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OVERVIEW ON ELEMENTAL KINETICS OF MICROBIAL GROWTH

Michele Vitolo*

School of Pharmaceutical Sciences, Department of Biochemical and Pharmaceutical Technology, University of São Paulo, Brazil.

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***Corresponding Author**
Michele Vitolo
School of Pharmaceutical Sciences, Department of Biochemical and Pharmaceutical Technology, University of São Paulo, Brazil.

ABSTRACT

This study discusses concepts of the evaluation of cell growth, product formation, and substrate consumption during batch, fed-batch, or continuous fermentation. The parameters considered were the average and/or instantaneous rates of change in cell density, product formation, and substrate consumption. Concepts of productivity, specific rate, and conversion factor were also discussed. Moreover, the study addresses the correlation between cell growth and product formation and the mechanisms of metabolic regulation and membrane transport.

KEYWORDS: Fermentation, prokaryotes, eukaryotes, microbial cell kinetics.

INTRODUCTION

Fermentation can be defined as a process carried out in the presence of either prokaryotic or eukaryotic cells, under controlled growth conditions.

The study of cellular kinetics aims to determine the rates of cell growth, substrate consumption, and product formation during fermentation. It also includes the influence of changing culture conditions on these rates and the derivation of equations to describe the process.

Fermentation can be envisaged as a process in which a cell receiving balanced amounts of nutrients and energy grows, multiplies, and generates one or more products (Figure 1). In an industrial scale, the process requires a series of unit operations as shown in Figure 2.

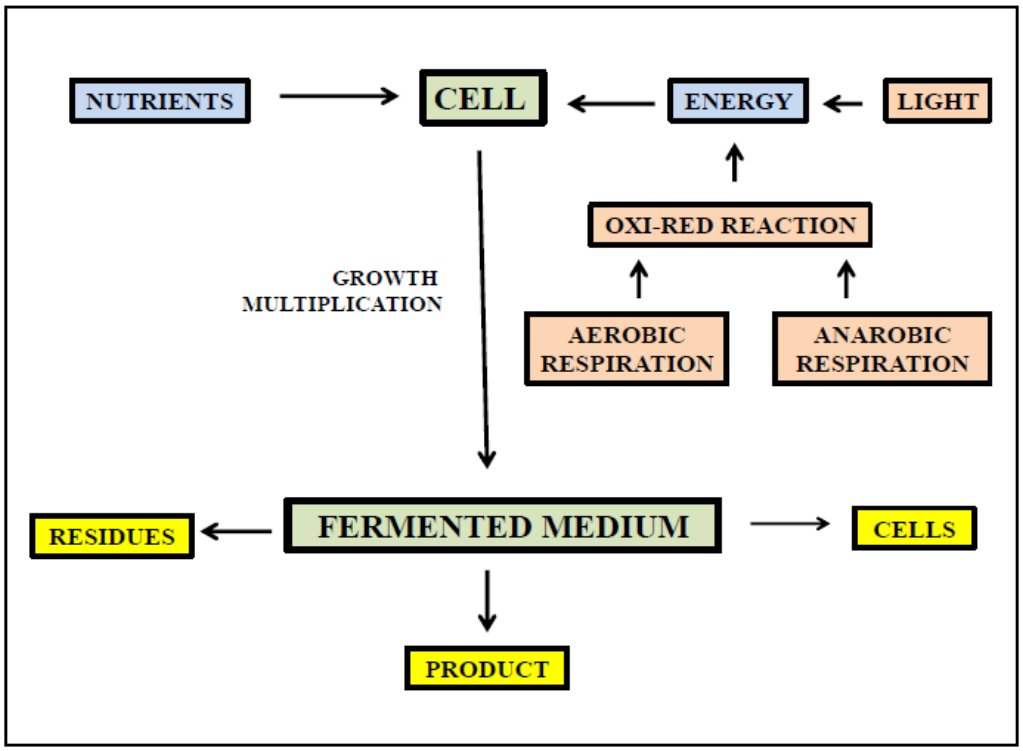

Figure 1: Sketch of the fermentation process.

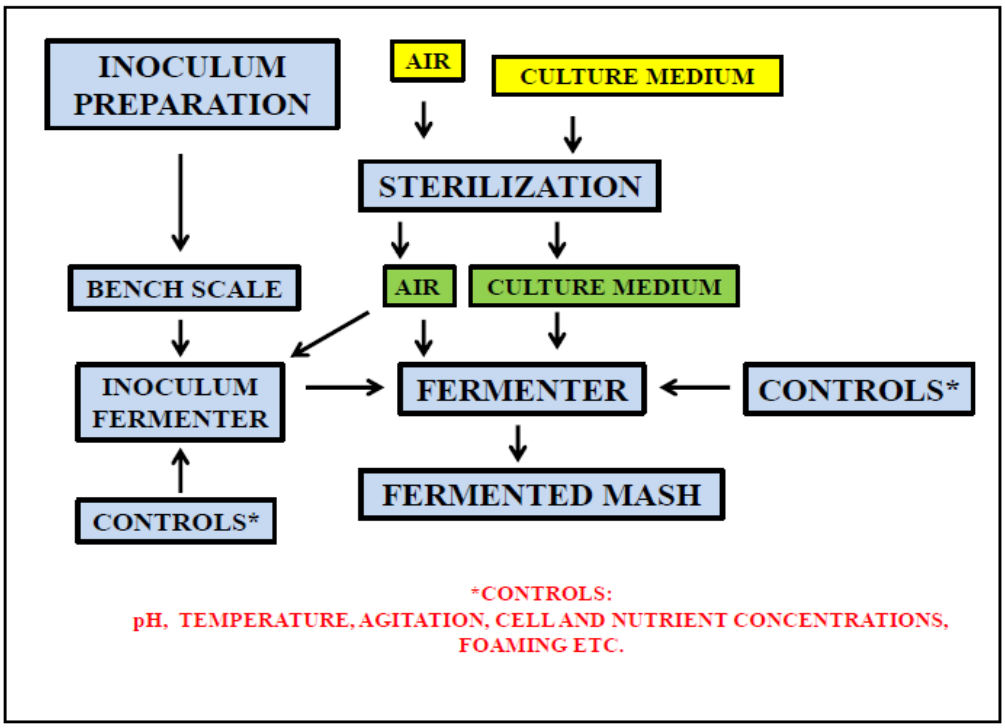

Figure 2: Sketch of industrial scale fermentation.

Monitoring of the fermentation process usually consists of measuring changes in pH, temperature, dissolved oxygen, cell density, substrate, and product in the culture medium.

Some methods for determining cell density in a culture are: **a) Dry weight:** a mash volume is filtered through a tarred membrane (pore diameter ≤ 0.45μm). The cake over the membrane is washed with distilled water, followed by placing the membrane in an oven at 105 ^{o}C to dry to a constant weight. Cell density is expressed as g/L; **b) Centrifugation:** a mash volume is centrifuged (3,000 *g*/10 min) in a graduated tube. The volume occupied by the cake (V_c) is compared with the total volume of the sample (V_t). Density is expressed as volume percent [(V_c/V_t).100]; **c) Optical density (O.D.):** A mash volume (1-3 mL) is placed into a cuvette and O.D. is measured at λ = 600nm using a spectrophotometer. In this case, the absorbance value must be compared with a standard curve correlating absorbance and cell density (expressed as dry weight); **d) Microscope counting:** a mash sample is diluted at least fifty times with distilled water. A volume of the diluted suspension is transferred to a Neubauer chamber (area = 1/400 mm^2; thickness = 0.100 mm) using a capillary tube, and the number of cells is counted using a light microscope.[1] Cell density is expressed as number of cells/mL. If the cell suspension is coloured with methylene blue, it is possible to determine the number of viable cells, i.e., those having full fermentative capability (these cells don't adsorb the dye); **e) Colony counting on solid medium:** a sample of the mash is adequately diluted and volumes of the suspension are spread over the surface of an agar medium. Then, agar plates are incubated, for example, at 37 ^{o}C for 24 h, and the number of colonies are counted, with the number of cells expressed as CFU/mL (CFU = **C**olony **F**orming **U**nits).

Specific components of the culture medium (substrate, product, among others) are determined in a cell-free sample using appropriate chemical procedures. For instance, if the substrate is glucose, the concentration can be determined using the Fehling titration method, dinitro salicylic acid, or the Somogyi method.

Rate measurements

For understanding a fermentation process, calculations of the average and/or instantaneous rates of changes in cell density, product formation, and substrate consumption are performed.

Average rates for changes in cell density, product formation, and substrate consumption are expressed as follows:

$P_x = (X_f - X_i)/T_f = (\Delta X)_c/T_f$ (Eq. 1)

$P_p = (P_f - P_i)/T_f = (\Delta P)_c/T_f$ (Eq. 2)

$P_s = (S_i - S_f)/T_f = (\Delta S)_c/T_f$ (Eq. 3)

Where P_x = cell productivity (g/L.h); P_p = product productivity (g/L.h); P_s = rate of substrate consumption (g/L.h); X_i = initial cell concentration (g/L); X_f = final cell concentration (g/L); P_i = initial product concentration (g/L); P_f = final product concentration (g/L); S_i = initialsubstrate concentration (g/L); S_f = final substrate concentration (g/L); T_f = time of fermentation (h); $(\Delta X)_c$ = variation of cell concentration due to cell growth (g/L); $(\Delta P)_c$ = variation of product concentration due to cell growth (g/L); $(\Delta S)_c$ = variation of substrate concentration due to cell growth (g/L).

It is well known that mash composition changes with time during fermentation due to cell metabolism, which leads to cell and product accumulation and substrate consumption (Figure 3). Average rates – despite being useful for giving a rough picture of the overall fermentation performance – don't explain how cell, substrate, and product concentrations vary during the process. It thus becomes necessary to determine instantaneous rates.

Figure 3 shows the profiles of cell (X), substrate (S), and product (P) concentrations during fermentation. The instantaneous rates, expressed as mass/volume x time (g/L/h, for instance), correlate with the inclination of tangents at each curve point, being mathematically represented as (dX/dt), (dS/dt), and (dP/dt).

If the mash volume remains constant during fermentation and there is no addition or loss of cells, substrate, or product in the fermenter, the variation rates of cell (dX/dt), substrate (dS/dt), and product (dP/dt) concentrations would only be due to cell growth. In this case, the following relationships can be considered:

$(dX/dt) = (dX/dt)_c$ (Eq. 4)

$- (dS/dt) = - (dS/dt)_c$ (Eq. 5)

$(dP/dt) = (dP/dt)_c$ (Eq. 6)

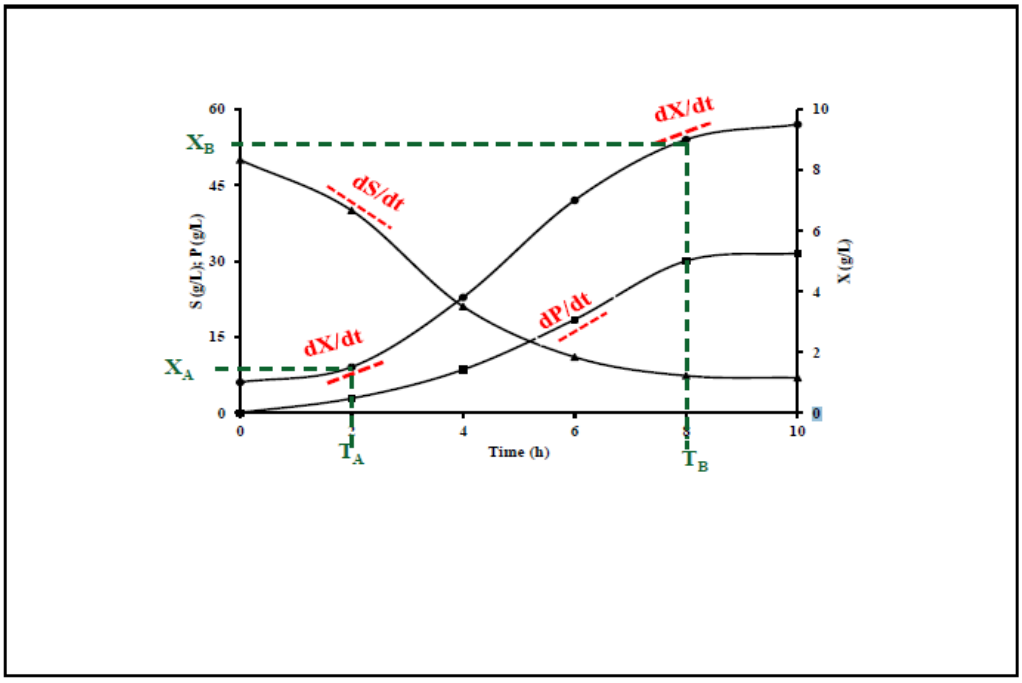

Figure 3: Variation of cell [X (●)], substrate [S (▲)], and product [P (■)] concentrations during batch fermentation.

Figure 3 shows that (dX/dt) has the same values at T_A and T_B since they have tangents with equal inclination. However, $X_B > X_A$ indicates that the variation rates would only be the same if cell growth at T_A is faster than at T_B. Thereby, to model this scenario, it is appropriate to now introduce the concept of specific growing rate (μ_x), which is defined as follows:

$\mu_x = (1/X).(dX/dt)_c$ (Eq. 7)

By analogy, the specific substrate consumption (μ_s) and specific product formation (μ_p) can also be defined as follows:

$\mu_s = - (1/X).(dS/dt)_c$ (Eq. 8)

$\mu_p = (1/X).(dP/dt)_c$ (Eq. 9)

The units μ_x, μ_s, and μ_p correspond to (g cells formed/g cells x h), (g substrate consumed/g cells x h), and (g product formed/g cells x h), respectively. The unit for specific rates is commonly written as h^{-1} for short.

It is assumed that all the substrate present in the mash will be converted by cells into product during fermentation, so it is appropriate to define the conversion factors related to S converted by the cells ($Y_{x/s}$) and into product ($Y_{p/s}$) as follows:

$Y_{x/s} = (X_f – X_i)/(S_i – S_f)$ (Eq. 10)

$Y_{p/s} = (P_f - P_i)/(S_i - S_f)$ (Eq. 11)

Equations 10 and 11 can be rewritten in terms of differentials so as to obtain the instantaneous conversion factors:

$Y_{X/S} = (X_f - X_i)/(S_i - S_f) = (dX)_c/(dS)_c = (dX/dt)_c/(dS/dt)_c = (1/X).(dX/dt)_c/(1/X)(dS/dt)_c = \mu_x/\mu_s$ (Eq. 12)

$Y_{P/S} = (P_f - P_i)/(S_i - S_f) = (dP)_c/(dS)_c = (dP/dt)_c/(dS/dt)_c = (1/X).(dP/dt)_c/(1/X)(dS/dt)_c = \mu_p/\mu_s$ (Eq. 13)

Types of fermentation

According to the physical nature of the mash to be fermented (liquid solution or solid mixture), fermentations can be categorized as submerged or semi-solid. Submerged fermentations are the most widely employed in microbial biotechnology. Focusing on submerged cultures, we have the following types: **a) Discontinuous (batch):** a microorganism is inoculated into a fixed volume of medium and, as growth takes place, nutrients are consumed and growth products (biomass, metabolites) accumulate. The nutrient environment within the fermenter is continuously changing, thus enforcing changes to cell metabolism. Eventually, cell division ceases because of an exhaustion or limitation of nutrient(s) and accumulation of toxic excreted waste products. The main characteristics of this process are time-variant reaction fluxes, discontinuous production, and downtime for cleaning and filling. The batch process can also be carried out with cell recycling, although being less common; **b) Semi-continuous (repeated fed-batch):** the fermenter is operated in batch mode for a determined period of time, followed by removing half of the fermented broth (sent to the downstream facility for separation of cells from products), which is replaced by an equal volume of fresh broth. In this case, cells may be recycled.[2,3] **c) Fed-batch:** the fermenter is filled with a small volume of broth that is inoculated with the microorganism. Volumes of fresh broth are added, step by step, up to a predetermined final volume. If the feeding is well planned, the end of fermentation and the final volume are reached simultaneously. Apart from normal sampling, no material is removed from the fermenter; therefore, the total quantity of material within the reactor will increase as a function of time. However if the feed is highly concentrated, then the reactor volume will not change much and can be regarded as essentially constant. The main characteristics of this process are an extension of batch growth or product production by additional substrate feeding, the possibility of operating with separate conditions for growth and production

phases, control possibilities using different feeding strategies, and development of high biomass and product concentrations.[4] **d) Continuous:** the fermenter is fed continuously with fresh medium, while at the same time depleted medium is continuously removed. The volume inside the fermenter is maintained constant by equalising addition/removal rates. This type of fermentation can be conducted either with one fermenter or by series or parallel coupling, with two or more fermenters. In both cases, cells may be recycled.

A general balance form can be derived by setting:

{Rate of accumulation}= {Input rate} - {(Output rate) ± {rate of production (+) /consumption (-)} and can be applied to the total volume (V) of the fermenter contents (Figure 4).

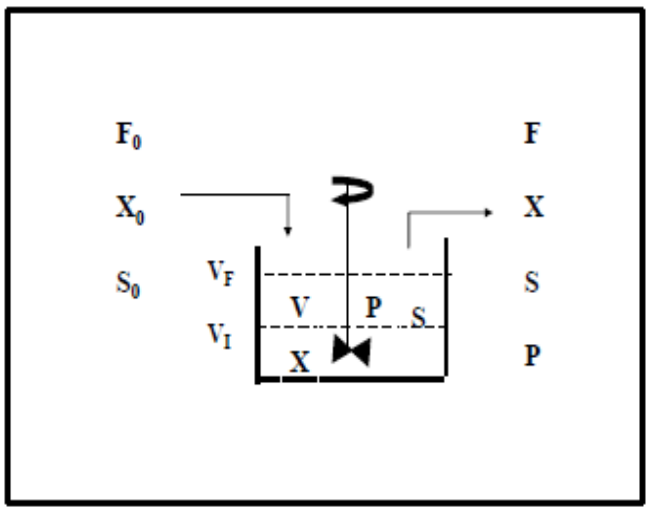

Figure 4: Sketch of continuous stirred fermenter that can be operated at batch ($F_o = F = 0$), fed-batch ($F = 0$; $\Delta V = (V_f - V_i)$, or continuous ($F_o = F > 0$) mode. Symbols: Input rate (F_o), initial concentrations of cells (X_o) and substrate (S_o), Output rate (F), final concentrations of cells (X), substrate (S), and product (P).

Batch Fermentation ($F_o = F = 0$)

Applying the biomass balance, we have:

$F_o.X_o - F.X + R_x = d(MX/dt)$ (Eq. 14)

As $F_o = F = 0$, then

$R_x = M.(dX/dt) + X.(dM/dt)$ (Eq. 15)

Considering the broth density (ρ=1) and the total biomass constant, the rate of biomass production (R_x) would be:

$R_x = V.(dX/dt)$ (Eq. 16)

Nevertheless, R_x is a parameter related to cell growth, so the equation can be written as:

$R_x = V.(dX/dt)_c$ (Eq. 17)

Equaling Equations (16) and (17) and dividing by cell concentration (X) results in:

$(1/X).(dX/dt) = (1/X).(dX/dt)_c$ (Eq. 18)

Considering the relation [$X = X_o.2^{t/\theta}$ (in which "t" is the culture time and "θ" the generation time)] and its derivation, we have:[5]

$(dX/dt)_c = X_o.2^{t/\theta}.(Ln\ 2)/\theta$ (Eq. 19)

Defining $\mu_x = (Ln\ 2)/\theta$ and $X = X_o.2^{t/\theta}$ (cell concentration at any moment of growth) results in:

$\mu_x = (1/X).(dX/dt)_c$ (Eq. 20)

The definition of μ_x (Eq. 7) is identical to that of Equation (20).

Comparing Equations (18) and (20), we have:

$\mu_x = (1/X).(dX/dt)$ (Eq. 21)

Therefore, in batch fermentation, biomass formation at any moment (dX/dt) is due only to specific cell growth rate.

The balances regarding substrate and product follow the same paradigm.

Continuous Fermentation ($F_o = F > 0$)

Applying the biomass balance, we have:

$F_o.X_o - F.X + R_x = d(M.X)/dt$ (Eq. 14)

Continuous fermentation is usually operated with sterile feed ($X_o = 0$); considering that broth density is not changed ($\rho = 1$; M=V), Equation (14) becomes:

$- F.X + R_x = V.(dX/dt)$ (Eq. 22)

Substituting R_x by Equation (17) and dividing both sides of Equation (22) by (V.X), we have:

$- (F/V) + (1/X).(dX/dt)_c = (1/X).(dX/dt)$ (Eq. 23)

Defining (F/V) = D (dilution rate) and substituting Equation (20), then Equation (23) becomes:

$\mu_x - D = (1/X).(dX/dt)$ (Eq. 24)

When the steady-state regimen is reached [(dX/dt) = 0], then:

$\mu_x = D = (1/X).(dX/dt)_c$ (Eq. 25)

Therefore, when the fermenter is operated continuously without cell recycling, the specific growth rate at steady-state is equal to the dilution rate.

Similarly, applying the substrate balance, we have:

$F_o.S_o – F.S – R_s = d(MS/dt)$ (Eq. 26)

Considering $\rho = 1$ ($M = V$), ($F_o = F$), $R_s = V.(dS/dt)_c$, and dividing both sides by V, then

$(F/V).(S_o – S) – (dS/dt)_c = (dS/dt)$ (Eq. 27)

Combining Equations (7) and (12) results in:

$(dS/dt)_c = (\mu_x.X)/Y_{x/s}$ (Eq. 28)

Where ($Y_{x/s}$) = substrate-to-cell conversion factor.

Substituting Equation (28) into Equation (27) gives:

$(dS/dt) = D.(S_o – S) – (\mu_x.X)/Y_{x/s}$ (Eq. 29)

At steady-state ($dS/dt = 0$ and $\mu_x = D$) and rearranging algebraically:

$S = S_o – (X/Y_{x/s})$ (Eq. 30)

Rewriting Equation (24) and assuming that $\mu_x = \mu_{max}$, then:

$(dX/dt) = X. \mu_{max} – DX$ (Eq. 31)

If ($D > \mu_{max}$), then (dX/dt) < 0. Thereby, the cell concentration inside the fermenter diminishes as the process goes on. This situation is known as **cell wash-out**.

Integrating Equation (31), we have:

$\text{Log } (X/X_i) = [t.(\mu_{max} – D)/2.303]$ (Eq. 32)

Where X_i = cell concentration before the steady-state is disrupted; X = cell concentration in the fermenter at time (t), counted from the moment in which the steady -state is disrupted.

The importance of Equation (32) is that it allows calculating μ_{max} for a continuous fermentation carried out under fixed conditions.

As cell and substrate concentrations are highly connected parameters in a continuous process, it is important to find a correlation with the dilution rate (D).

Monod demonstrated experimentally the equation.[5]

$\mu_x = (\mu_{max}.S)/(K_S + S)$ (Eq. 33)

Where K_S = Monod coefficient (g/L) [corresponding to the value of the limiting nutrient concentration which results in a growth rate of half the maximum value].

At steady-state, Equation (33) can be written as:

$S = (D.K_S/\mu_{max} – D)$ (Eq. 34)

When $D << \mu_{max}$, Equation (34) becomes:

$S = (D.K_S)/ \mu_{max}$ (Eq. 35)

So, the substrate concentration varies linearly with D.

Now, what happens with cell concentration (X) at $D << \mu_{max}$?

For that, one must rewrite Equation (30) as:

$X = Y_{x/s}.(S_o – S)$ (Eq. 35a)

At low D, practically all substrate fed into the fermenter is consumed so that $S \cong 0$, then cell concentration is approximately constant and equal to $(S_o.Y_{x/s})$. Conversely, when D approximates μ_{max}, then S tends to S_o and X tends to zero (wash-out conditions).

It can be seen that the dilution rate has a great influence on continuous fermentation. Using this kind of fermentation, the maximum substrate concentration can be converted to cell biomass and product over a long period of fermentation time. Establishing how productivity (DX) and dilution rate are related provides an estimate of the performance on a larger scale of continuous steady-state fermentation.

Rewriting Equation (20) at steady-state mode and coupled with Equation (35a) gives:

$(dX/dt)_c = D.Y_{x/s}.(S_o – S)$ (Eq. 36)

Substituting Equation (34), then:

$(dX/dt)_c = D.Y_{x/s}.\{S_o – [(D.K_S)/(\mu_{max} – D)]\}$ (Eq. 37)

Plotting $(dX/dt)_c$ or (DX; see Eq. 25) against D results in Figure 5. As can be seen, there is an optimal dilution rate after which the wash-out takes place. Conversely, for D lower than $D_{optimal}$, then $\mu_{max} > D$. Working at the optimal dilution rate, it is quite probable that the steady-state will be disrupted due to variations in feeding pump performance. Therefore, it is advisable to work at dilution rates at least 5% lower than the optimal rate.

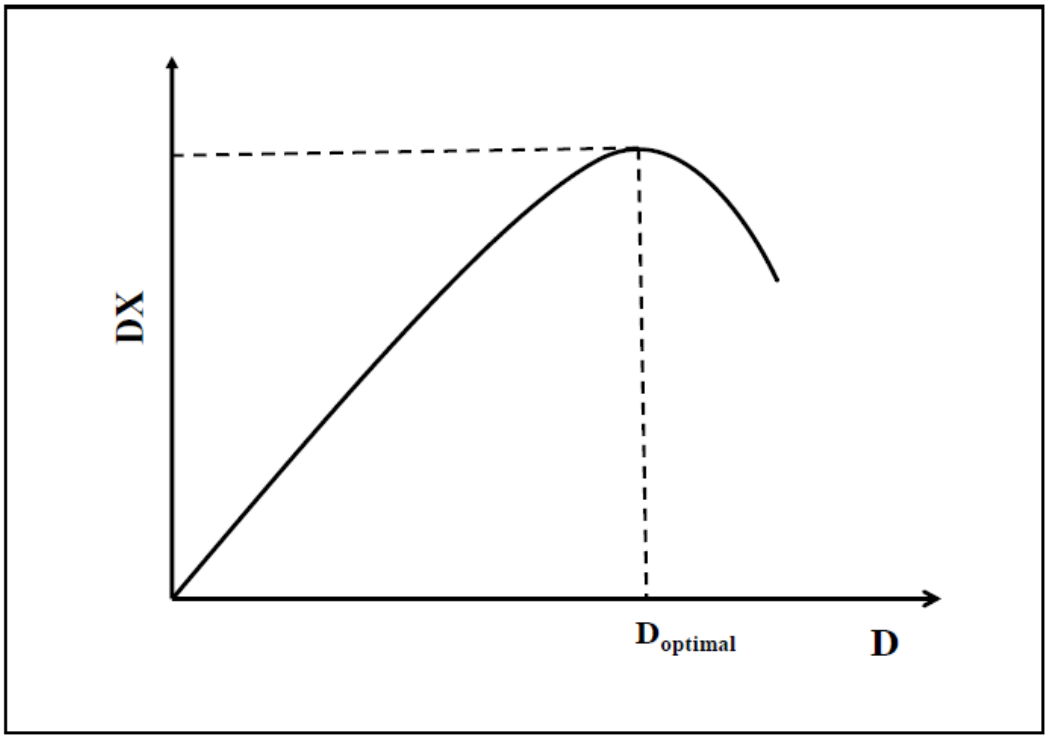

Figure 5: Variation of productivity (DX) against D for continuous fermentation.

Fed-Batch

As shown in Figure 4, the outlet is zero for a fed-batch fermenter, and the inlet flow (F_o) may be variable. As a result, the reactor volume will change with time. The addition of broth can be continuous – in this case the feeding obeys a particular addition law (exponential decreasing, linear increasing, among others)[1] – or by pulse (the substrate is added at high concentration and the volume of broth inside the fermenter doesn't vary significantly).

As the volume is variable in a fed-batch process, so the cell concentration (X) is a function of cell mass (M_x) and volume (V) as follows:

$X = M_x/V$ (Eq. 38)

As cell mass varies with time (dM_x/dt) in the fermenter, mass variation is equivalent to cell growth $(dM_x/dt)_c$.

Applying the biomass balance ($\rho = 1$), we have:

$d(V.X)/dt = V.(dX/dt)_c$ (Eq. 39)

Expanding the differential terms gives:

$(dX/dt) = (dX/dt)_c - (F_o.X)/V$ (Eq. 40)

The residual substrate concentration is also a function of residual substrate mass (M_{rs}) and volume (V), as follows (written in differential terms):

$(dS/dt) = (dM_{rs}/dt)/V$ (Eq. 41)

Applying the substrate balance ($\rho = 1$), we have:

$d(V.S)/dt = F_o.S_o - (dM_{rs}/dt)_c$ (Eq. 42)

Expanding the differential terms and substituting Equation (41) gives:

$(dS/dt)= (F_o/V).(S_o - S) - (dS/dt)_c$ (Eq. 43)

Considering the particular conditions under which the fed-batch process is performed, Equations (40) and (43) can only be solved analytically. Such a procedure is mathematically complicated, so that a practical solution is preferred to operate this kind of process under optimal conditions. For example, continuing the fed-batch by varying the volume in the fermenter from an initial volume (V_i) up to a final volume (V_f) under a defined type of feeding law (exponential, constant, linear etc.). The density of cells, amount of product formed, and the mass of substrate consumed would be recorded as the process proceeds.

As an example, one could set a linear increasing feeding law for addition of a substrate solution into a fermenter. The main starting equations are:

$F_o = dV/dt$ (Eq. 44)

$F_o = F^* + k.t$ (Eq. 45)

Where F_o = feeding rate (L/h), F^* = initial feeding rate (L/h), k = constant for addition (L/h^2), and t = addition time (h).

Equaling Equations (44) and (45), we have:

$dV/dt = F^* + k.t$ (Eq. 46)

Expanding the differential terms and making algebraic rearrangements gives:

$(V - V_i) = F^*.t + (k.t^2)/2$ (Eq. 47)

When $t = \theta$, then $V = V_f$ so Equation 47 becomes:

$(V_f - V_i) = F^*.\theta + (k.\theta^2)/2$ (Eq. 48)

Where θ = filling time (h).

As the feeding obeys an increasing linear law, so F^* is zero. After all, the addition must be made from a starting point, which can only be zero. Therefore, Equation (47) becomes:

$V_{ad} = (V - V_i) = (k.t^2)/2$ (Eq. 49)

Just to give an idea about the practical application of the above equations, let us consider as follows:[6] hydrolysis of sucrose (64 g/L) by invertase (780 UI) at pH 5.0, 100rpm, and 37 ^{O}C. The bioreactor has a capacity of 500 mL, and it is filled with a sucrose solution every 0.1 h from V_i = 100 mL up to V_f = 400 mL at θ = 1 h.

Substituting the values of parameters [($V_f - V_i$) = 0.3 L, F* = 0, and θ= 1 h] into Equation (48) gives k = 0.6 L/h^2. Introducing k into Equation (49), then:

$V_{ad} = 0.3t^2$ (Eq. 50)

By applying Equation (50), we have the results in Table 1.

Table 1: Volume of sucrose solution added (V_{ad}), volume to be added at each interval of 0.1 h (V'), and mass of sucrose (M_{suc}) added because of sucrose hydrolysis by invertase.

t (h)	V_{ad} (mL)	V' (mL)	M_{suc} (g)
0	3	3	0.192
0.1	12	9	0.576
0.2	27	15	0.960
0.3	48	21	1.344
0.4	75	27	1.728
0.5	108	33	2.112
0.6	147	39	2.496
0.7	192	45	2.880
0.8	243	51	3.264
0.9	300	57	3.648
1	-	-	-

Correlation between cell growth and product formation

Inside a normal microbial cell, thousands of reactions (constituting what is called metabolism) occur simultaneously and in an integrated manner. By integrated, we mean that molecules are made in the correct proportions and valuable nutrients are not wasted. This generates thousands of different low molar weight compounds (amino acids, vitamins, ribonucleotides, deoxyribonucleotides, fatty acids, acids, sugars, sugar alcohols, sugar acids, among others) and high molar weight compounds (proteins, nucleic acids, and polysaccharides, for instance). These molecules are then used to form cell structures such as nuclei, ribosomes, flagella, cell walls, membranes, and mitochondria. During the course of a microbial fermentation, all of these structures must be assembled within a maximum period of 1-3h (the average interval between each microbial generation time).

During fermentation, external parameters (pH, temperature, dissolved oxygen, concentrations of cells, nutrients, among others) related to the culture medium change. This change will

affect some cell metabolic pathways so as to over-produce a specific primary or secondary metabolite, i.e., the desired product. Microbes are amazingly flexible in their ability to alter their composition and metabolism in response to environmental changes. The environment does not change the genetic makeup of the cell, but markedly affects the phenotypic expression of the genes. However, over producing a desired product may require circumventing metabolic regulation mechanisms such as induction, catabolite regulation, feedback regulation, regulation in branched pathways, amino acid regulation of RNA synthesis, and energy charge regulation. Moreover, control of cell permeability must also be considered (mainly the mechanisms involved in the transport of substances across the cytoplasm membrane).

Induction is a metabolic process in which a specific enzyme is synthesized when a particular substance, the inducer (normally its natural substrate), is present in the culture medium. A closely-related compound can also stimulate the rate of enzyme synthesis. Catabolite regulation is the decrease in the rate of synthesis of a specific enzyme, caused by exposure of the cell to a rapidly assimilated carbon source. An example of an enzyme that undergoes this type of regulation is invertase from *Saccharomyces cerevisiae* (Figure 6). Fed-batch fermentation is a useful tool for circumventing catabolite regulation (Figure 7).[7] Feedback regulation is a control mechanism for biosynthetic enzymes that convert metabolic intermediates to the building blocks of macromolecules. There are two types of feedback regulation, i.e., feedback inhibition (phenomenon by which the final metabolite of a biochemical pathway inhibits the action of an enzyme involved earlier in the pathway – usually catalyzing the first reaction in the pathway) and feedback repression (refers to the inhibition of enzyme biosynthesis, i.e., the repressor acts at the level of transcription). Both mechanisms act to adjust the rate of product formation by a pathway and thereby the rate of synthesis of macromolecules. Regulation in branched pathways is needed when more than one end product arises from a common metabolic sequence that does branch at one or more points. The presence of several end products at the same time, if not managed properly, would surely bring about a chaotic situation for the cell. There are three regulatory devices for controlling this situation, i.e., differential regulation by isoenzymes (multiple enzymes are produced, each of which catalyzes the same reaction but is controlled by a different end product), concerted feedback regulation (only one enzyme is involved, but more than one end product must be present in excess to inhibit or repress the activity. An individual end product produces little to no negative effect), and cumulative feedback regulation (each end product is

capable of only a small degree of inhibition or repression by itself, even when added in excess. Combinations of end products show cumulative effects. Thus, if one effector causes 55% inhibition and other causes 30%, the combination could lead to 66.5% inhibition). Amino acid regulation of RNA synthesis occurs when an amino acid-requiring strain exhausts the supply of the amino acid in its growth medium; in this situation, not only does protein synthesis stop, but so does RNA synthesis. Energy charge regulation refers to the relative balance of ATP, ADP, and AMP inside the cell. Energy charge regulates the activities of both catabolic (leading to ATP formation) and biosynthetic (leading to ATP depletion) enzymes. Permeability control is a selective mechanism due to the presence of the cytoplasmic membrane – a thin delicate structure containing proteins and lipids – that defines the shape and size of all microbial cells. The thick cell wall surrounding the membrane of bacterial and fungal cells provides rigidity, but excludes only large molecules, leaving most of the selective activity to the plasma membrane. Whereas metabolic regulation prevents over-synthesis of metabolites and macromolecules essential to the life of the cell, the permeability barrier allows cells to retain concentrated solutions of these same molecules and to selectively bring essential nutrients into the cell. Culture conditions exercise a strong control over permeability.

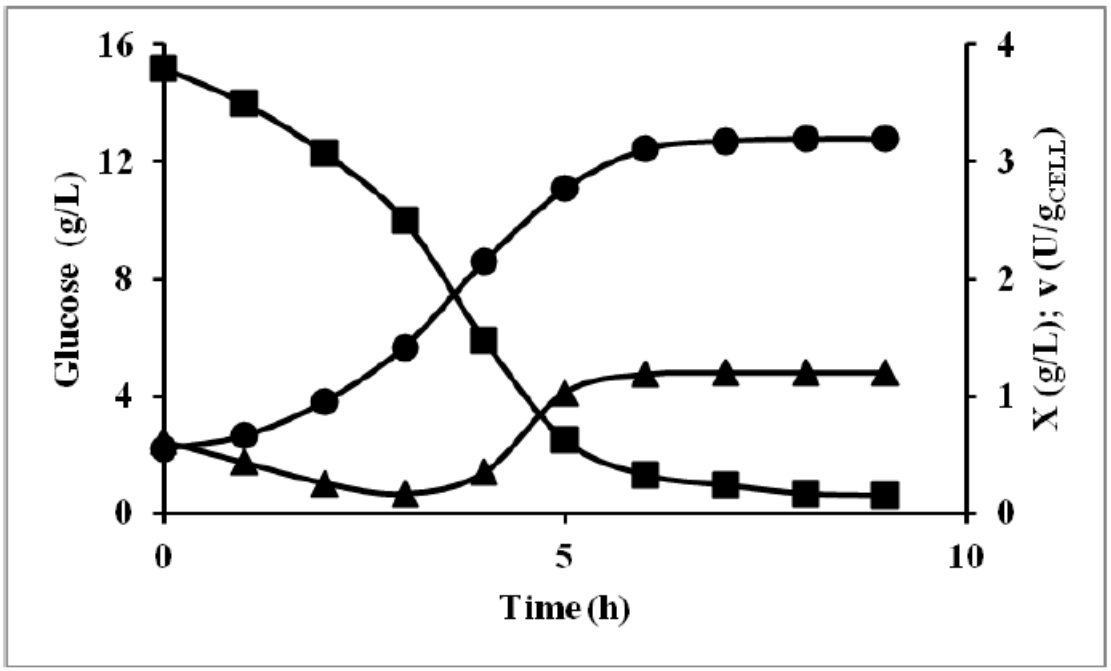

Figure 6: Catabolite regulation of invertase (▲) by glucose (■) in a batch process carried out at pH 4.0, 35 °C, 500 rpm, and aeration rate (1 L/L.min). Cell concentration (X) (*S. cerevisiae*) (●) [After t = 4 h, catabolite regulation was over].[8]

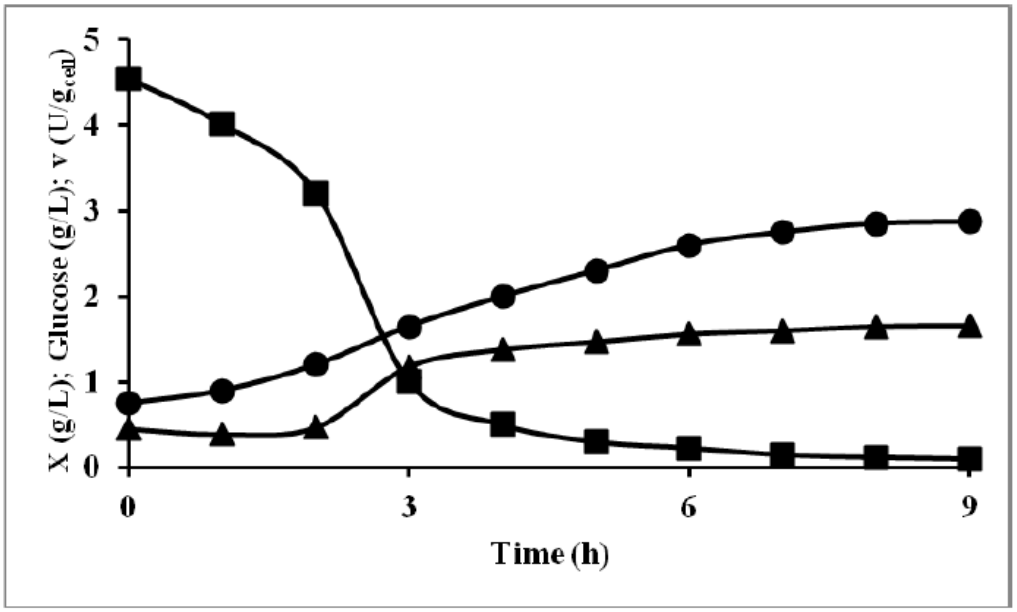

Figure 7: Catabolite regulation of invertase (▲) by glucose (■) in a fed-batch process (linear increasing mode) carried out at pH 4.0, 35 ^{O}C, 500 rpm, k = 0.13 L/h^2, θ = 4 h, ΔV = 0.3 L, and aeration rate (1 L/L.min). Cell concentration (X) (*S. cerevisiae*) (●) [After t = 2 h, catabolite regulation was over].[8]

As the main goal of any fermentation is the over-production of a primary or secondary metabolite, so the wild-type strain must be altered in some manner to overcome the tight control mechanisms described above. This can be achieved by using mutant strains – resulting from submitting the wild strain to chemical or physical mutagenic agents[9] – or by genetic engineering through modern techniques of recombinant DNA manipulation, and CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) or TALEN (Transcription Activator-Like Effector Nuclease) methods.[10]

The plasma membrane (PM) – a hydrophobic structure constituted by a phospholipid bilayer, proteins, and cholesterol - of any cell acts as a selective barrier between intra- and extracellular environments, being responsible for the permeability of different molecules (Figure 8).

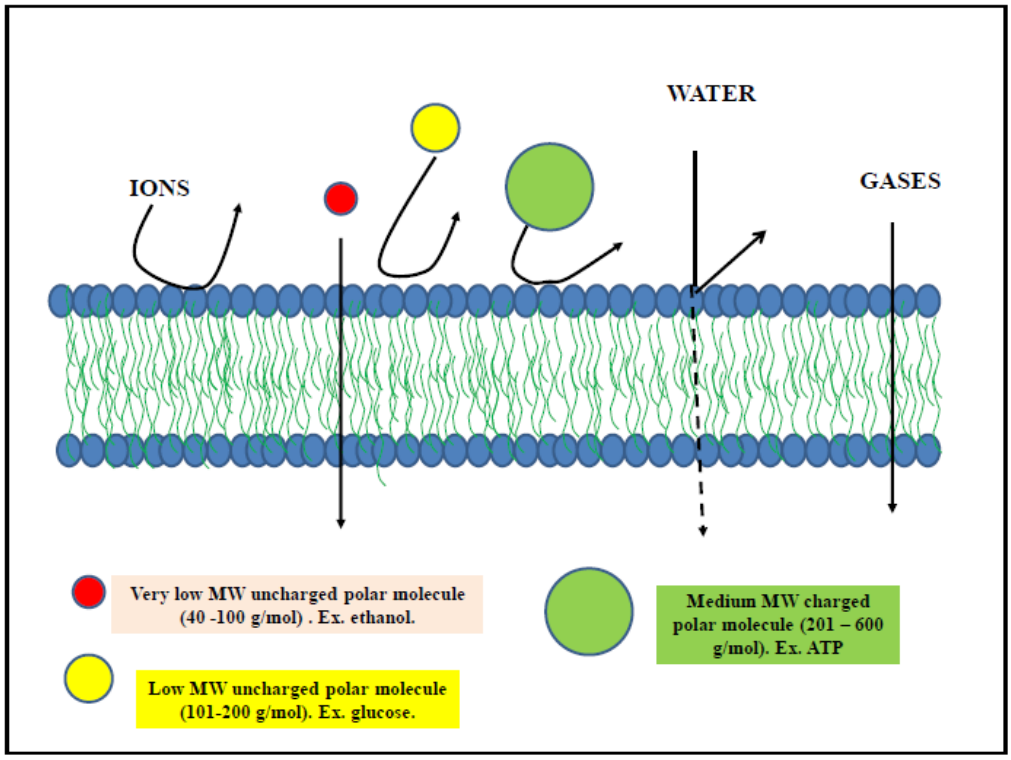

Figure 8: Selective permeability of the membrane phospholipid bilayer.

The transport across PM can be intermediated or not by proteins. Except for passive diffusion and osmosis, all other transport mechanisms involve proteins.

In the transport process called passive diffusion, a low MW molecule (ethanol and gases in general) in aqueous medium dissolves into the lipid bilayer, crosses it, and then dissolves into the cytoplasm. Therefore, no protein is involved and the diffusion driving-force is due to the hydrophobicity and concentration gradient of the molecule. In aerobic cell fermentation, the transfer of oxygen from the bubble air to the cell follows the passive diffusion pattern (Figure 9). Osmosis occurs when a solvent crosses a semi-permeable membrane from a diluted phase to a concentrated one, and the driving-force is called osmotic pressure. Almost all cells and cell organelles are subjected to osmosis since the water movement can follow: (environment ⇆ cell cytoplasm) and/or (organelles ⇆ cytosol).

The so-called facilitated diffusion is intermediated by a membrane protein (anion exchange protein, for instance) that, in the presence of the molecule to be up-taken, undergoes conformational change leading to the interchange between outward- and inward-facing conformations (Figure 10A). This type of transport does not require energy consumption

because the mechanism is thermodynamically favored, i.e., the Gibbs free energy of the process is negative.[11]

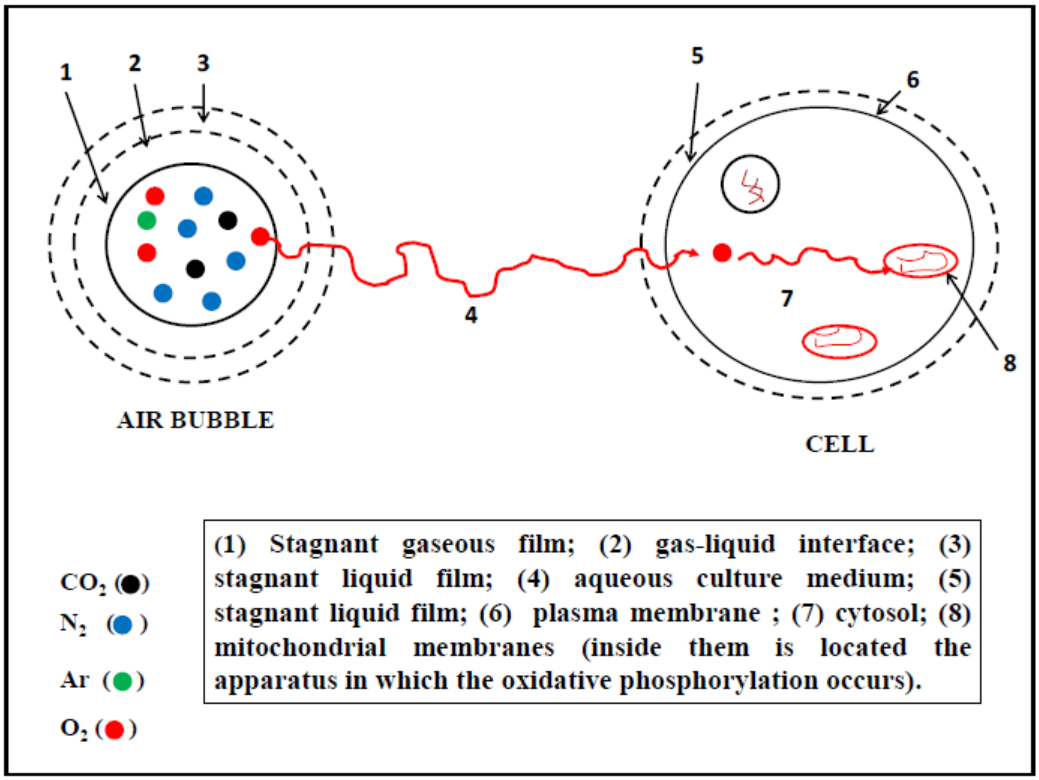

Figure 9: Oxygen transfer from the air bubble to the mitochondria. The barriers to be crossed are: diffusion from the bubble to the medium (1, 2, and 3), diffusion through the medium until the plasma membrane (4 and 5), and diffusion through the cytosol until the mitochondrial membranes (6, 7, and 8).

The active transport across PM promoted by membrane proteins can be coupled or not with ATP hydrolysis. In all situations that an energetically unfavorable movement of ions or molecules must occur, the energy needed for moving the protein inside PM comes from the reaction: ATP → ADP + Pi (ΔG = - 7.7 Kcal/mol). Moreover, there are membrane protein transporters not linked to ATP hydrolysis, called uniporter, symporter, and antiporter (Figure 10B). Uniporters catalyze only the energetically favorable (downhill) movement of substances, while symporters and antiporters couple the downhill movement of one substance to the uphill movement of another one.

Despite all equations in fermentation technology being deduced assuming the cell to be a black box, professionals must always remember that experimental deviations, usually not

rare, may be due to permeation mechanisms of the plasma membrane and/or organelle membranes.

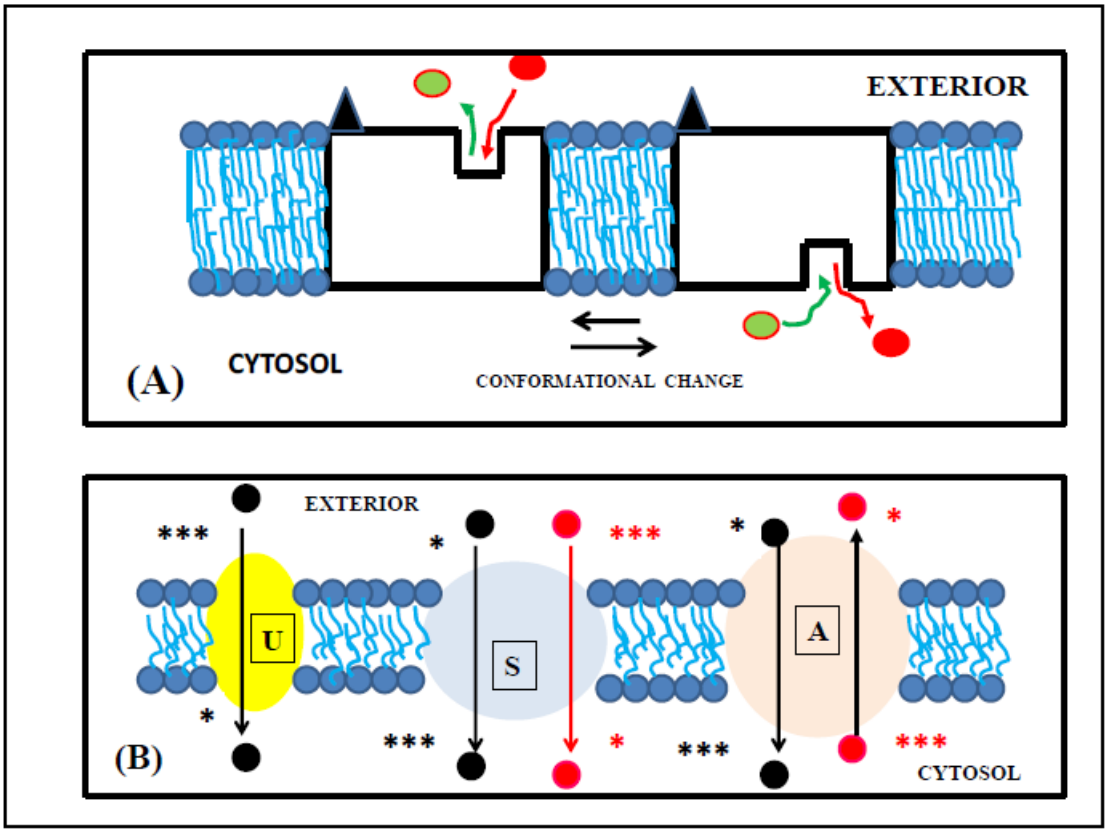

Figure 10: Sketch illustrating the action of membrane transporters. (A) Facilitated transport: the transport protein suffers only a reversible conformational changing; (B) The three groups of transporters: uniporter (U), symporter (S), and antiporter (A). Gradients of concentration and/or electrical potential are indicated by asterisks (*low and ***high).

The dependence of microbial fermentation kinetics on the composition and physicochemical conditions of the culture medium as well as on the complexity of the permeation through plasma membrane and intracellular environment can be envisaged by graphically comparing cell and product formation (Figure 11). Three situations arise from observing Figure 11: a) product and cell formation are associated (for example, ethanol and amino acids fermentation); b) product and cell formation are not associated (antibiotic fermentation, for instance); c) product and cell formation are partially associated (for example, organic acids fermentation).

The situations cited occur in batch fermentations which follow, by nature, a transient regimen. Notwithstanding, enzyme activity varies even in continuous fermentation at steady-state (Figure 12). At first glance, the cell and substrate concentrations would indicate that the metabolism of cells in a chemostat at steady-state would be in equilibrium with the conditions of the culture medium. However, this is not observed when a specific product is formed during the steady-state phase – such as invertase formation by *Saccharomyces cerevisiae*, which oscillates along this phase (Figure 12). Clearly, the complex intracellular metabolic apparatus acts independently of the extracellular environment.

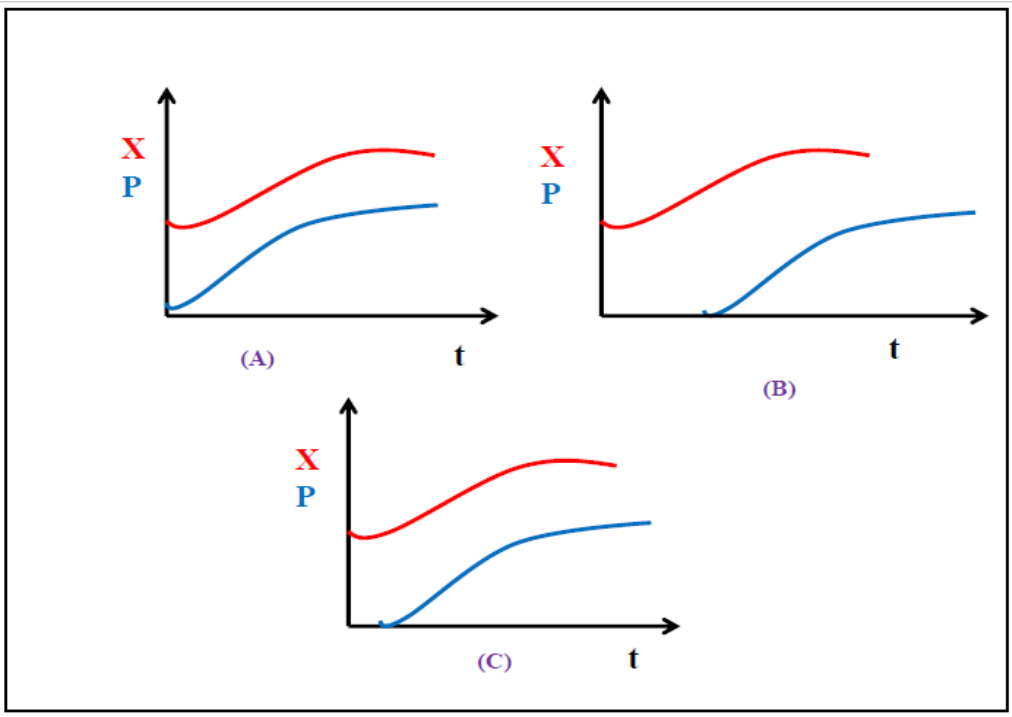

Figure 11: Formation of cells (X) and product (P) against time: (A) Coupled product and cell formation; (B) Uncoupled product and cell formation; (C) Partially coupled product and cell formation.

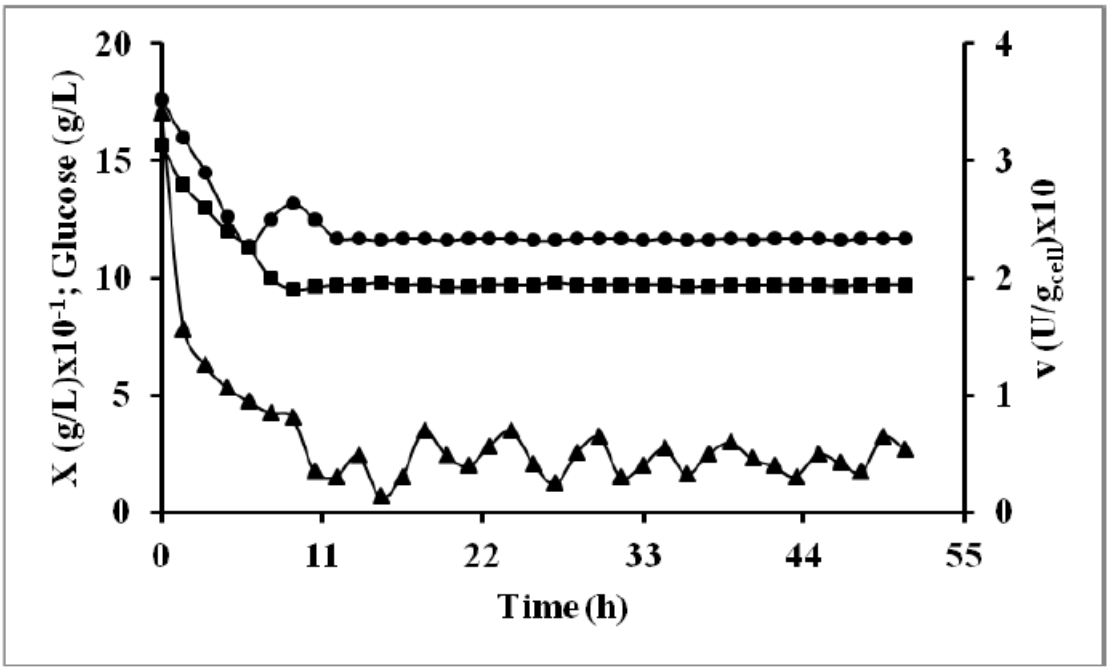

Figure 12: Invertase activity (▲), concentration of glucose (■) and cells (●) in steady-state continuous fermentation of *Saccharomyces cerevisiae* cultivated in blackstrap molasses. Culture conditions: Agitation = 500 rpm, D = 0.23 h^{-1}, air bubbling = 1.0 v/v/m, pH = 4.0, and 30 ^{O}C.[12]

CONCLUSIONS

In the study of microbial growth kinetics during fermentation, the cell is taken as a black-box in order to derivate equations that allow quantifying the fermentation process. Considering the general balance relation written as [RATE OF ACCUMULATION] = [INPUT RATE] – [OUTPUT RATE] + [RATE OF PRODUCTION], it is possible to deduce the operational equations for batch, fed-batch, or continuous fermentation. For example, in continuous mode, it is possible to set the dilution rate that leads to the highest product productivity. Moreover, product formation and cell growth during fermentation can be total, partial, or not correlated depending on both the metabolic regulation under which the product is submitted and the availability of key nutrients to the cell. The latter, in turn, depends on the molecular transport across the plasma membrane and/or organelle membranes.

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