Laboratório de Bioprodutos
ICB - USP

USP
UNIVERSIDADE DE SÃO PAULO

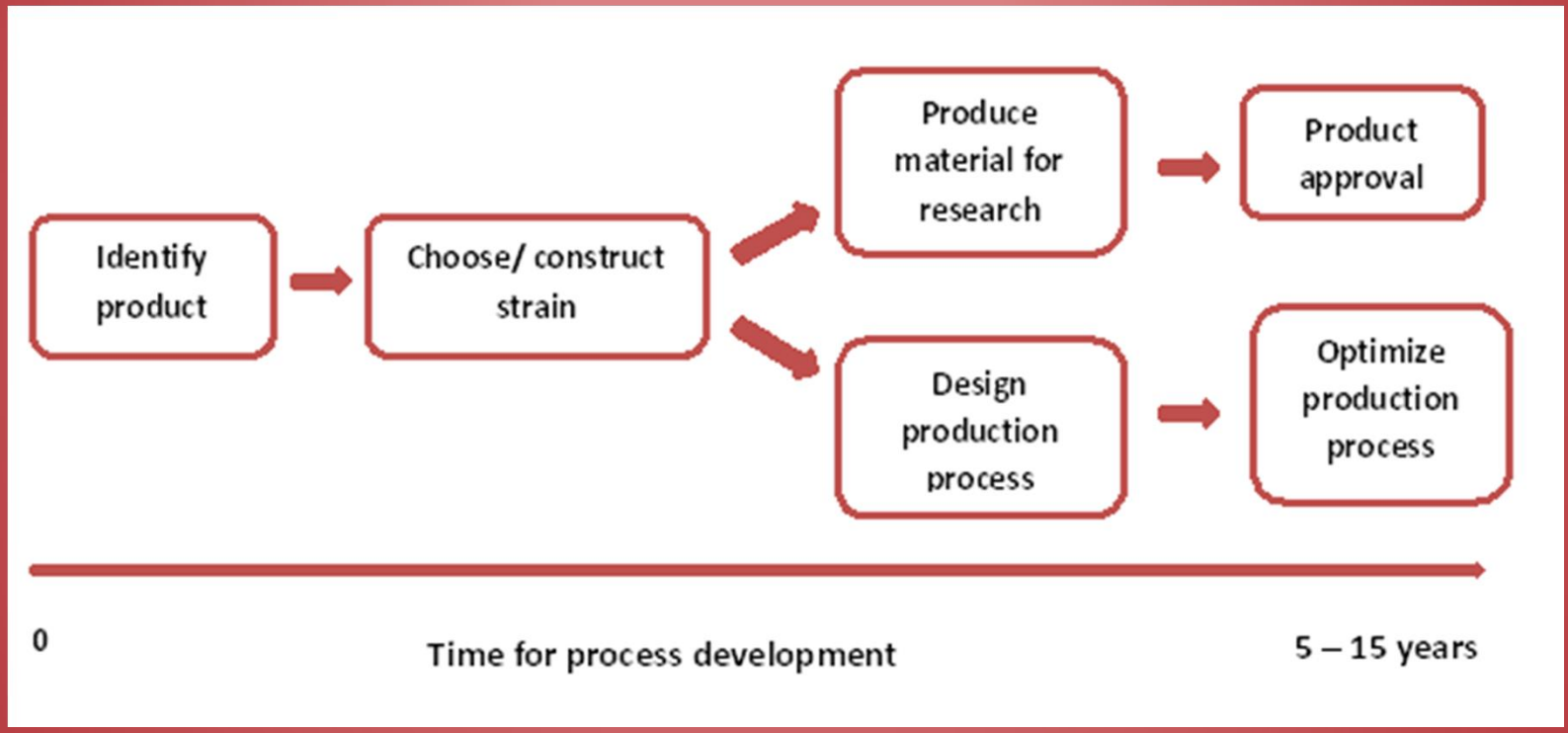
MICROBIAL BIOPROCESSES

DEPARTAMENTO DE MICROBIOLOGIA
UNIVERSIDADE DE SÃO PAULO

March/2018

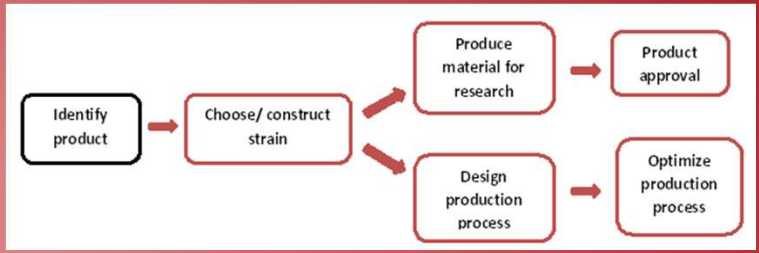
Agenda:

- Introduction to Microbial Bioprocesses
- Bioprocesses Kinetics
- Bioprocesses Analysis



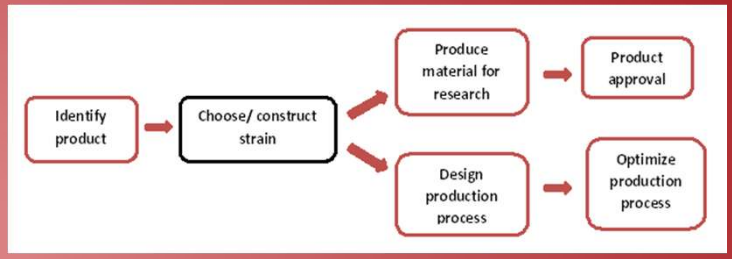
Phases in the development of a microbial bioprocesses (Bioreaction Engineering Principles - Nielsen, J. Villadsen, J., Lidén, G. cap.2, p.38 – 2nd edition).

PRODUCT IDENTIFICATION



- selecting metabolites with therapeutic or other effects;
- new product with known application;
- a new enzyme or even a certain protein using bioinformatics from sequenced genomes.

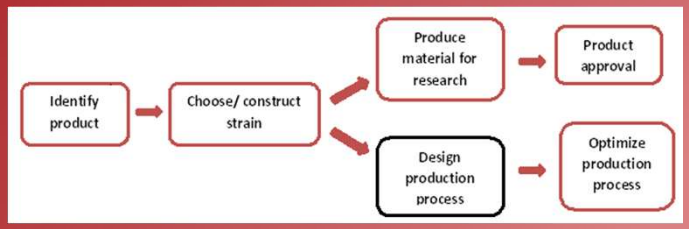
CHOOSE OR CONSTRUCT A STRAIN

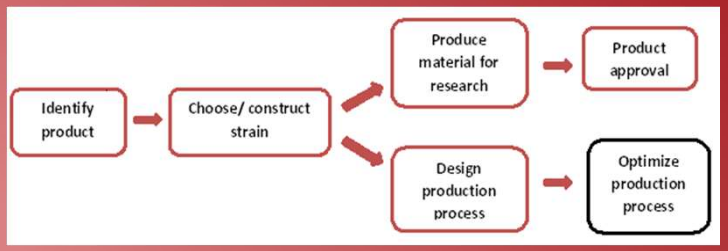


- Culture collection (ATCC, DSM, etc);
- Isolation and screening;
- Genetic improvement.

Important: choose a good host system from the beginning

- process improvement continues even after large-scale production starts;
- drugs: introduction of new strains requires a new process licensing and costs can make it unfeasible even though it is an economically better process (+ efficient, < cost) .





PROCESS DESIGN AND OPTIMIZATION

- Selection of the appropriate medium;
- mode of operation (batch, fed-batch or continuous);
- optimal operating conditions.

- **Selection of the appropriate medium**

It should contain:

- Carbon, nitrogen and energy sources;
- All essential minerals required for growth;
- Factors to ensure rapid growth and high yields of the desired product;
- Consistent quality and availability;
- Minimum problems in the downstream processing and on gas-liquid mass transfer.

It should consider:

- Purpose

- Price

- Complex media X Chemical defined media

CRITERIA FOR DESIGN AND OPTIMIZATION

- **Depends on the product**

- high volume/low value added product;
- Low volume/high value added product.

Produced in large quantities and low value added ("commodities"): most primary metabolites, many secondary, industrial enzymes, many polysaccharides, cell is the product.

Produced in small volumes and high added value: some drugs and specialties.

LARGE QUANTITIES AND LOW VALUE ADDED PRODUCTS

- **$Y_{p/s}$** (g of product per g of substrate) - significant part of the total cost (raw material, source of C);
- Productivity (g of product per L of reactor volume per time) - efficiency of production capacity utilization, investment costs;
- Final concentration of product or titre (g of product per reactor volume) - separation and purification costs ($\$ \uparrow$).

SMALL VOLUMES AND HIGH VALUE ADDED PRODUCTS

- **Product quality;**
- **Time-to-market;**

+ important than the previously mentioned design parameters (Ys, Prod e P_F).

Initial stage of project: Ys, Prod e PF should be considered

P_F Downstrean costs often > than 90% total costs of production.

Changes in process after implementation: requirement of a new approval (FDA).

BIOPROCESS KINETICS

1. **Bioreactor: type, design and function**
2. **Bioreactor operating modes**
3. **Cellular growth**
    - **3.1 Specific rates**
    - **3.2 Yields**
    - **3.3 Modeling of growth kinetics**

1. Bioreactor: types, designs and function

BIOREACTORS

On the basis of the agent used:

- **Biochemical / Enzymatic Reactors**

- **Biological Reactors (living cells)**
  - Unicellular organisms,
  - Filamentous fungi,
  - Animal Cells,
  - Plant cells, etc.

GENERAL CLASSIFICATION

1. Reactors in aqueous phase (submerged process)

1.1 Free cells / enzymes

- CSTR reactor (Continuous stirred tank reactor)
- Pneumatically agitated reactor

1.2 Cells / enzymes immobilized on supports

- Fixed bed reactor
- Fluidized bed reactor

2. **Non-aqueous phase reactors (semi-solid process)**

- Static reactors (Reactors with trays)
- Reactor with shaking (Rotary drum)

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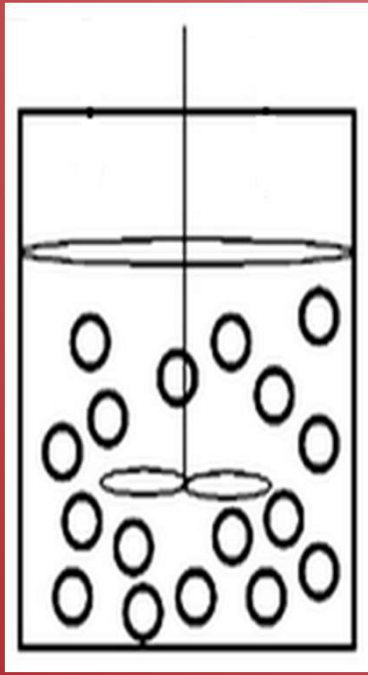
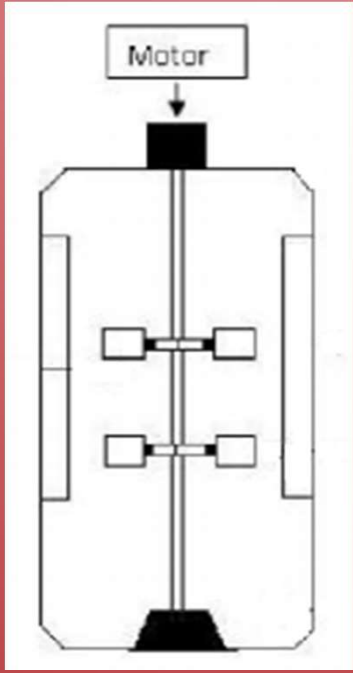
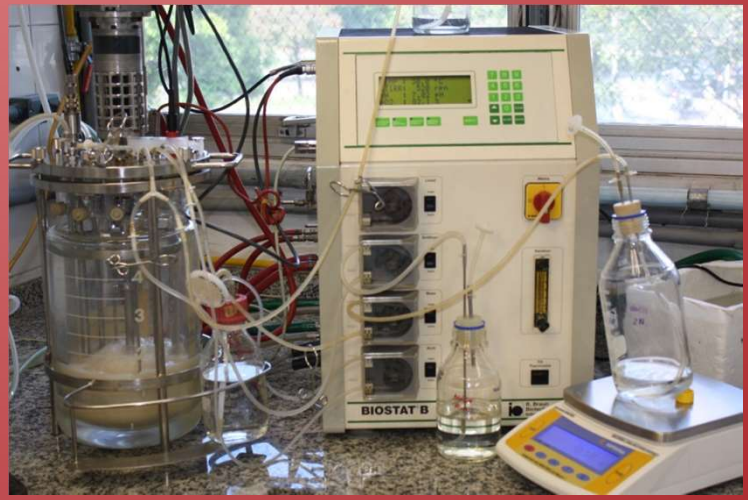
1. REACTORS IN AQUEOUS PHASE (SUBMERGED PROCESS)

STR reactor (*Stirred tank reactor*)

- Mechanically stirred







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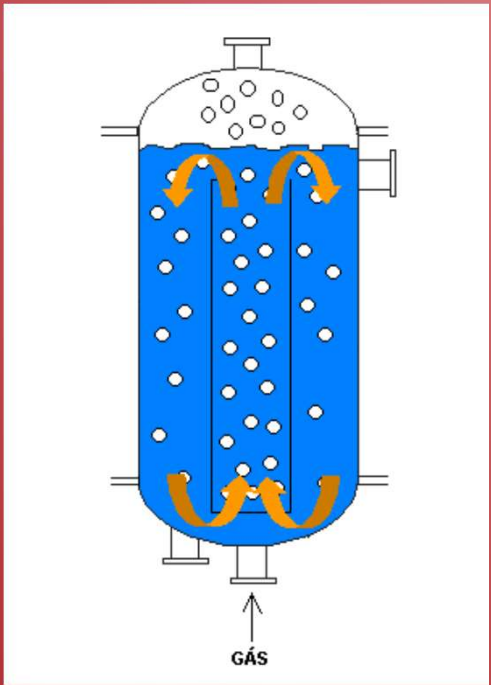
1. AQUEOUS PHASE REACTORS (SUBMERGED PROCESS)

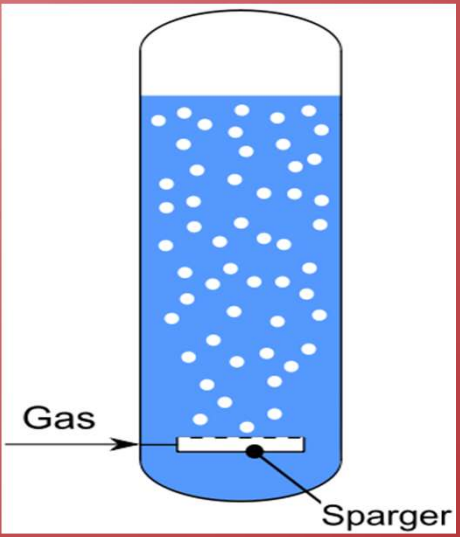
1.2 CELLS / ENZYMES IMMOBILIZED ON SUPPORTS

- Pneumatically agitated reactor

Air-lift reactor
"loop reactors"



Tower type reactor
(Bubble column)



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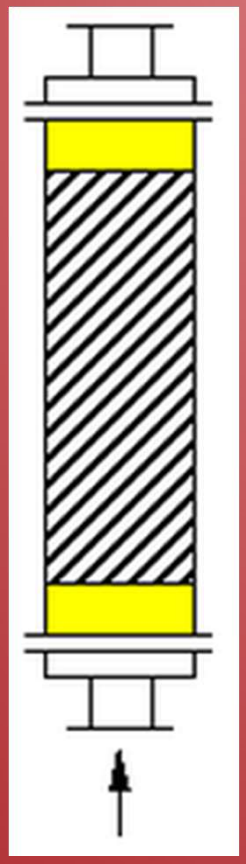
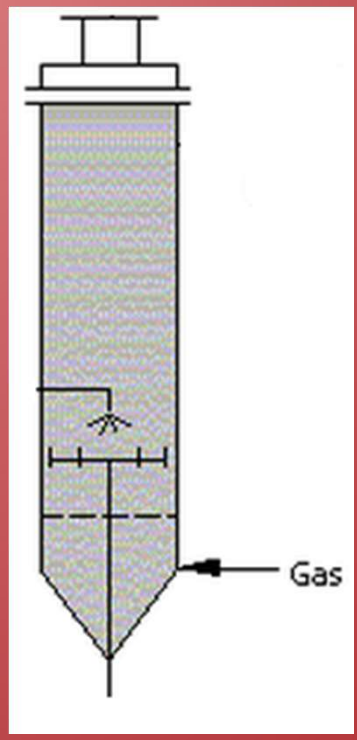
1. AQUEOUS PHASE REACTORS (SUBMERGED PROCESS)

1.2 CELLS / ENZYMES IMMOBILIZED ON SUPPORTS

Reactor with fixed bed



Reactors with fluidized bed



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2. **Non-aqueous phase reactors (semi-solid process)**

- Static reactors (Reactors with trays)
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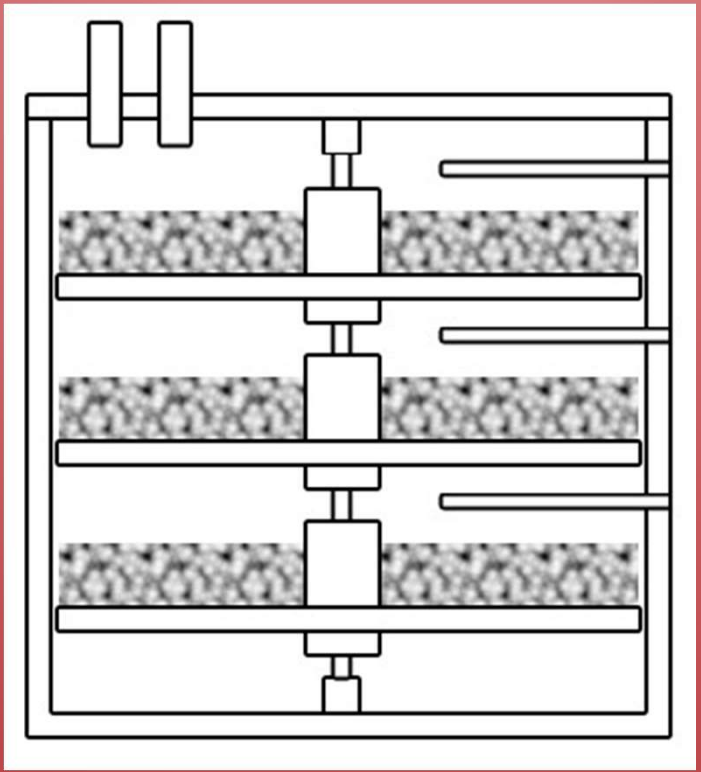
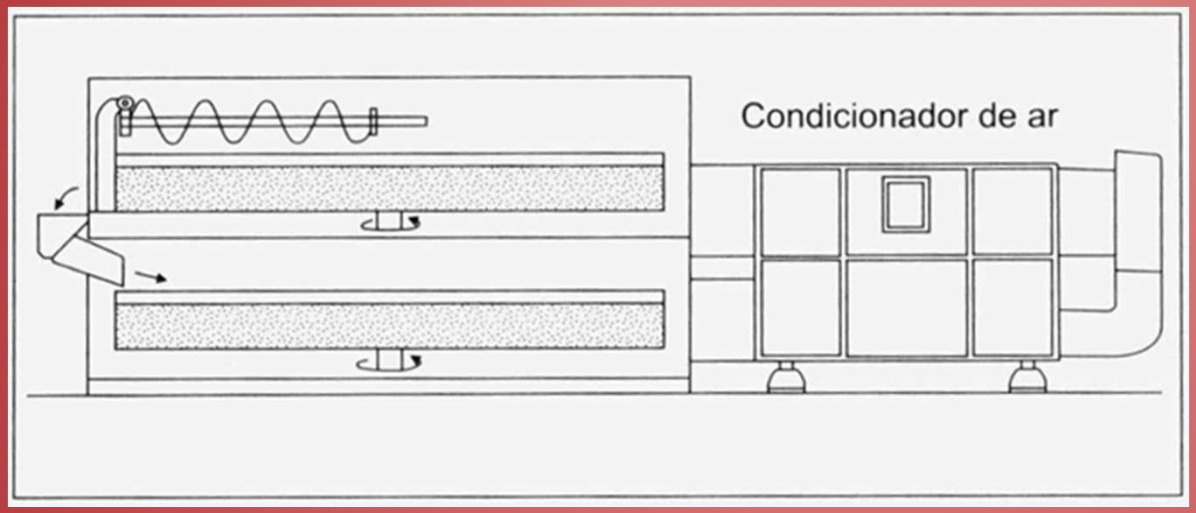
2. NON-AQUEOUS PHASE REACTORS (SEMI-SOLID PROCESS)

STATIC REACTORS / REACTORS WITH TRAYS
(STATIONARY TRAYS)

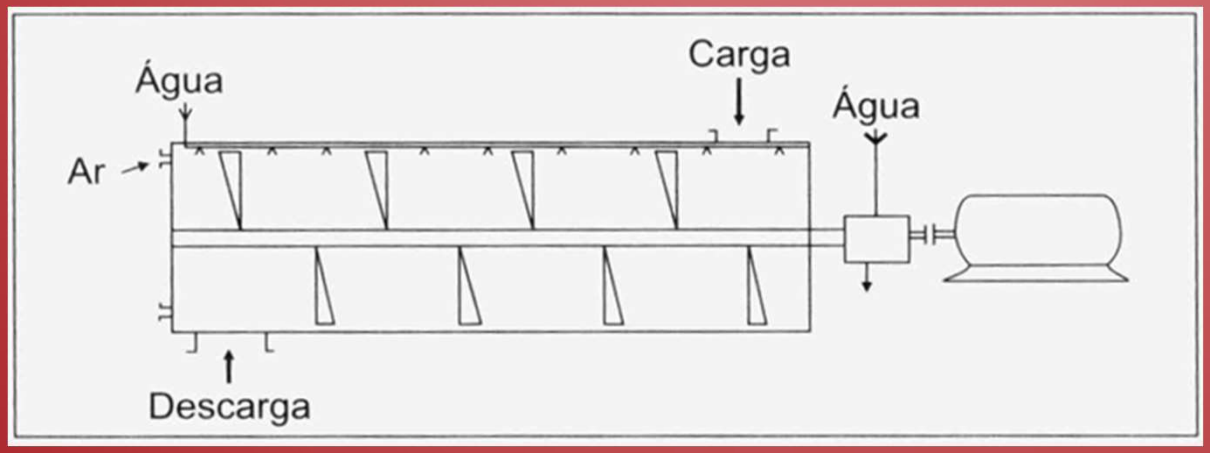


2. NON-AQUEOUS PHASE REACTORS (SEMI-SOLID PROCESS)

Reactor with shaking (Rotary drum)



Circular tanks



Tubular horizontal reactor with internal agitation

⇨ **All bioreactors deals with heterogeneous systems with 2 or more phases (liquid, gas, solid)**

⇨ **Optimal conditions: efficient mas transfer, heat and *momentum* from one phase to another.**

2. BIOREACTOR OPERATING MODES

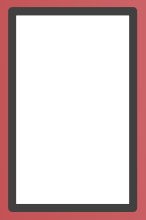
OPERATING MODES

1. **Discontinuous**
   - Batch
   - Fed-batch
     - With or not recirculation of cells

2. **Continuous**
   - With or not recirculation of cells
   - **Perfusion** (cell imobilized)

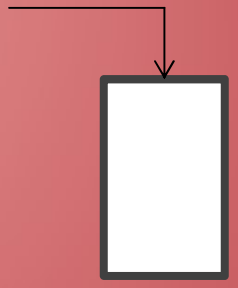
DISCONTINUOUS

Batch



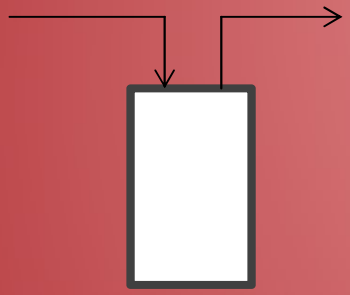
1. Media/bioreactor esterilization
2. Full charge of culture medium
3. Charge of inoculum
4. process time
5. discharge and cleaning

Fed-batch

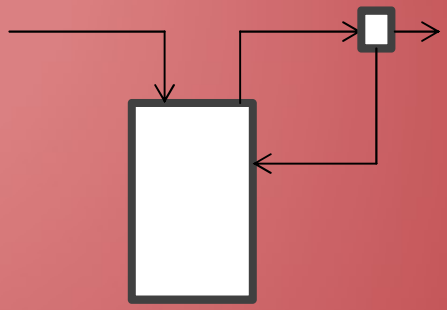


1. Media/bioreactor esterilization
2. Charge of inoculum
3. Controlled charge of medium / addition of component
4. process time
5. discharge and cleaning

CONTINUOUS

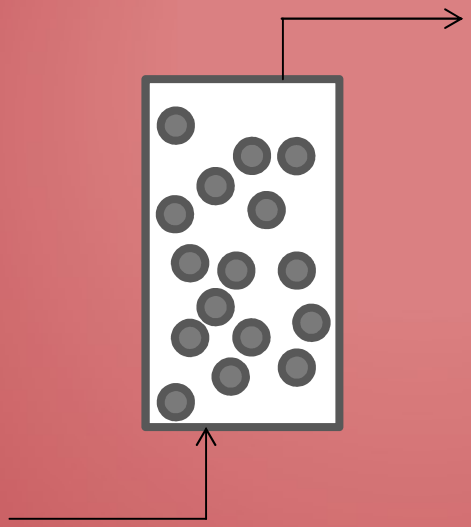


1. Media/bioreactor esterilization
2. Charge of inoculum
3. Continuous charge of the medium
4. Continuous discharge of product and medium



1. Media/bioreactor esterilization
2. Charge of inoculum
3. Continuous charge of the medium
4. Continuous discharge of product and medium
5. Partial biomass recicle

PERFUSION (cell imobilized)



1. Media/bioreactor esterilization
2. Charge of inoculum
3. Continuous charge of the medium
4. Continuous discharge of product and medium
5. Full biomass in bioreactor

BIOREACTOR CULTIVATIONS

On the basis of the product:

- Those that aim to produce biomass;
- Those where the product is produced intra or extracellularly such as an enzyme or metabolite;
- Those that modify a compound, known as biotransformation.

EXAMPLES (MICROBIAL BIOPROCESSES**)**

- ✓ Food industry
- ✓ Enzymes
- ✓ Antibiotics
- ✓ Vitamins
- ✓ Organic acids
- ✓ Solvents
- ✓ Treatment of industrial or domestic organic waste

- ➢ Viral vaccines
- ➢ Monoclonal antibodies
- ➢ Hormones
- ➢ Growth factors

- ❖ Active drug principles
  - -Morphine
  - -Quinina
- ❖ Cosmetic industry products

EXAMPLES

Food industry (Batch processes)

Yogurt
Sauerkraut
Pickles

Mix of processes

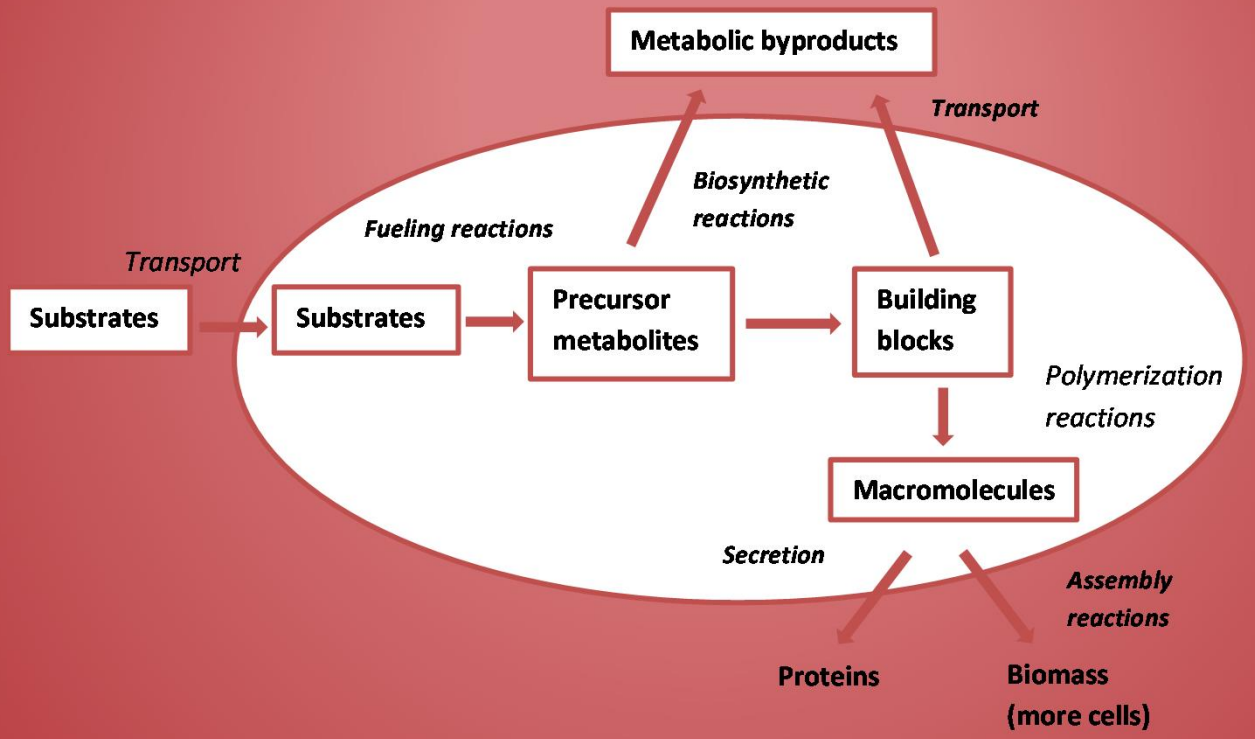
- **Beer** (cell recycle)
- **Vine**

(Recycling of cells – Fed-batch – Batch)

- **Ethanol**

(Cells / immobilized enzymes - continuous process – discontinuous)

3. Cellular growth



Overview of reactions and processes involved in cellular growth and product formation (Bioreaction Engineering Principles - Nielsen, J. Villadsen, J., Lidén, G. cap.2, p.38 – 2nd

Nutrients must be available to ensure cell growth (roughly):

i) **Carbon source;**

ii) **Energy source;**

iii) **Nitrogen source;**

iv) **Minerals;**

v) **Vitamins.**

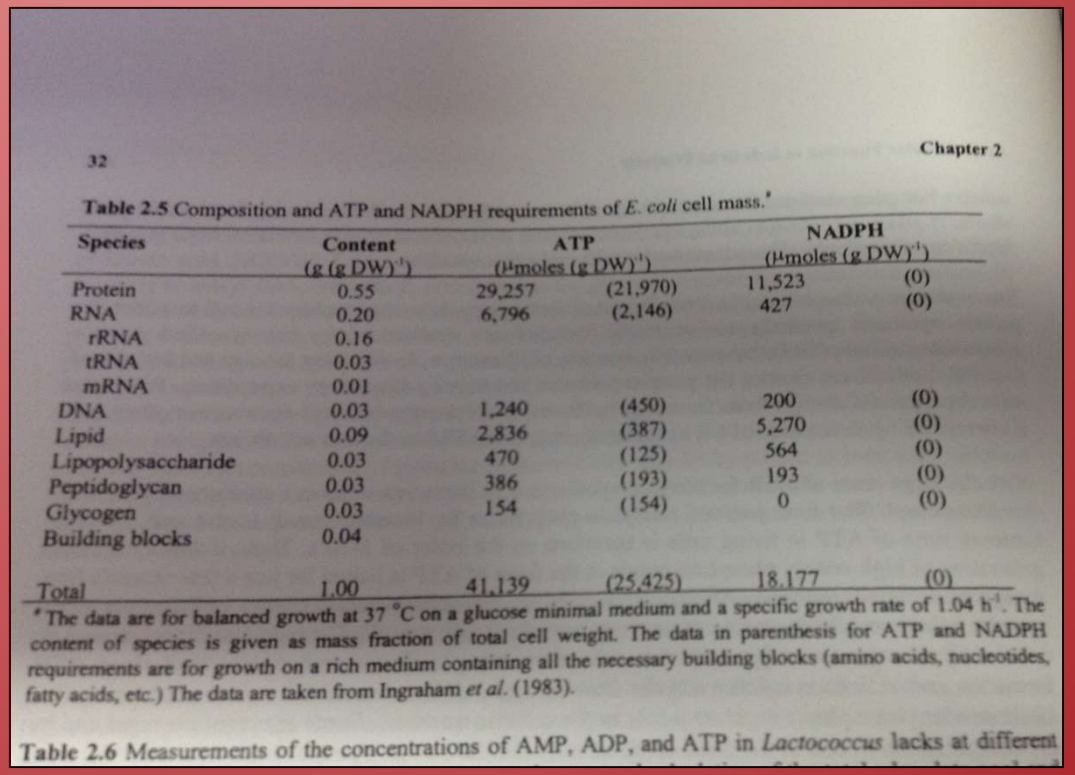


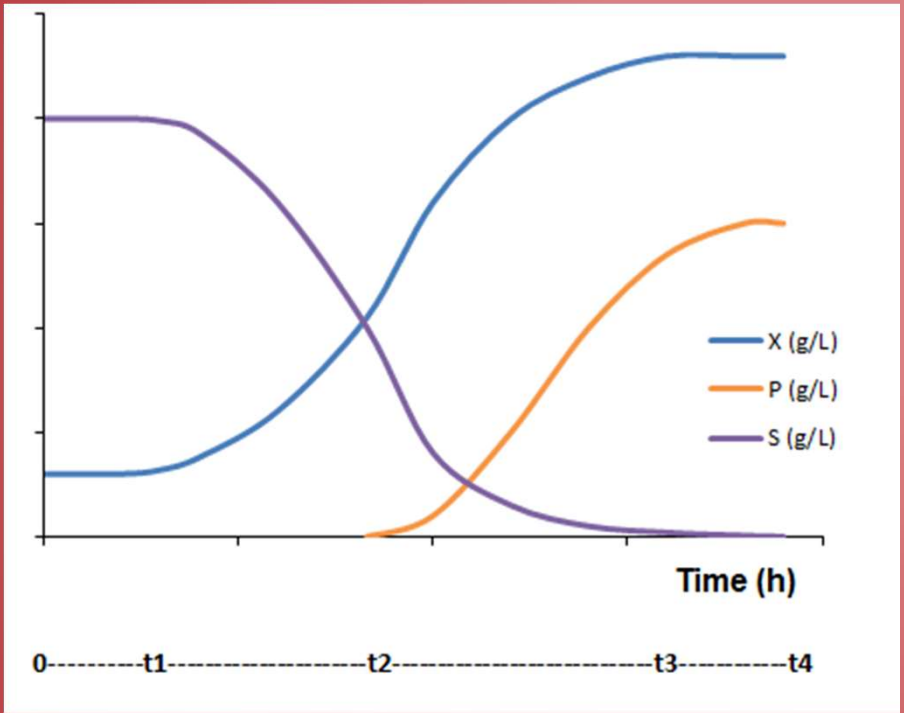
Table 2.5 Composition and ATP and NADPH requirements of *E. coli* cell mass.[a]

Species	Content (g (g DW)$^{-1}$)	ATP (μmoles (g DW)$^{-1}$)		NADPH (μmoles (g DW)$^{-1}$)	
Protein	0.55	29,257	(21,970)	11,523	(0)
RNA	0.20	6,796	(2,146)	427	(0)
rRNA	0.16				
tRNA	0.03				
mRNA	0.01				
DNA	0.03	1,240	(450)	200	(0)
Lipid	0.09	2,836	(387)	5,270	(0)
Lipopolysaccharide	0.03	470	(125)	564	(0)
Peptidoglycan	0.03	386	(193)	193	(0)
Glycogen	0.03	154	(154)	0	(0)
Building blocks	0.04				
Total	1.00	41,139	(25,425)	18,177	(0)

[a] The data are for balanced growth at 37 °C on a glucose minimal medium and a specific growth rate of 1.04 h^{-1}. The content of species is given as mass fraction of total cell weight. The data in parenthesis for ATP and NADPH requirements are for growth on a rich medium containing all the necessary building blocks (amino acids, nucleotides, fatty acids, etc.) The data are taken from Ingraham *et al.* (1983).

Table 2.6 Measurements of the concentrations of AMP, ADP, and ATP in *Lactococcus* lacks at different

BATCH CULTIVATION



Lag phase: 0-t1: adaptation

Duration depends on:

- differences in composition of medium, pH, temperature;
- different C sources require other enzymes.

⇨ monitoring methodology: sensitivity to detect

Inoculum fraction

- permeation of vitamins, cofactors and activators require the presence of these substances

Inoculum age

- permeation of toxic and transient metabolites in response to induction by higher nutrient concentrations for older inoculum**.**

Minimization of lag phase **:**

- Similar media and conditions
- volume ratio inoculum / bioreactor medium> 5%
- inoculum in the exponential phase

Exponential phase (Log): t1 – t2

- Unrestricted availability of nutrients and low content of inhibitory metabolites.

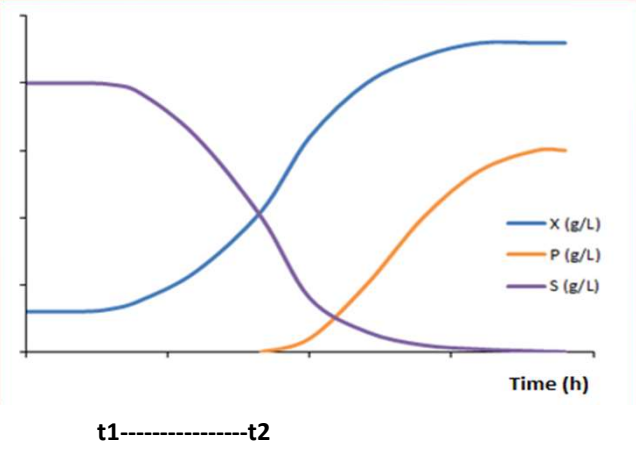
  Thus, it can be assumed that the specific growth rate is constant and maximum:

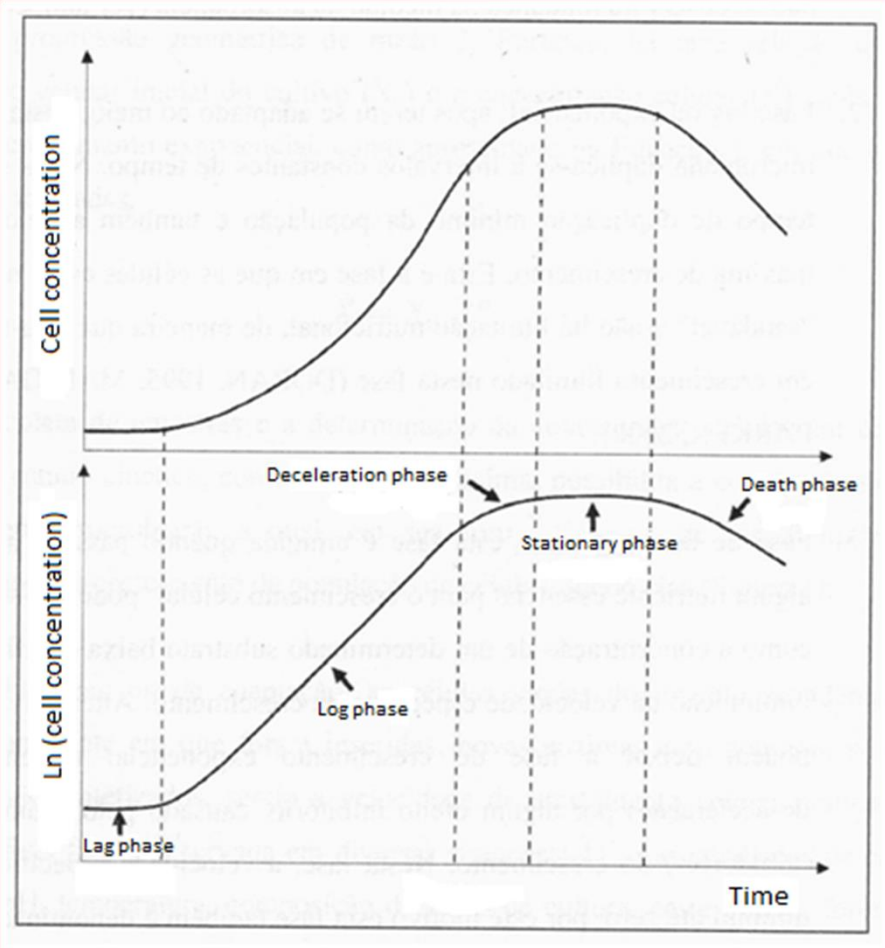
$$\mu_X = \frac{1}{X}\frac{dX}{dt} = \mu_{MAX}$$

$$\int_{X_0}^{X} \frac{dX}{X} = \mu_{MAX} \int_{t_0}^{t} dt$$

$$\ln \frac{X}{Xo} = \mu_{MAX} * (t - to)$$

$$X = Xo * \exp\left[\mu_{MAX} * (t - to)\right]$$



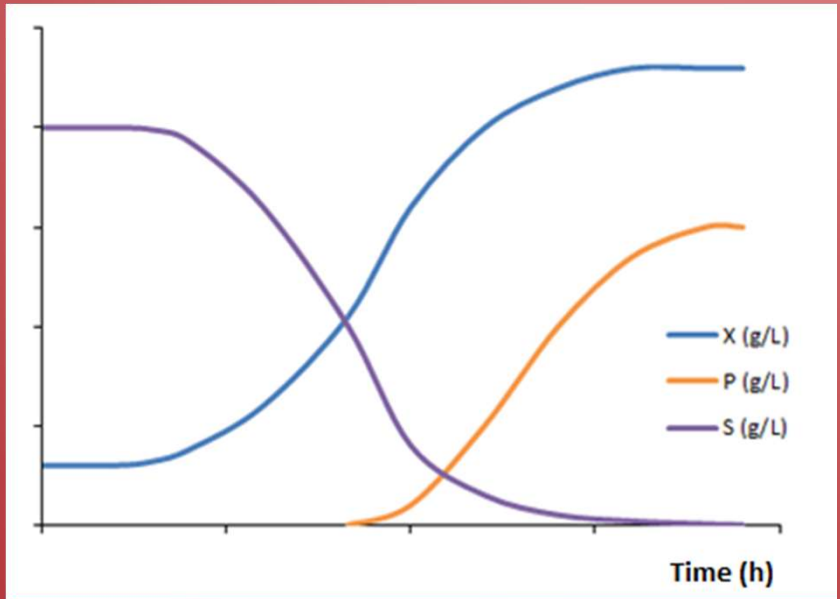


Deceleration and Stationary Phase: t2-t4,

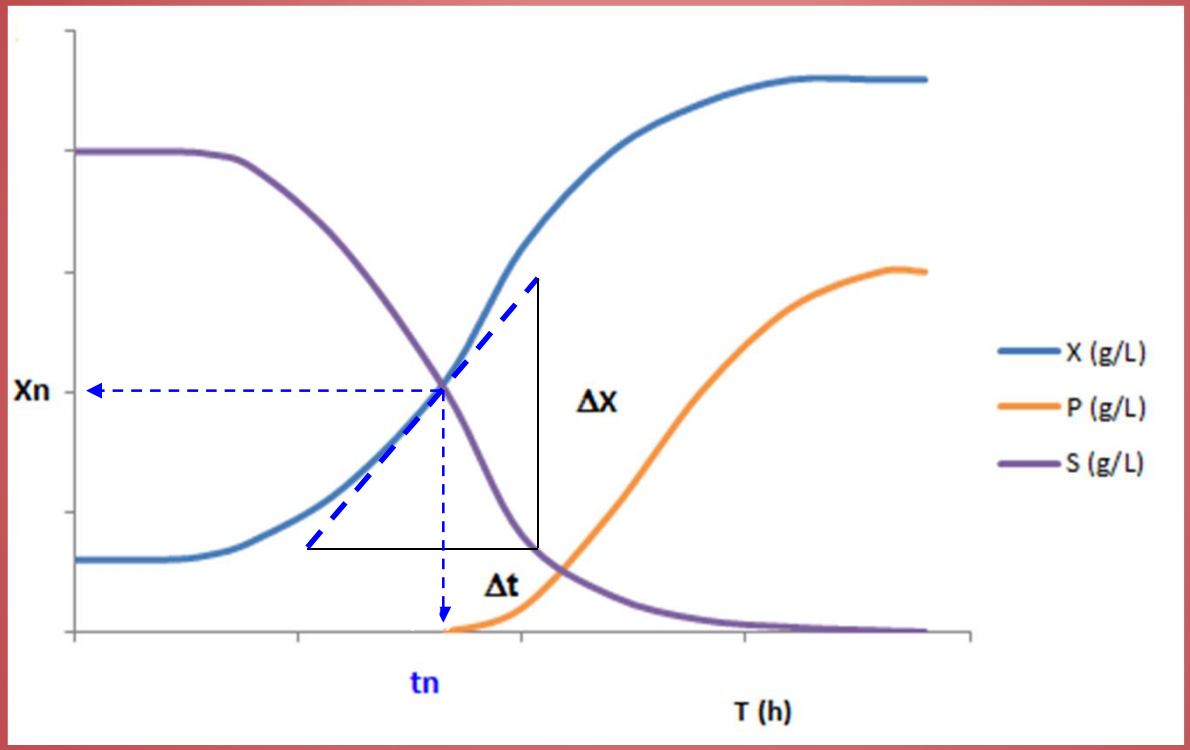
Occurs when:

- **concentration of toxic metabolites (inhibition)**
- **nutrient concentrations reach values that prevent the population from growing on μ_{max} (limitation)**

⟹ **maximum cell concentration, Xmax**



3.1 Specific rates



- **Specific growth rate**

$$\mu_X = \frac{1}{X}\frac{dX}{dt} \qquad (3)$$

- **Specific substrate consumption rate**

$$-\mu_S = \frac{1}{X}\frac{dS}{dt} \qquad (4)$$

- **Specific product formation rate**

$$\mu_P = \frac{1}{X}\frac{dP}{dt} \qquad (5)$$

Example: calculating μ_{max}

$$\mu_X = \frac{1}{X}\frac{dX}{dt} = \mu_{MAX}$$

$$\int_{X_0}^{X} \frac{dX}{X} = \mu_{MAX}\int_{t_0}^{t} dt$$

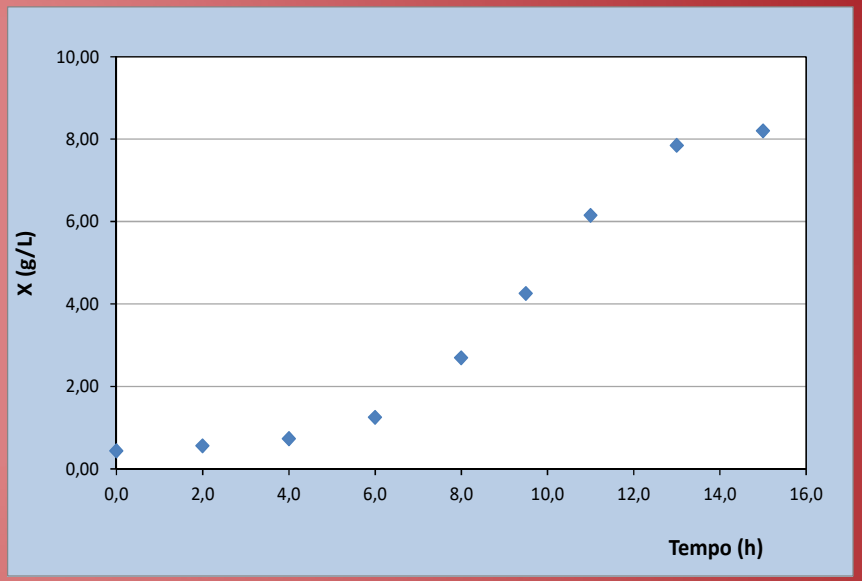
$$\ln\frac{X}{Xo} = \mu_{MAX} * (t - to)$$

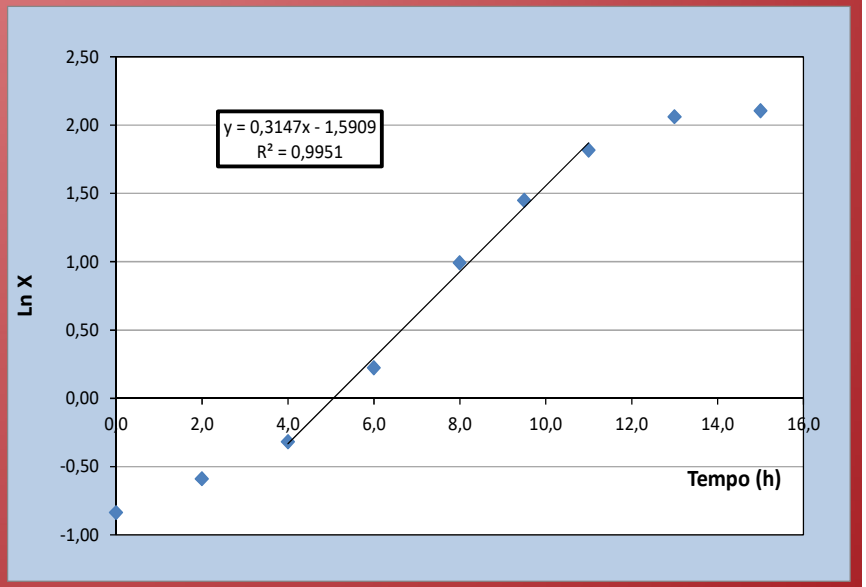
$$\ln X - \ln Xo = \mu_{MAX} * (t - to)$$

$$\ln X = \ln Xo + \mu_{MAX} * (t - to)$$

$$X = Xo * \exp[\mu_{MAX} * (t - to)]$$

T(h)	X (g/L)	Ln X
0,0	0,43	-0,84
2,0	0,55	-0,59
4,0	0,73	-0,32
6,0	1,25	0,22
8,0	2,69	0,99
9,5	4,25	1,45
11,0	6,15	1,82
13	7,85	2,06
15,0	8,20	2,10





3.2 Yields

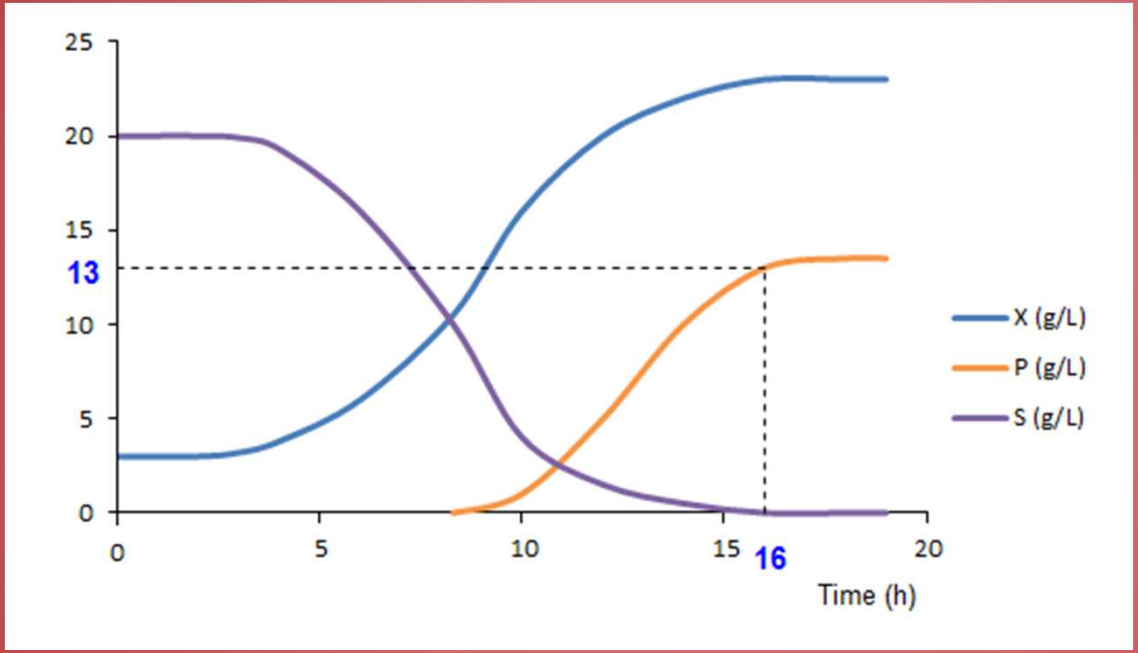
- Yield of substrate to cell

$$Y_{X/S} = \frac{X - Xo}{So - S} \qquad (6) \qquad = \mu x/\mu s$$

- Yield of substrate to product

$$Y_{P/S} = \frac{P - Po}{So - S} \qquad (7) \qquad = \mu p/\mu s$$

Example: calculating Yields (Y)



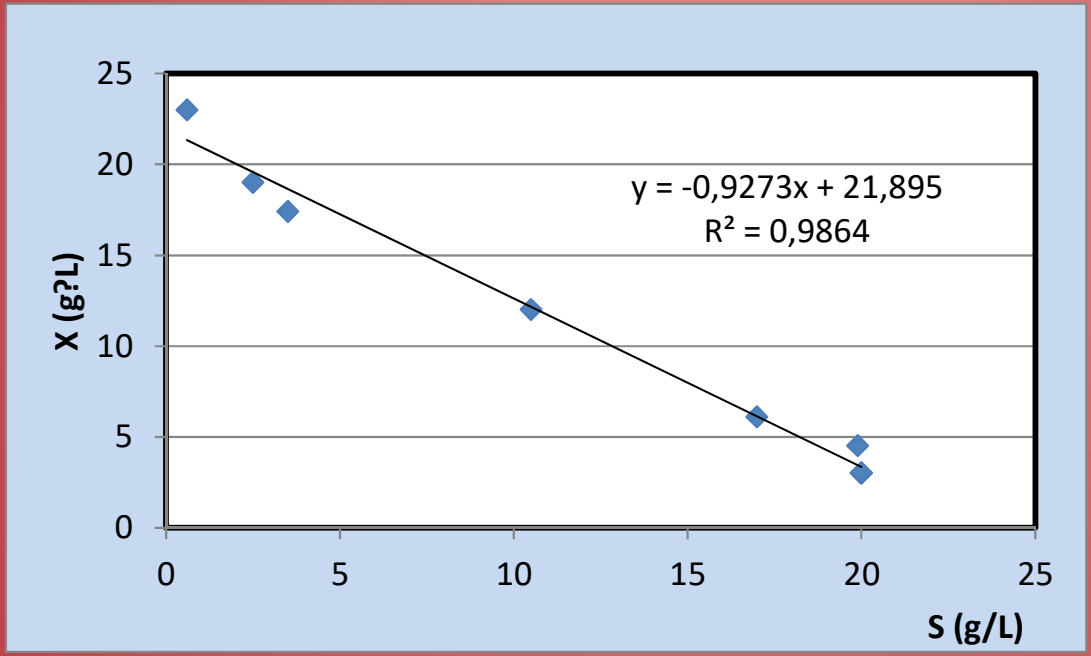
For t = 16 h

$Y_{P/S} = (13 - 0)/(20 - 0) = 0{,}65$ g/g

For t = 15 h

$Yx/s = (24 - 3)/(20 - 0) = 1{,}05$ g/g

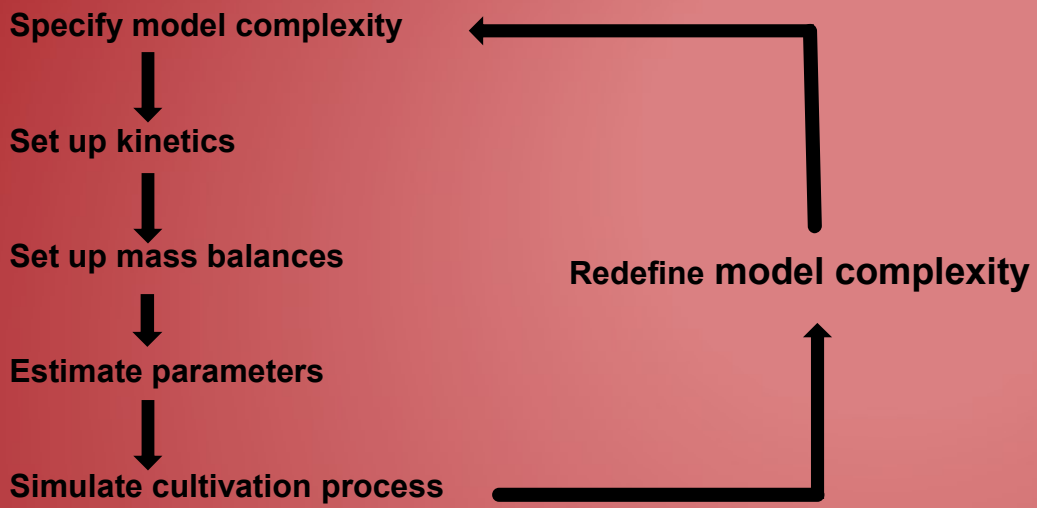
Example: calculating Ys/x by linear adjustment



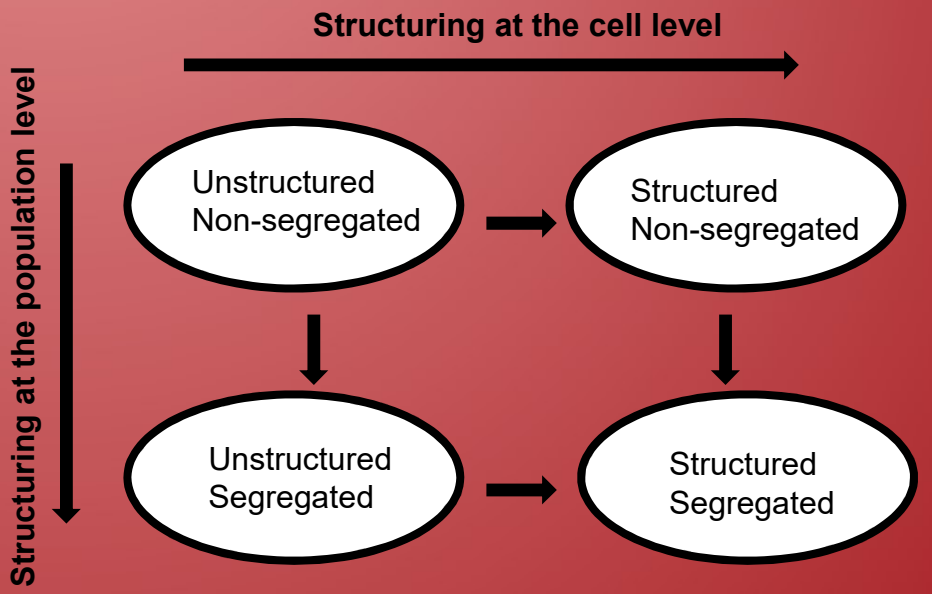
Yx/s = 0,93 g/g

Difference ?

3.3 Modeling of growth kinetics



Interpretation of cell growth with
+ biochemistry
+biology



Bioreaction Engineering Principles - Nielsen, J. Villadsen, J., Lidén, G. cap.2, p.38 – 2nd edition

KINETIC MODELS (unstructured – single limiting substrate)

Monod (1940):

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

Substrate inhibition:

$$\mu = \mu_{\max} \frac{S}{S + Ks + \frac{S^2}{Ki}}$$

Inhibition by product:

$$\mu = \mu_{\max} \frac{S}{S + \left(1 + \frac{P}{Kp}\right) Ks}$$

$$\mu = \mu \max \left(1 - \frac{P}{P \max}\right)^n$$

Product formation

Classification according to Gaden (1955):

- primary metabolite
- secondary metabolite
- mixed

Kinetics Models

- **Associated with growth**

$$\mu_p = \alpha . \mu_x$$

- **Not associated with growth**

$$\mu_p = \beta . X$$

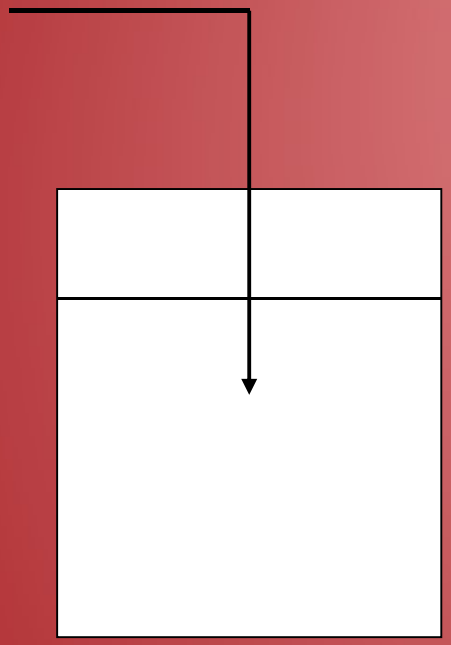
- **Mixed**

$$\mu_p = \alpha . \mu_x + \beta . X$$

BIOPROCESS ANALYSIS

1. Batch cultivation
2. Fed batch cultivation
3. Continuous

1. Batch



$V_o = V$

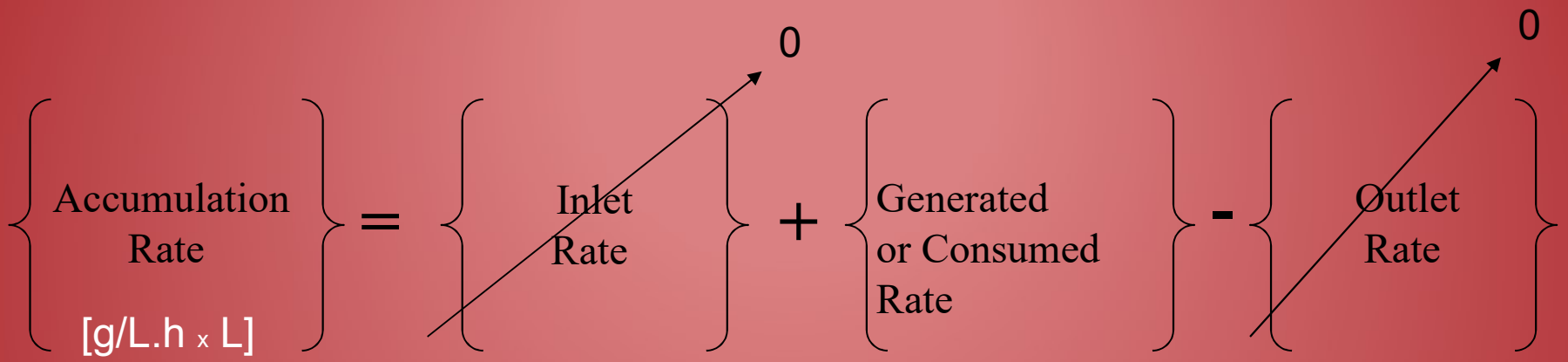
- Medium preparation
- Inoculation
- Development until S limiting exhaustion
- Discharge and cleaning

Batch

+ Easy operation;
+ variable controls (pH, temperature, dissolved O2);
+ accumulated knowledge;

- non-stationary environment requires constant population adaptation;
- inhibition by the substrate;
- low cell concentration;
- downtime of charging, discharging and cleaning;
- low productivity.

Mass Balance Equations - Batch

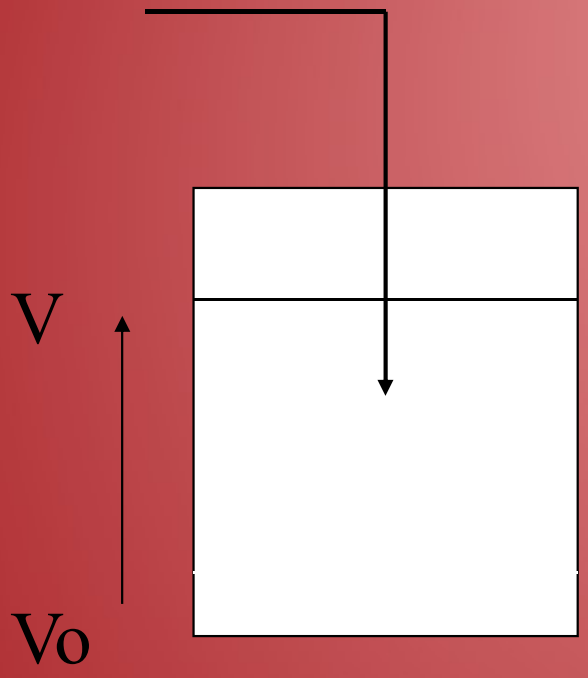


Not considering neither death nor cell maintenance

- **Biomass:** $dx/dt = \mu X$
- **Product:** $dp/dt = \alpha \ dX/dt$
- **Substrate:** $-ds/dt = (1/Yx/s)\ \mu X + (1/Yp/s)\ \alpha\ \mu X$

Part of the substrate spent: cell maintenance (high densities).

2. Fed- batch

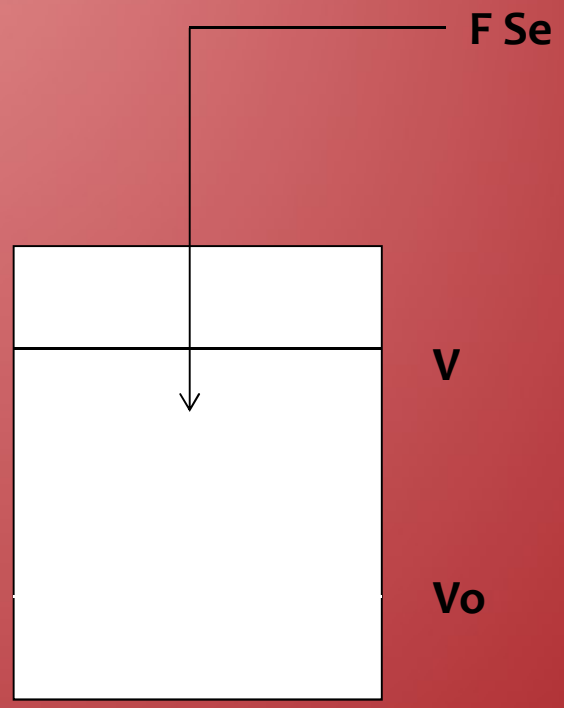


- Medium preparation
- Inoculation
- Development until reactor filling
- Discharge and cleaning

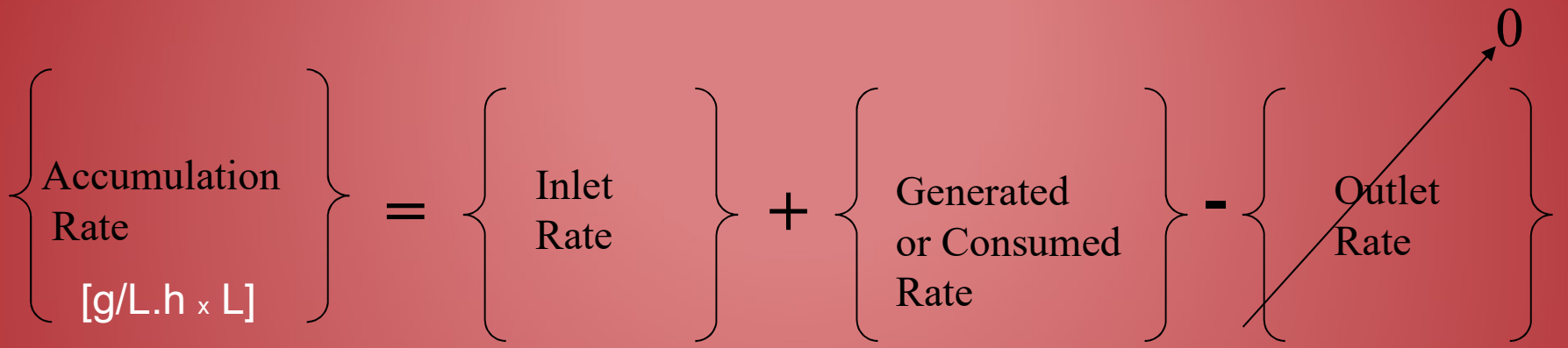
+ Minimizing effects of cell metabolism control (catabolic repression, substrate or precursor inhibition, toxic products formation);
+ *High cell density;*
+ *Variables control (pH, temperatura, O2 dissolvido);*
+ *High produtivity;*
+ Process adequacy to operational conditions (addition of unstable nutrient in the environment condition, replacement of evaporative losses, etc.);
+ Overcoming stability problems that can occur in the continuous process;

- Non-stationary environment forces the population to constantly adapt;
- More complex operation;
- Downtime in charging, discharging and cleaning .

F	volumetric feed rate (inlet)
Se	concentration of S in feed
Xo, So, Po	concentrations for time t = to
X,S,P	concentrations for time t = t
Vo	initial volume
V	final volume
x = XV	mass of cells
s = SV	mass of substrate
p = PV	mass of produto



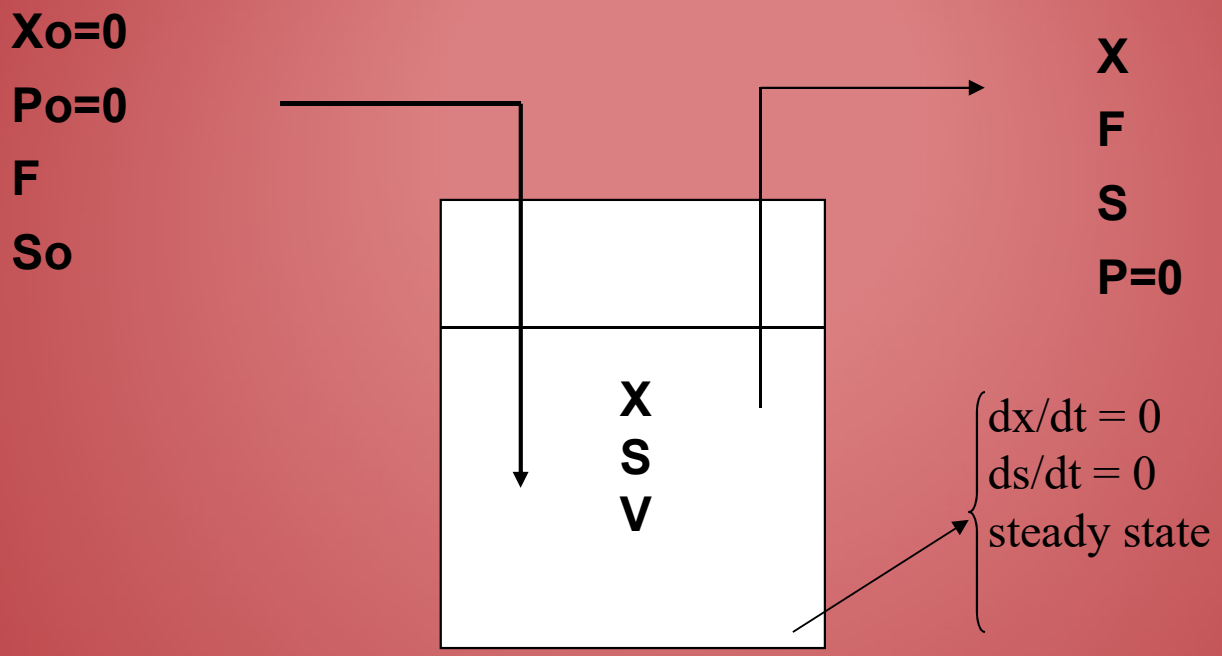
Mass Balance Equations – Fed Batch



Not considering neither death nor cell maintenance

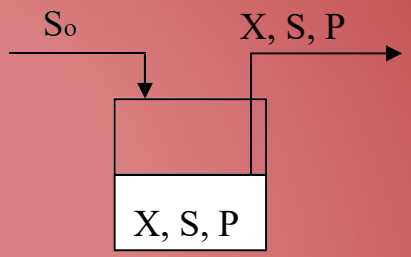
- **Biomass:** $dx/dt = \mu x$
- **Substrate:** $-ds/dt = F Se - (1/Yx/s) \mu x$
- **Product:** $dp/dt = \mu p x$

3. Continuous (without recycle)



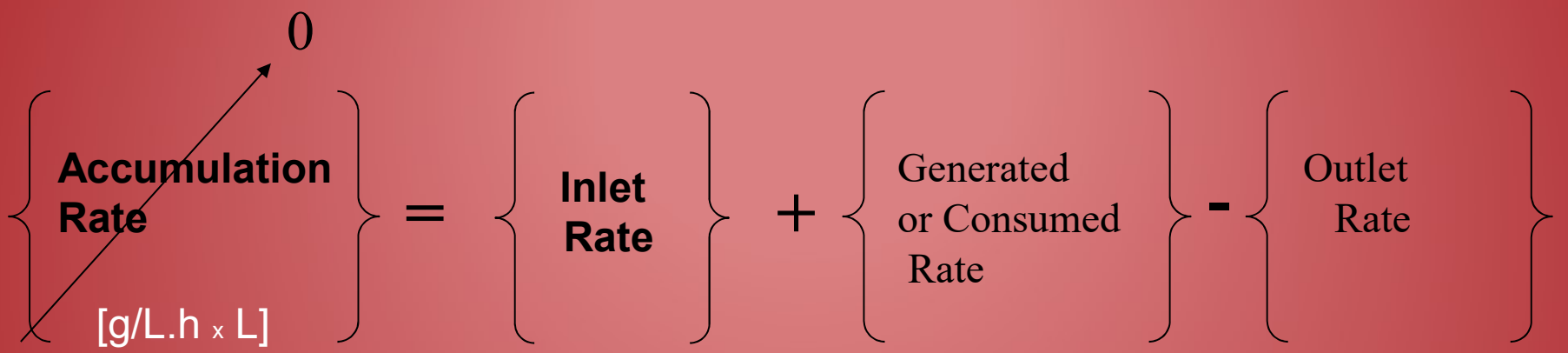
- Medium preparation
- Inoculation
- Continuous charge of limited S
- Continuous dischage of processing material

Continuous



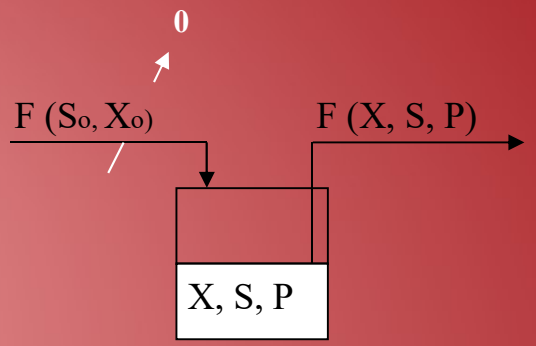
+ reducing downtime (Prod ⇧);
+ uniform broth (“downstream” + easy);
+ cells in the same physiological state (metabolism study, medium optimization);
+ enables to associate other continuous operations in production
+ enables to use advanced controls (+ easy);
+ employment < manpower;
- spontaneous mutations may result in selection;
- requires + care with contaminations (open system);
- homogeneity in low flows (problems);
- operation difficulties: foam, cell growth on wall / inlet / outlet.

Mass Balance Equations



Not considering neither death nor cell maintenance

Equation: ideal reactor



$$MB: \quad VdX/dt = FXo - FX + V(dX/dt)_{growth}$$

Variation of cell mass | Inlet | Outlet | produced

steady state: X, S, P = cte

Growth rate: $V(dX/dt)_{growth} = \mu X$

Dilution rate: $D = F/V \ (h^{-1})$

Residence time: $\theta = 1/D$ (samples: 3-4 θ stead ystate)

$$dX/dt = D(Xo - X) + \mu X$$

(dX/dt: 0; Xo: 0)

steady state

sterilized medium.

$$\Rightarrow \quad \mu X = DX \quad \therefore \quad \mu = D$$

MiniBio: 500 ml (400 – 100 ml volume útil)

Video
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3864403/

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