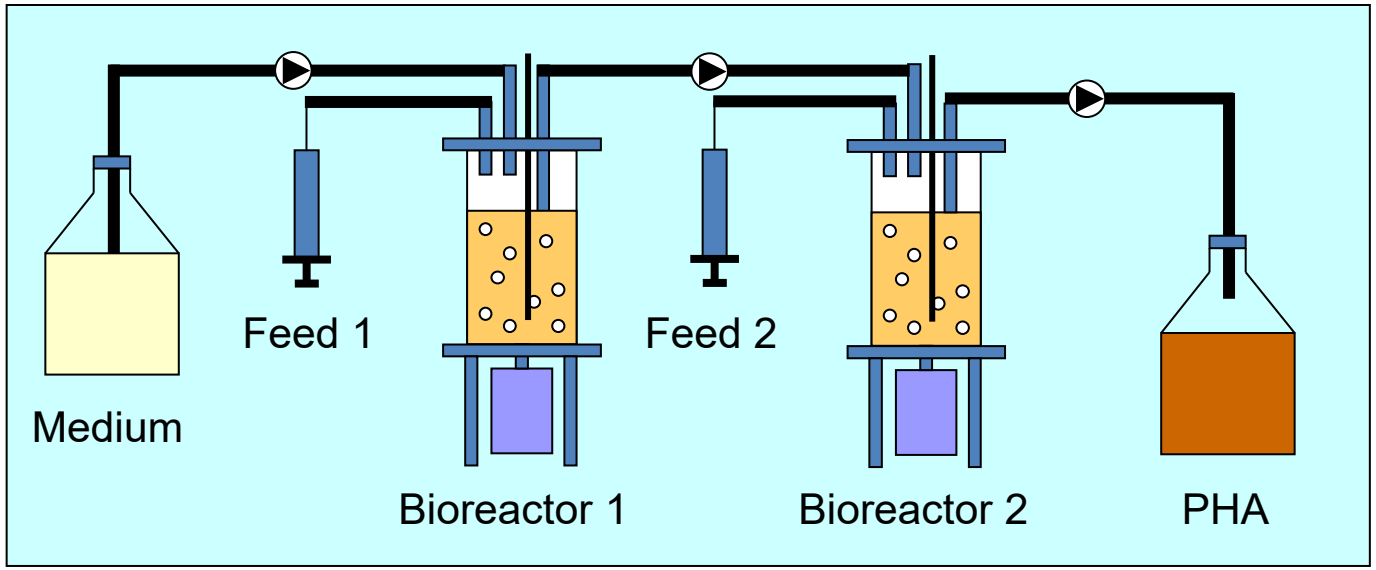
Simplified two-stage chemostat



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

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

Two-stage chemostat in praxis



R. Hartmann *et al.*, *Biomacromolecules*, 2010, 11 (6), pp 1488–1493

Two-stage chemostat with *P. putida* GPo1

Experimental conditions								
Fermentation	**A**		**B**		**C**		**D**	
Bioreactor	1	2	1	2	1	2	1	2
Dilution rate (h^{-1})	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.2
C/N (g g^{-1})	10.9[a)]	20.7[b)]	13.4[a)]	19.8[b)]	15.9[a)]	19.1[b)]	9.7[a)]	14.7[b)]
Biomass and PHA production								
Cell dry weight (CDW) (g L^{-1})	1.13	1.53	1.26	1.48	1.35	0.78	1.22	1.32
PHA content during steady state (% CDW)	24.2	52.4	32.6	50.3	31.4	53.8	22.7	38.8
PHA composition [mol%]								
3-Hydroxyalkanoate								
3-hydroxyoctanoate	85.6	16.8	86.8	30.5	84.7	58.5	83.7	33.8
3-hydroxyhexanoate	14.4	2.6	13.2	4.9	15.3	9.1	16.3	6.2
Σ 3-hydroxyalkanoates	**100**	**19.4**	**100**	**35.4**	**100**	**67.6**	**100**	**40**
3-Hydroxyalkenoates								
3-hydroxy-10-undecenoate	0	24.1	0	19.1	0	8.9	0	19.6
3-hydroxy-8-nonenoate	0	48.0	0	38.1	0	19.8	0	35.8
3-hydroxy-6-heptenoate	0	8.5	0	7.4	0	3.7	0	4.6
Σ 3-hydroxyalkenoates	**0**	**80.6**	**0**	**64.6**	**0**	**32.4**	**0**	**60**

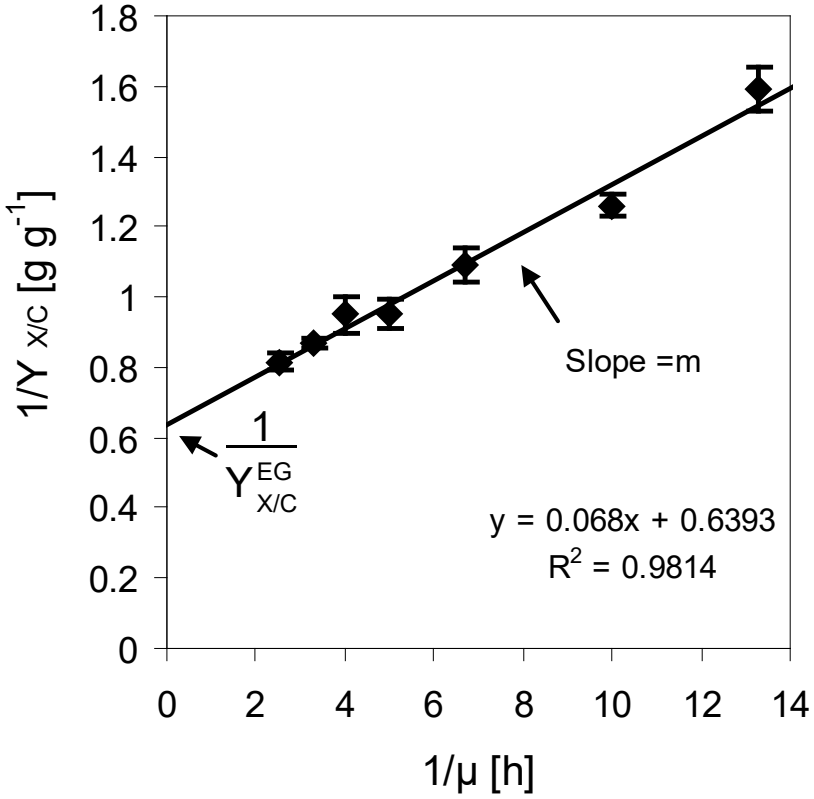
What's the reason for the decrease in biomass?

a) Cells were supplied with minimal medium (N_0 = 150 mg N L^{-1}) and octanoic acid in R1.
b) Culture broth transferred from R1 was supplied with only 10-undecenoate which lead to a higher C_0/N_0 ratio in R2 (N_0 = 150 mg L^{-1}).

R. Hartmann *et al.*, *Biomacromolecules*, 2010, 11 (6), pp 1488–1493

Pirt parameters on octanoic acid

$$\frac{1}{Y_{X/S}} = \frac{1}{Y^{G}} + \frac{m_s}{\mu}$$



Cell maintenance energy cannot be neglected!

63.9 mgC g^{-1} h^{-1}

R. Hartmann *et al.*, *Biomacromolecules*, 2010, 11 (6), pp 1488–1493

By now you ought to be the master of the …

…happy fermentations!

- The chemostat cultivation technique is the most controlled way to study cell physiology at a specific growth rate during steady-states.
- The volumetric productivity of a chemostat is always larger than repetitive batch cultures.
- The law of the minimum is not valid for boundary zones between two nutrient limitations where the cell composition is changing.
- Multiple nutrient limited growth can be used to tailor material properties of the bioplastic polyhydroxyalkanoate.
- Automated control of nutrient supply as an auxostat enables fast growth without wash-out.

Task 19

Steady-state concentration in a chemostat

Zymomonas mobilis is used for a chemostat culture in a 60 m^3 fermenter. The feed contains 12 g L^{-1} glucose; Ks for the organism is 0.2 gL^{-1}.

a) What flow rate is required for a steady-state substrate concentration of 1.5 g L^{-1}?

b) At the flow rate of (a), what is the cell density?

c) At the flow rate of (a), what concentration of ethanol is produced?

Following is known:

$Y_{X/S}$ = 0.06 g g^{-1}; $Y_{P/X}$ = 7.7 g g^{-1}; μ_{max} = 0.3 h^{-1}; K_s = 0.2 g L^{-1};
s_0 = 12 g L^{-1}; V = 60 m^3

Task 20

Application of chemostat

A bacterium is used to absorb (uptake) uranium from contaminated water in a continuous process operated at steady state. It incorporated uranium into cell mass, which is then removed from the bioreactor as solid waste. The advantage of the process is in converting a diluted contaminated water into a solid form for easier disposable.

Methanol is added to the feed water stream at a concentration of 5 g/l, which also contains 10 mg/l of uranium. The biomass yield on methanol is 0.6 g dry mass/g methanol. The Ks for methanol is 0.1 g/l, while the maximum specific growth rate is 0.345 hr^{-1}. The absorption of uranium follows zero order kinetics with respect to uranium concentration. The absorption kinetics can thus be described as R=-kx

Where R is the absorption rate and x is the cell concentration. Because of the environmental concern the discharge methanol concentration has to be no higher than 0.05 g/l, while the target uranium concentration at discharge is 0.5 mg/l. It has been assumed that about 0.5 g uranium can be bound by one gram biomass.

What is the value of the reaction rate constant for absorption that will allow the uranium to be discharged in a continuous culture?
As it turns out, the absorption rate constant is only half of the needed to accomplish the decontamination process. What will you do to meet the discharge regulation?

Task 21

Substrate conversion and biomass productivity in a chemostat

A 5 m^3 fermenter is operated continuously with a feed substrate concentration of 20 kg m^{-3}. The genetically engineered microorganism cultivated in the reactor has the following characteristics:

μ_{max} = 0.45 h^{-1}; K_s = 800 g m^{-3}; $Y_{X/S}$ = 0.55 kg kg^{-1}

a) What feed flow rate is required to achieve 90% substrate conversion?
b) How does the biomass productivity at 90% substrate conversion compare with the maximum possible?
c) What is the biomass concentration in case a and at the optimal dilution rate?

Task 22

Substrate conversion and biomass productivity in a chemostat

The specific growth rate for inhibited growth in a chemostat is given by the following equation:

$$\mu = \mu_{max}\, s /(K_s + s + I\, K_s / K_i)$$

Where

$s_0 = 10$ g L^{-1}, $K_s = 1$ g L^{-1}; $I = 0.05$ g L^{-1}, $Y_{xs} = 0.1$ g g^{-1}
$x_0 = 0$ g L^{-1}, $K_i = 0.01$ g L^{-1}, $\mu_{max} = 0.5$ h^{-1}

a) Determine X and S as function of D when I = 0
b) With inhibitor added to a chemostat, determine the effluent substrate concentration and x as function of D
c) Determine the cell productivity, Dx, as a function of dilution rate

Task 23 Pirt Equation & Lineweaver-Burk Plot

A new strain of yeast is being considered for biomass production. The following data were obtained using a chemostat. An influent substrate concentration of 800 mg/l and an excess of oxygen were used at a pH of 5.5 and T = 35°C. Using following data, calculate μ_{max}, K_s, $Y_{X/S}$, k_d and m_s, assuming $\mu = \mu_{max}\, s / (K_S + s) - k_d$

D [h^{-1}]	C-source Concentration in steady-state [mg L^{-1}]	Cell concentration [mg/l]
0.1	16.7	366
0.2	33.5	407
0.3	59.4	408
0.4	101	404
0.5	169	371
0.6	298	299
0.7	702	59