Chapter 6

Bioprocess Modeling and Control

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Additional information is available at the end of the chapter

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1. Introduction

The bioprocess advancement is determined by the living cells capabilities and characteristics, the bioreactor performance as well as by the cultivation media composition and the main parameters evolution. The high metabolic network complexity inside the cells often determine very sophisticated, non-linear growth and product formation kinetics, with further consequences on the bioprocess behavior, but at the same time on the product quality and yield.

The key issue of this rather complicated situation is the use of modeling and further on of computer assisted control as a powerful tool for bioprocess improving. The process models, as relationships of the input, output and inner variables, though incomplete and simplified, can be effective to describe the phenomena and the influences of great importance for control, optimization and better theoretical knowledge. The function of any biological model is to describe the metabolic reactions rates and their stoichiometry on the basis of bioreactor conditions, with the main difficulties-the identification of principal factors affecting cellular growth and bioproduct formation, and the building up of a suitable model structure for the intracellular processes.

Moreover the scheduling, supervision and automatic control in modern bioprocessing is done by advanced process control systems, where all the functions are implemented in software (in accordance with the Figure 1). The main bioprocess control attributes are: handling of off-line analyses; recipe and scheduling; high level overall control; state and parameters estimation; simulation; prediction; optimization.

For the industrial developments the central and manifold objective of the computer control is the realization of the economic interests in assuring high operational stability, process reproducibility and increased product yield together with the maintaining of rigorous safety and the implementation of the GMP or environmental regulations, important requests in modern biomanufacture imposed by the product quality improving needs.

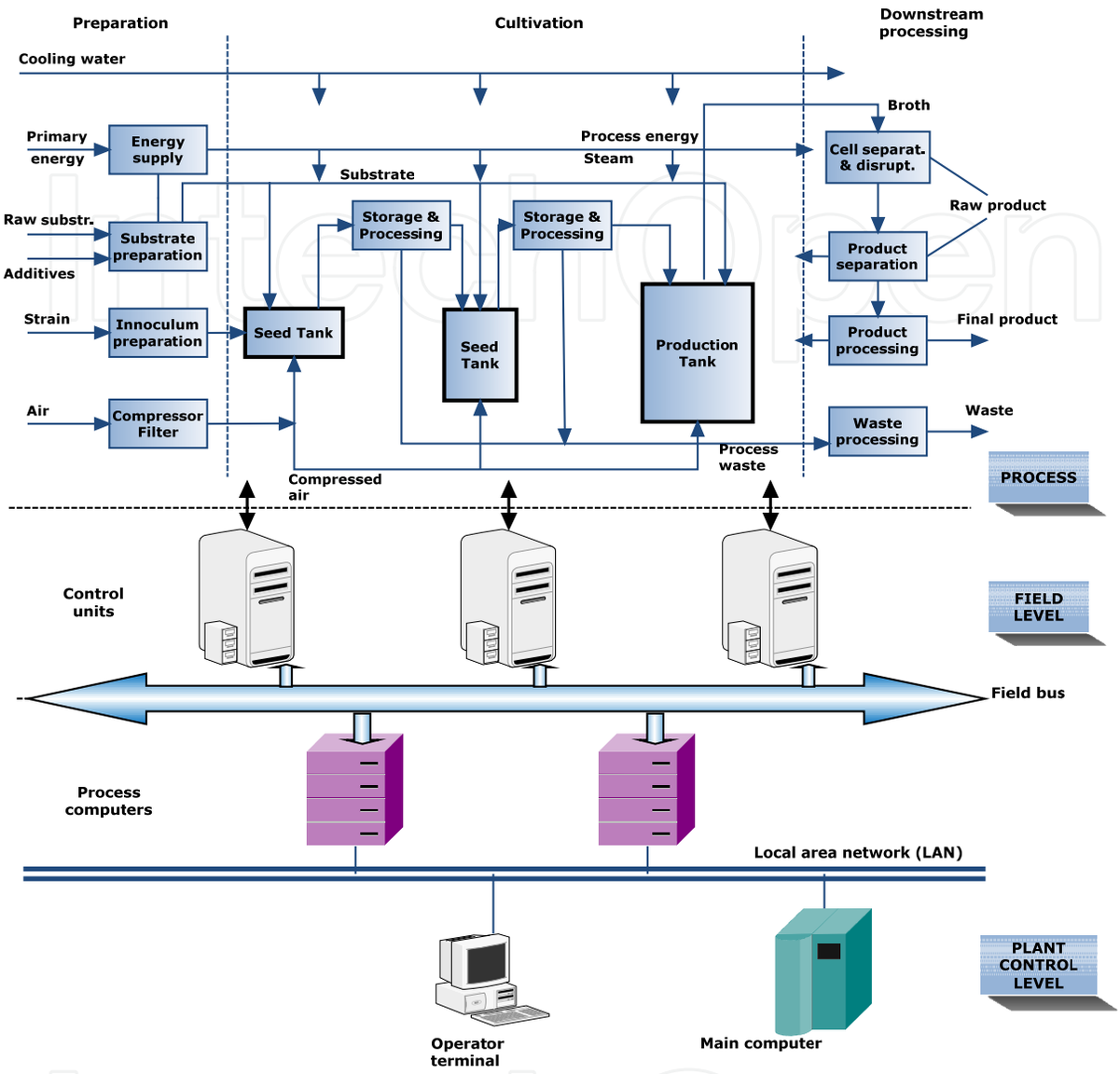


Figure 1. General presentation of the computerized bioprocess control [2]

2. Instruments and techniques for bioprocess variables determination and monitoring

To achieve the biological potential of cells, the optimal environmental conditions must be maintained in the bioreactor for cell growth / product formation, at least with regard to the key parameters. Generally speaking, biological systems are influenced by different process variables, which have a direct influence on cell metabolism. Sensors for these variables are (typically) inserted into specially designed ports on the bioreactor. As bioreactors increase in size (i.e. in the industry field), the mixing problems become usual and probe location becomes problematic. To accurately outline large fermenters, probes may be collected from several locations.

A. Direct physical determinations

The existence of defined and optimal environmental conditions for biomass and product formation means that different physical and chemical parameters require to be kept constant or conforming to an optimal evolution trend during the process, i.e. any deviation from a specified optimum might be corrected by a control system.

The standard direct physical determinations are[3]: (1) temperature; (2) pressure (over pressure); (3) agitator shaft power and rate of stirring; (4) foam; (5) gas and liquid flow; (6) weight.

Temperature determination is important for bioprocess evolution as well as other process operations (i.e. sterilization, concentration, and purification). The temperature measurement is made in the range +20^{o}C to +130^{o}C through mercury-in-glass thermometers, bimetallic thermometers, pressure bulb thermometers, thermocouples, metal-resistance thermometers or thermistors; all of them must be steam-sterilizable at 120^{o}C. The most popular are the Pt100 resistance thermometers.

Pressure measurements may be needed for several reasons; the most important of them is the safety. Industrial and laboratory equipment is designed to withstand a specified working pressure plus a factor of safety. Also, the measurement of pressure is important in media sterilization. Moreover, the pressure will influence the solubility of gases and contribute to the maintenance of sterility, when a positive pressure is present. The standard measuring sensor is the membrane pressure gauge based on strain or capacitance measurements.

The formation of **foam** can create serious problems in no controlled situations: loss of broth, clogging of gas analyzers, infections, etc. It is a common practice to add an antifoam agent when the culture starts foaming above a certain predetermined level. A standard foam sensing consists in an electrical conductivity / capacitance / heat conductivity probe.

A number of mechanical antifoam devices have been made, including discs, propellers, brushes attached to the agitator shaft above the surface of the broth. Unfortunately, most of the mechanical devices have to be used in conjunction with an antifoam agent, without negative influence on the bioprocess behavior.

B. Direct chemical determinations

The regular chemical determinations are [3]: (1) pH; (2) redox potential; (3) dissolved oxygen concentration (pO_2); (4) exit-gas analysis; (5) on-line analysis of other chemical factors (ion-specific sensors, enzyme electrodes, microbial electrodes, mass spectrometers, fluorimeters).

In most processes there is a need for **pH** monitoring and control if maximum yield of a product is to be obtained. The pH may be further controlled by the addition of appropriate quantities of alkaline or acid solutions, depending of the characteristic pH trend evolution. Normally, the pH drift is only in one direction. pH measurement is carried out using a combined glass reference electrode that will withstand repeated sterilization at temperature of 120^{o}C and pressures of 138kN/m^2.

In most aerobic fermentations it is essential that the **dissolved oxygen concentration** does not fall below a specified minimal level. If in small fermenter the most used electrodes are galvanic, the polarographic electrodes are more commonly used in pilot or production bioreactors. For an increase of precision, they are both pressure and temperature compensated.

C. On-line analysis of other chemical compounds

Ion-specific sensors have been developed to measure NH_3^+, Ca^{2+}, K^+, Mg^{2+}, PO_4^{3-}, etc. However, none of these probes are steam sterilizable.

The **mass spectrometer** [4] can be used for in-line analysis since it is very versatile, but unfortunately expensive. It does allow for monitoring of gas partial pressures (O_2, CO_2, CH_4, etc.), dissolved gases (O_2, CO_2, CH_4, etc.), concentrations of volatiles (methanol, ethanol, acetone).

The **fluorimetric measurements** [5,6] are very specific and rapid, but their use in bioprocess is quasi-limited nowadays. Hence, the measurement of NAD (provided it remains at a constant concentration in cells) would be an ideal method for continuous measurement of microbial biomass concentration.

The **biosensor** [7] is based on a biological receptor, which is coupled to an electronic transducer that converts the biological signal into an electrical signal by measuring voltage, current, light, temperature. Biosensors can be used to measure the concentration of different substrates / metabolites in the culture broth. In order to avoid the possible effects on growth / product formation (i.e. inhibition), the biological receptor can be immobilized on a separate membrane or on the transducer surface. **Enzyme electrodes** are the most applied, normally for in-line determinations (no steam sterilizable). The specific enzyme is immobilized on a membrane held in close contact to a pH or oxygen electrode. Also **microbial electrodes** using immobilized whole cells have been used for determination of sugars, acetic acid, ethyl alcohol, vitamin B, nicotinic acid, glutamic acid and cephalosporin. Generally speaking, the biosensors have been investigated with limited success. Hence, only the glucose biosensors have been fully applied. The main difficulties in developing an on-line biosensor are the thermal stability of the immobilized biomaterial, i.e. inactivation during sterilization, and the limited linear range inherent to biologic species.

It is to consider two most recent directions of development regarding the bioprocess variables monitoring and control [8]: (a) **realization of miniaturized sensors for the *in situ* measurement** of temperature, pH or dissolved oxygen; (b) **use of analyzers** not yet applied for on-line process monitoring in biotechnology, but of real interest: the continuous air-segmented flow analyzer (**CFA**); the flow injection analyzer (**FIA**); and the High Performance Liquid Chromatography (**HPLC**).

3. The mathematical modeling of the aerobic bioprocess

The aerobic bioprocess modeling is an useful tool to accomplish several important tasks [2]: (a) it can be the basis for adequate optimization and control technique applications; (b) it can

provide the necessary information about the features of the chosen bioprocessing system; (c) it synthesizes the characteristics of the specified living cells' evolution and hence, it is the best technique to predict the process efficiency.

The models show the complex biosystems attributes; so they must be as possible as extensive and non-speculative. Moreover the models are an acceptable compromise between the presentation of processes in detail, with considerable number of parameters, and the use of few parameters, easy to apply and estimate.

Most important properties of a biological mathematical model were defined in the Edwards and Wilke' postulates [2]: (a) it is capable to represent all the culture phases; (b) it is flexible enough to approximate different data types without the insertion of significant distortions; (c) it must be continuously derivable; (d) it must be easy to operate, once the parameters evaluated; (e) each model parameter is to have a physic significance and must be easy to evaluate.

The attempts to realize high global models were not successful: firstly, due to the impossibility to measure on-line the great number of bioprocess parameters, and secondly, due to the high degree of complexity. Finally several types of models can represent the evolution of the aerobic bioprocess. The most important categories will be presented further on.

1. The unstructured global models are in use nowadays as the main tool for both the bioprocess modeling, but also for being applied in overall computer control [2]. Their limit is they are a simplified representation of the bioprocess behavior: conforming to this concept the bioprocess evolution depends directly and only on the macroscopic variables representing the working conditions in the bioreactor. Therefore the unstructured models are essentially kinetic equations that describe the variation of substrate or product concentrations and of a unique biological state variable-the cell concentration, and can also express the influences of some important process variables (pH, pO_2, temperature, and others), and only sometimes they are balance equations.

Generally speaking [9], one considers that the specific growth rate ($\mu = \frac{1}{X}\frac{dX}{dt}$) is the key variable for cell growth, substrate consumption and product formation. The specific growth rate is time dependent and dependent on different physical, chemical and/or biological parameters (substrate concentration-S, cell concentration-X, product concentration-P, pH, temperature-T, dissolved oxygen concentration-C, and different inhibitors-I).

Conforming to the literature assumptions [10], the specific growth rate dependence upon different process parameters can be considered as follows:

$$\mu = f\left(S, X, P, pH, C, I, ..., t\right) \tag{1}$$

a. μ=μ(S) Kinetic models with growth limitation through substrate concentration (without inhibition) Main model equations [2, 11] are presented in Table 1.

Model equation	Constants	Authors	Comments
$\mu(S)=\frac{\mu_{\max}S}{K_S+S}$ (2)	μmax=max specific growth rate [1/h] KS = saturation constant [g/L]	Monod equation (1942, 1949)	Empirically derived from the Michaelis & Menten equation
$\mu(S)=\frac{\mu_{\max}S^n}{K_S+S^n}$ (3)		Moser equation (1988)	Analogy with a Hill kinetic (n>0)
$\mu(S)=\mu_{\max}\frac{S}{K_S+K_D+S}$ (4)	KD=diffusion constant	Powell equation (1958)	Influence of cell permeability, substrate diffusion and cell dimensions through KD parameter

Table 1. Models $\mu=\mu(S)$

There are also some models, which utilize the substrate concentration in more complex structures. Nyholm (1976) introduces a dual function for substrate utilization: consumption (including assimilation and dissimilation in the liquid phase) and growth (substrate utilization for growth):

$$S \xrightarrow{k_{e\lim}} S_e \xrightarrow{k_{\deg rad}} S_a \tag{5}$$

S_e is the substrate for growth and S_a the substrate used for consumption. The growth rate is linked to the intracellular concentration of limiting substrate (S_{int}/X) and to *preserved* substrates (i.e. inorganic ions or vitamins, not decomposed through cell metabolism) with application in wastewater bio treatment:

$$\mu=\mu\frac{\frac{S_{\text{int}}}{X}}{\frac{dS_{\text{int}}}{dt}}=r_{S_{e\lim}}-r_{S_{\deg rad}} \tag{6}$$

b. μ=μ(X, S) The influence of cell and substrate concentrations upon the specific growth rate[2, 11]

Model equation	Constants	Authors	Comments
$\mu(X)=\mu_{\max}(1-k_xX)$ (7)	kX=kinetic constant	Verhulst (1845)	It is known as growth logistic model
$\mu(X,S)=\mu_{\max}\frac{S_0-\frac{X}{Y}}{K_S+S_0-\frac{X}{Y}}$ (8)	So=substrate initial concentration Y=substrate/cell yield.	Meyrath (1973)	It is based on Monod kinetics.

$N = N_0 \exp(\mu_{max} t) = \frac{N_0 \mu_{max}^0 \exp(\mu_{max}^0 t)}{\mu_{max}^0 + m_x N_0 (\exp(\mu_{max}^0 t) - 1)}$ (9)	N=population density m=limiting size of the population (the carrying capacity)	Verhulst – Pearl kinetics	Logistic growth: combination between the population trend to growth according to a geometric progression and the environment tendency to limit the excessively high densities of the population
$\mu = \mu_{max} \frac{S}{K_X X + S}$ (10)	KX=kinetic constant	Contois (Contois – Fujimoto) equation (1959):	If S = constant, the only dependence remains μ = f(X).

Table 2. Models $\mu = \mu(X, S)$

c. Growth kinetics with substrate inhibition

In most cases, the kinetic model equations are derived (like the Monod model) from the inhibition theory of enzymatic reactions. Consequently they are not generally valid and can be applied in connection with experimental acceptability [2, 11].

Model equation	Constants definition	Authors name	Comments
$\mu = \mu_{max} \frac{1}{1 + \frac{K_S}{S} + \frac{S}{K_i}} = \frac{S}{K_S + S} \frac{1}{1 + \frac{S}{K_i}}$ (11)	Ki = inhibition constant	Andrews model (1968)	Substrate inhibition in a chemostat
$\mu = \mu_{max} \frac{S(1 + \frac{S}{K_S^l})}{S + K_S \frac{S^2}{K_S^l}}$ (12)	Ksl= inhibition constant	Webb model (1963)	
$\mu = \mu_{max} \frac{1}{1 + \frac{K_S}{S} + \sum_j (\frac{S}{K_{i,S}})^j}$ (13)	Ki,S= inhibition constant	Yano model (1966)	
$\mu = \mu_{max} \frac{S}{K_S + S} e^{-\frac{S}{K_{i,S}}}$ (14)		Aiba model (1965)	

Table 3. Growth kinetics with substrate inhibition

d. μ = f(S, P) Growth kinetic with product inhibition [2, 11]

Hinshelwood (1946) detected product inhibition influences upon the specific growth rate: linear decrease, exponential decrease, growth sudden stop, and linear/exponential decrease

in comparison with a threshold value of P. The first type (Hinshelwood - Dagley model):

$$\mu(S,P) = \mu_{\max}\frac{S}{K_S + S}(1 - kP) \tag{15}$$

where: k = inhibition constant (considering the product concentration influence).

Model equation	Constants definition	Authors name
$\mu(P) = \mu_{\max} - K_1(P - K_2)$ (16)	K1, K2 = constants (>0)	Holzberg model (1967)
$\mu(P) = \mu_{\max}(1 - \frac{P}{P_{\max}})$ (17)	Pmax = maximum product concentration.	Ghose and Tyagi model (1979)
$\mu(P) = \mu_{\max}e^{-K_1P(t)}$ (18)	K1 = constant	Aiba (1982):
$\mu(S,P) = \mu_{\max}\frac{S}{K_S + S}e^{-KP}$ (19)		Aiba and Shoda model (1989)

Table 4. Models $\mu = f(S, P)$

e. The influence of dissolved oxygen (as a second substrate) upon the specific growth rate

In some cases it is needed to consider the dissolved oxygen as a second substrate. The most used equation is the kinetic model with double growth limitation, μ(S, C) [2, 11]

i. Olsson model:

$$\mu(S,C) = \mu_{\max}\frac{S}{K_S + S}\frac{C}{K_C + C} \tag{20}$$

where: K_C = oxygen saturation constant.

ii. *Williams' model*, which also quantifies the P influence (K_P=P saturation constant; K_1, K_2, K_3, K_4=modeling constants):

$$\mu(S,C,P) = \left(\frac{K_1S}{K_S + S}\cdot\frac{K_2P}{K_P + P}\right)\cdot\left(\frac{C}{K_C + C} + K_3C - K_4\right) \tag{21}$$

f. μ(S_1, S_2) Kinetic models based on different substrates

Besides the case when the dissolved oxygen is considered as a second substrate, there are many cases when two or more carbon sources are taken into consideration. There are two typical situations: (1) the cells grow through the sequential (consecutive) substrate consumption (diauxic growth), where a simple Monod model can be applied; (2) the cells grow through the simultaneous consumption of substrates (e.g. wastewater treatment); in this case, the mathematical modeling is more complex.

g. Unstructured kinetic models for product formation

The product formation kinetic is taken into account in conjunction with the growth kinetic. Nowadays, the Gaden [3] classification is still useful. Based on this categorizing, four kinetic types can be defined:

Type 0: This production type occurs even in resting cells that use only a little substrate for their own metabolism. The microbial cells function only as enzyme carriers. Some examples are provided by steroid transformation and vitamin E synthesis by *Saccharomyces cerevisiae.*

Type 1: Type-1 situations include processes in which product accumulation is directly associated with growth; in this case the product formation is linked to the energy metabolism. Examples include fermentation to produce alcohol and gluconic acid and situations in biological wastewater treatment.

Type 2: Type-2 bioprocesses include fermentations in which there is no direct connection between growth and product formation (for example, penicillin and streptomycin synthesis).

Type 3: This production type includes those having a partial association with growth and thus, an indirect link to energy metabolism (e.g. citric acid and amino acid production)

Afterward there are now more advanced models, the structured and the segregated models.

2. In case of the structured models [12, 13] the biotic phase is not any more viewed as a homogenous component, but they provide information about the physiological state of the cells, their composition and regulatory adaptation to the environment. Conforming to this concept the cell mass is structured in several intracellular compounds and functional groups, which are connected to each other and to the environment by fluxes of material and information. The structured models can be: multi compartment models, genetically structured models, and biochemical structured models.

A case study of **the biochemical structured model is the modeling of Penicillin V biosynthesis:** The model of *Penicillin V* biosynthesis [2] is a tool for both: the understanding of the kinetic function of the precursors, the dissolved oxygen, enzymes activities, formation of metabolic intermediates and by-products (the determination of the metabolic step responsible for the global rate limitation can be a basis for the genetic engineering modification of the enzyme expression involved in this metabolic reaction); the bioprocess computer control.

First it is the metabolic pathway with the L-Cysteine, L-Valine and α-Aminoadipic Acid (AAA) as the initial substrates, which can form together Tripeptide ACV (α-α-aminoadipyl-L-cysteinyl-D valine). The further cyclisation reaction of Tripeptide ACV to Isopenicillin N (IPN) is oxygen dependent. The following reactions can be done directly in one step or in two steps. In this second case the intermediate is the 6-Aminopenicillanic Acid (6-APA), with the precursors Phenylacetic Acid (PAA) for Penicillin G or Phenoxyacetic Acid (POA) for Penicillin V, to be incorporated into the Penicillin molecule during the last step. It is also possible in parallel with Penicillin G and Penicillin V formation that 6-APA is alternatively

carboxylated with CO_2 to form 8-HPA (8-hydroxy-Penicillinic Acid).The model for Penicillin V biosynthesis is presented in Table 5.

Metabolic step	Kinetic equation
ACV formation by ACV Synthetase	$r_1 = k_1 X_{ACVS} \cdot \frac{1}{\left(1 + \frac{K_{AAA}}{C_{AAA}} + \frac{K_{CYS}}{C_{CYS}} + \frac{K_{VAL}}{C_{VAL}}\right)} \cdot \frac{1}{1 + \frac{C_{ACV}}{K_{ACV}}}$ (22)
Isopenicillin N formation by IPN Synthetase	$r_2 = k_2 X_{IPNS} \cdot \frac{C_{ACV}}{C_{ACV} + K_o(1 + \frac{C_{Glut}}{K_L})C_o}$ (23)
Formation of 6-APA from IPN by Isopenicillin N Amidohydrolase (IAH)	$r_3 = k_3 X_{IAH} \frac{C_{IPN}}{C_{IPN} + K_{IPN}}$ (24)
Formation of Penicillin V from activated side chain precursor and 6-APA by Acyl-CoA and 6-APA Acyltransferase (AT)	$r_4 = k_4 X_{AT} \cdot \frac{1}{1 + \frac{K_{6APA-POA}}{C_{6APA}} + \frac{K_{POA}}{C_{POA-CoA}}}$ (25)
One step conversion of IPN to Penicillin V	$r_5 = k_5 X_{AT} \cdot \frac{1}{1 + \frac{K_{IPN-POA}}{C_{IPN}} + \frac{K_{POA}}{C_{POA-CoA}}}$ (26)
Carboxylation of 6-APA to 8-HPA (first order kinetics if CO2 concentration is considered as constant)	$r_6 = k_6 X_{AT} . C_{6APA}$ (27)
Cleaving of Penicillin V to 6-APA and Phenoxyacetic Acid by Penicillin Amidase (PA) (reversible reaction of Penicillin formation)	$r_7 = k_7 X_{PA} \cdot \frac{C_{PenV}}{C_{PenV} + K_{PenV}}$ (28)

where X=the activity of the corresponding enzyme

Table 5. Model for Penicillin V biosynthesis

The parameters values from the above model were determined in a fed-batch bioprocess; it was found that the IPNS enzyme is metabolic flux limiting and further on the ACVS enzyme. As the IPN formation from Tripeptide ACV is dependent on the O_2 concentration, the dissolved oxygen concentration superior to 45% from the saturation can increase productivity.

3. The segregated models [12, 13] can describe more complex phenomena like: alterations or disturbances in the physiology and cell metabolism; cells 'morphological differentiation; genome mutations; spatial segregations of growth regions; cells aggregation; mixed cultures

(including the competition between two or more species for the same substrate). On the contrary the unstructured and structured models have the limit to consider a homogenous population of cells and only one species in the bioreactor. The segregated models can be built by using ordinary differential equations to describe the behavior of several classes of independent/correlated cells. Each cell class behavior can be described by both unstructured and structured models.

4. Metabolic modeling

The most sophisticated modeling tool is that introduced by the metabolic engineering. This approach relies upon the concept of metabolic pathways as sequences of specific enzyme-catalyzed reaction steps converting substrates into cells' products. The manipulation of metabolic pathways to improve the cellular properties and especially the yield or the productivity of some important metabolites is of interest. So the metabolic engineering is recently developed with the purpose of generating information for the oriented modification of the enzymatic, regulatory or transport activities of the cells. The information will be used to build upgraded cells by the further application of the recombinant DNA technology.

The determination and the correct interpretation of the structure and the control mechanisms of metabolic networks are the first critical tasks of the metabolic engineering in order to fulfill the goal of rational pathway manipulation [14]. The main accent is towards considering the metabolic network as a whole and not the individual reactions. Due to the increased complexity of these networks and of the corresponding regulatory mechanisms the physiological state (metabolic steps characteristics at specific genetic and environmental conditions) of the cells is determined by the *in vivo* metabolic fluxes and their control.

The flux can be defined as the rate of material processing through a whole metabolic pathway. The value of the flux does not introduce information about the activity of the enzymes from the considered pathway, but it represents only their contribution regarding the substrate conversion into the final metabolite of this pathway.

The quantification of the metabolic fluxes is the principal objective realized by the techniques of **Metabolic Flux Analysis** (MFA) [15]. Metabolite balancing is the first operation in the determination of fluxes, done with the major hypothesis that the intracellular fluxes can be evaluated by measuring the extracellular fluxes. The metabolite balancing is performed by using a stoichiometric model for the intracellular reactions and by applying a mass balance around each intracellular metabolite, without any enzyme kinetic information. The general defining relationship is of matrix form:

$$S \cdot \underline{v} = \underline{r} \tag{29}$$

where: S=stoichiometric matrix of the metabolic network

$\underline{v}$=vector of unknown fluxes

$\underline{r}$=vector of measured metabolite extracellular concentrations, whereas the metabolite intracellular concentrations is 0.

The rows number is representing the number of metabolites in the pathway and the number of columns is equal to the unknown number of fluxes at steady state condition. The resulting system is normally underdetermined as the number of reactions is normally greater than the metabolite number. There are also various network structure characteristics (metabolic branch, reversible reactions, and metabolic cycles) that can increase the system degree of freedom.

So beside the metabolic balancing constraints additional constraints are needed to solve the equations system. If finally there are more constraints than the freedom degree, the system becomes over determined and redundant equations are to be used to test the consistency of the overall balances. The supplementary constraints can be obtained by using other information regarding the intracellular biochemistry and/or by applying others techniques [15].

So, another tool to perform MFA is the *Linear Programming* (LP) [14, 16]. Conforming to this method the metabolic fluxes are determined by simultaneously accomplishing 2 conditions: to be in line with the metabolic balances constraints and to optimize a certain objective function. So it is to formulate the mathematical problem:

$$\text{Minimize } \underline{c \cdot v} = \sum c_i v_i$$

$$\text{Subject to } S \cdot \underline{v} = \underline{r} \tag{30}$$

where: c= vector of the weight factors of fluxes in the objective function.

The objective functions can be: maximize the metabolite production rate or cells' growth rate; minimize the ATP production rate or substrate uptake rate; maximize growth rate for a given metabolite formation rate.

Another source of additional constraints is usually the introduction of certain types of supplementary measurements. The most useful tool of this type is the **application of isotopic tracer methods**. In isotopic tracer techniques there is a substrate in the cells labeled with an easily detectable isotope of a specific atom, normally ^{14}C , but especially ^{13}C , stable and non radioactive isotopes, to be detected by Nuclear Magnetic Resonance (NMR).

The isotope distribution among the metabolites from a network for a certain labeled substrate and known biochemistry is a function of the *in vivo* metabolic fluxes. This distribution can be obtained by studying the NMR spectra or by measuring the mass isotopomer (the molecules of the same metabolite, but with different labeling characteristics) distribution by Gas / Liquid Chromatography coupled with Mass Spectrometry (GC / LC-MS).

The general model for the determination of metabolic flux distribution is presented in the Fig. 2. The implementation of such flux quantification methods seems simple, but due to the high integrated networks complexity is rather an intensive computer application. There are now important studies of metabolic modeling used to improve the metabolite production in aerobic bioprocesses [17-22].

4. Bioprocess control

The bioprocess control has different goals and objectives, function of bioprocess characteristics and imposed performances. In spite of high non-linearity linear control theory and basic controllers (on/off, PID) are still applied in most industrial applications.

More sophisticated control should rely on models able to correctly represent the biosystems behavior. Due to the complexity of the biological systems, basic models, which are nice to use and help to simplify the underlying mathematics, are not able to reflect the real situations. The large sets of parameters from the complex models need to be experimentally identified, and consequently the e, and consequently the experiments should be carefully designed to provide this valuable information. Taking into account the time-to-market, which must be as short as possible the accepted control solution could be suboptimal based on classical robust control.

Bioprocess reproducibility and living cell systems variability reduction from run to run is to be carefully studied. The media composition optimization and the successful application of PAT (process analytical technologies combining the techniques for in-process monitoring, data-based modeling process control) will contribute to the quality of production improvement.

In bioindustry, bioprocesses are subject to a number of local and / or supervisory control structures. Local controllers are used to get the set-point control of different physical / chemical parameters (e.g. temperature, pH and dissolved oxygen concentration), while supervisory control is necessary for optimizing the feed in a fed batch process or the dilution rate in a continuous one [23].

Various simple feed-control strategies have been applied in the past [12, 24, 25]: (a) *Simple indirect feedback methods:* nutrients (indirect variable) are fed to the bioreactor by an on-off controller when a direct (on-line measured) variable deviates from its set point, e.g., feeding of ammonium by monitoring the pH (pH-stat), or nutrient feeding to keep the dissolved oxygen concentration constant (DO-stat). (b) *Predetermined feeding strategies;* this is a feed-forward strategy based on prior process knowledge, e.g., exponential feeding to grow at a constant biomass-specific growth rate. (c) *Direct feedback;* a substrate concentration can be directly controlled by nutrient feeding when it is measured on-line by sensors inside or outside the bioreactor. (d) *Feed control by state estimation;* the estimation of key-process parameters from on-line measurements can be applied and the control is based on the evolution of the growth rate or the substrate concentration.

Other advanced feed-control strategies may be applied when additional process information is available: *feed-forward model-based control; feedback model-based control* (an extended Kalman filter simultaneously estimates a state variable and adapt the controller); *fuzzy control; neural-network control* (for predictive control); *expert systems* (for supervisory control).

A. Bioprocess control with a priori model (model based process control)

The bioprocess control based on *a priori* model (BCAPM) can be seen as the on-line application of optimal control, where control actions are regularly re-calculated based on a

global process model and process information. The global model is used to calculate optimal control actions by a prediction of future outputs over a limited time horizon.

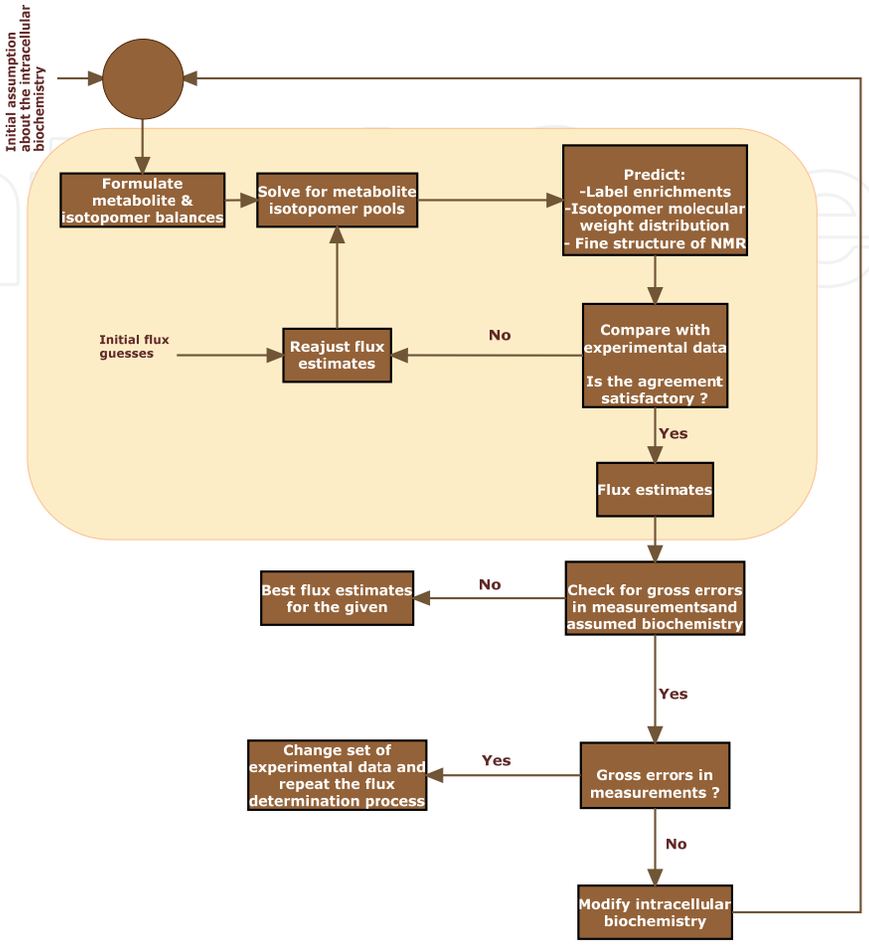


Figure 2. Determination of metabolic flux distribution [14]

For the time being, the unstructured deterministic models (the cells are considered as black-box units) are very used in the bioprocess control [26]. In the future an increase of the structured models role is expected, as a consequence of modern analysis methods development, as well as of the capacity to more adequately describe the phenomena.

The basic concepts of BCAPM consider two main ideas [27, 28]: (1) the explicit use of an *a priori* model to predict the process output(s); (2) the calculation of the future control actions by minimizing a global objective function.

The problem can be solved in different ways: (a) for a linear, time-invariant model, and in the absence of constraints, an explicit analytic solution of the above optimization problem can be obtained; (b) with linear constraints, the above optimization problem is a Quadratic-Programming problem, which can be numerically solved; (c) in the presence of a nonlinear model or nonlinear constraints, a non-convex optimization problem must be solved at each sampling period. So iterative optimization algorithms, (e.g. the Nelder-Mead method) can be used in order to converge to local minima.

There are two major problems which limit the application of BCAPM to bioprocesses [29]: (1) the model must predict the process variables evolution with sufficient precision; (2) given a nonlinear process model, the nonlinear optimization problem is solved for each (sampling) period; hence, the bioprocess model must be linear during these time periods.

The first item obstructs the application of BCAPM to complex or partially known systems, without defined global models. The second item blocks the application to performable systems; otherwise the control techniques are not properly used, due to the short sampling time periods (the second issue can be avoided by reason of large time constants characteristic to bioprocesses).

Recent developments in on-line measurement techniques, parameter and state estimation, in addition to the search of improved quality control, motivated the development of BCAPM. Now the technique was upgraded with better results. For instance [30] the applied BCAPM for feed control in the production of monoclonal antibodies allows to improve the yield with 43%.

B. Bioprocess adaptive control

When the process characteristics change during time, the operation conditions must also be changed: controller parameters and set point values. Moreover, optimal bioprocess evolution is commonly determined off-line, the process conditions are not perfectly known, and the process model is not well defined. Furthermore, it can be a lot of changes in process conditions in conjunction with different microorganisms' life cycles (when the cell concentration increase in time in a batch bioprocess, the oxygen set point must be increased). Hence, there is a need for some feedback mechanisms based on on-line measurements. On-line adaptation is possible when the state variables can be measured online[10] (directly using *hardware sensors* or indirectly by *soft sensors* [31, 32]).

The adaptive control structures are based on the design of different estimation algorithms which are able to determine the off-line parameter values. Many control algorithms were developed based on *minimal* knowledge about bioprocess kinetics (the *minimal modeling* concept) [33-36].

A typical adaptive control system is presented below:

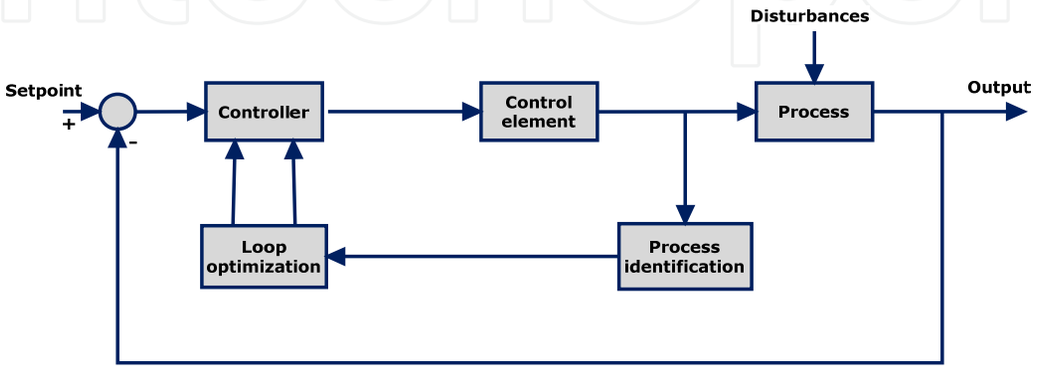


Figure 3. Adaptive control structure

There are two classes of adaptive control (where the adaptation is attained on the basis of on-line parameter observers) [37]: (1) the process changes can be measured – therefore it is possible to systematically adjust the controller settings, based on the measured / anticipated bioprocess changes; (2) the process changes cannot be measured / predicted – hence the controller settings are automatically adjusted by a loop optimizer.

C. Bioprocess control using Artificial Intelligence (AI)

The limitations of the bioprocess control systems do not concern only the measurements or models, but at the same time much valuable human knowledge is only available in a qualitative heuristic form.

Hence, it has been found that the knowledge-based control structures using the human decisional factor (i.e. a subjectively element) offer sometimes better results. Moreover, the computer performances are developed in the detriment of the general knowledge concerning life phenomena and do not promote advanced comprehension upon the metabolic routes of bioprocesses. Consequently, the intelligent techniques (i.e. neural nets, fuzzy structures, genetic algorithms or expert systems) are capable of simulating human expert-like reasoning and decision making, dealing with uncertainties and imprecise information [24].

As the human perception about the bioprocess is commonly altered by the psychological factors, the intelligent control systems founded (only) on the human subjective knowledge is less valuable than the control systems who utilize the objective information fitted by a conceptual model. Hence, the literature recommends the intelligent control techniques utilization only if the control structure based on quantitative models fails.

Frequently, different process parameters are controlled in order to follow predefined transitory trajectories. Such control strategies can be designed by a *trial-and-error* approach in combination with operator's experience and statistical analysis of historic data.

a) One method for automatic bioprocesses control using AI is based on *expert systems (ES)* that reproduce the human operator' rules of action. The literature presents several examples how to transfer the knowledge from operators into knowledge-rule bases [38]. An ES conceptual architecture is presented in Figure 4.

The most used ES systems in bioprocess control are applied for supervisory control, or process monitoring and diagnosis.

Moreover, the ES logic is used to translate human language into a mathematical description. The parameters tuning is then regulated by phase detection based on *if...then* rules, conditional statements representing heuristic reasoning in which *if* expresses the condition to be applied and *then*-the action to be done. Of course, at the same time, it is not possible to calculate optimal parameter' values with this method. For example [39] an ES was developed in order to supervise a conventional control system applied to fed-batch baker's yeast cultivation and to surmount its limitation. Expert system BIOGENES can execute standard process control tasks, but also advanced control tasks: process data classification;

qualitative process state identification (metabolic state, process phase, substrate feeding); supervisory control through corrective actions.

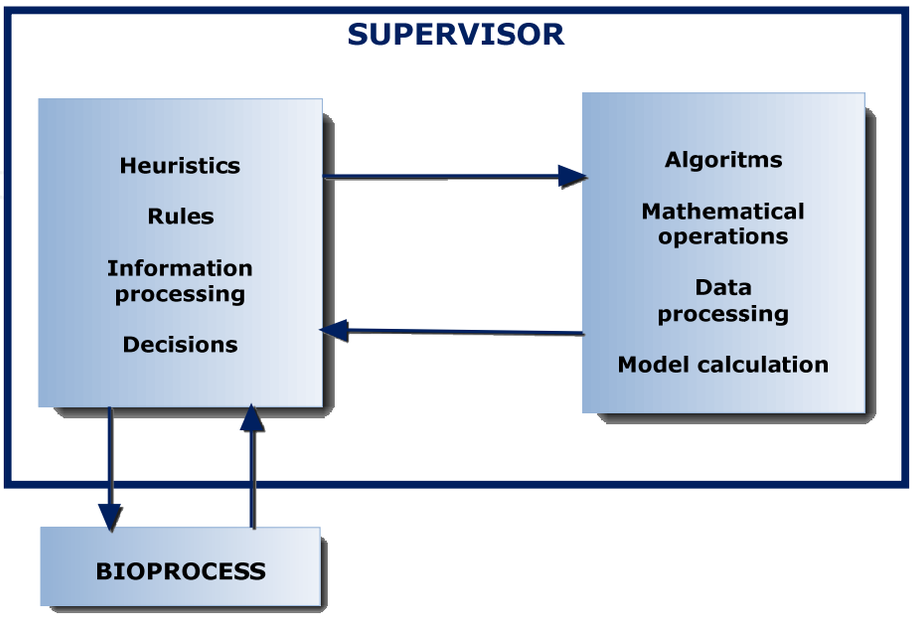
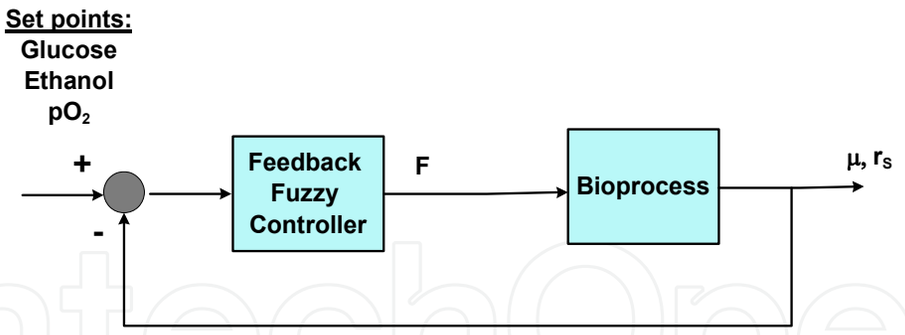


Figure 4. The expert system conceptual architecture

One of the main limits in developing ES is the knowledge acquisition process due to: (1) the linguistic rules formulation by human experts, i.e. the analytical description of their actions during the bioprocess (manual) control; (2) the loss of information during the transfer between the bioprocess expert and the IT specialist; (3) the subjectivity of human expert regarding own decision / rules.

b) Another AI technique, the *fuzzy approach* is based on fuzzy sets and fuzzy reasoning. In actual AI systems, fuzzy rules are often applied together with different types of models / parameter / state estimators. These fuzzy rules can be regarded as problem specific basis function system [40]. Any variable can be a fuzzy variable, particularly recommended when it is not possible to define its value in a given situation. One define fuzzy sets in the form of membership functions (between 0 and 1) in order to express what is likely to be considered as degree / level for a certain characteristic (high, medium, low). Relationships between fuzzy variables can be formulated with fuzzy logic operators (and, or, not) and processed by fuzzy logic. Fuzzy rules reflect the rules of thumb used in everyday practice and can be processed as if…then expressions. With a set of fuzzy rules, considered as universal process approximates, the behavior of a system can be described quite accurately. There are many applications: (a) hierarchical fuzzy models within the framework of orthonormal basis functions41 (Laguerre and Kautz functions); (b) several important use of fuzzy control in the Japanese bioindustry by the companies Ajinomoto, Sankyo or Nippon Roche [42]; (c) the control of the α-amylase fed batch bioprocess with the recombinant E. coli to maintain glucose and ethanol at low concentrations with 2 fuzzy controllers for feed rate control: feed forward and feedback [43] (see Figure 5 below):



where: F = real glucose feed rate [L/h]
μ = specific growth rate [h^{-1}]
r_s = specific glucose consumption rate [g/L/s]
$Y_{X/S}$ = cellular yield [g/g]
pO_2 = dissolved oxygen concentration [mg/L]

Figure 5. Schematic presentation of the fuzzy control structure

c) One can use *artificial neural networks* (ANN) to get predictions about biosystem behavior. The traditionally used format of ANN is the feed forward. Given a set of process measurements, the output of ANN can be estimated parameters or process variables. The weights applied to the process measurements as inputs are determined through the "training process" of the ANN [44]. To train the ANN it is to get complete process information, corresponding to the NN inputs and outputs, from the data gathered in a set of fermentation runs. This set defines "an experimental space" and the ANN will predict outputs accurately only within this range and not beyond it.

Various applications were studied: (a) biomass and recombinant protein concentration estimation via feed forward NN for a fed batch bioprocess with a recombinant *E. coli* [45, 46]; (b) two types of NN (input/output and continuous externally recurrent) can control the batch and fed batch piruvat production from glucose and acetate with a recombinant strain of *E. coli* [47]; (c) two NN also to control the submerged bioprocess of *Monascus anka* fungus cultivation (the temperature and the dissolved oxygen are the inputs and the controlled outputs are the glucoamylase activity and the concentration of the red pigment [48]; (d) NN based soft sensor for online biomass estimation in fed bioprocess for polyhydroxibutirate production [49]; (e) media formulation optimization with genetic algorithm evaluated by ANN [50].

d) Because all types of information must be used in order to improve the bioprocess control: mathematical / deterministic models, heuristic knowledge, rule-based reasoning, a new control structure is developed in the last years – i.e. *hybrid control system* (HCS). HCS acts on both parts of bioprocess control: conventional control systems (i.e. based on a priori model) merge with AI techniques, in a complementary way: if a priori (mathematical) model exists, it will be preferred; else the linguistic rules (i.e. expert systems / fuzzy techniques) will be used.

Generally it is necessary to design a control system, which can to choose the (intelligent) control strategies, based on analytical models, in order to improve the control performances.

This is an Intelligent Control Structure (ICS) based on Hybrid Control Techniques (HCT). The most widely used hybrid structure combines balance equations with ANN: (a) balance equations for substrate and cell concentrations coupled with ANN for growth rate model in case of bakers 'yeast fed batch cultivation [51]; (b) ANN is responsible for modeling the unknown kinetics in applications with the yeast *Saccharomyces cerevisiae* or activated sludge urban wastewaters bio treatment [52]; (c) batch bioprocess of animal cells [53].

5. Case study [54]

The research objective of this case study was to develop an appropriate control method for a bioprocess and to implement it on a laboratory plant, namely the control of the fed batch cultivation of *Hansenula polymorpha* yeast for alcoholoxydase-containing biomass. At first, the process is described and a mathematical model is proposed and then the control strategy is defined and the intelligent control structure is designed. Finally, the control performances are tested through real data.

A discontinuous fed-batch bioprocess for alcoholoxydase-containing biomass with the methylotrophic yeast *Hansenula polymorpha* CBS - 4732 was operated in an airlift lab - bioreactor The intracellular enzyme, to be separated further on, is used for obtaining a high-specialized kit for methanol/ethanol determination. The yeast was cultivated on a complex medium with $(NH_4)_2SO_4$, KH_2PO_4, Na_2HPO_4, $MgSO_4*7H_2O$, $CaCl_2$, yeast extract or autolysed residual beer yeast as organic N source and microelements (Fe, B, Cu, I, Mn, Zn, Mo).

$$\begin{aligned}
\frac{dV}{dt} &= -\frac{E_S}{\rho_S} - \frac{E_M}{\rho_M} \\
\frac{dX}{dt} &= \frac{\mu_{\max} S}{K_S + S} X + \frac{X}{V}\left(\frac{E_S}{\rho_S} + \frac{E_M}{\rho_M}\right) \\
\frac{dS}{dt} &= -\frac{\mu_{\max} S}{K_S + S}\frac{X}{Y_{X/S}} - \frac{E_S \rho_S}{V} + \frac{S}{V}\left(\frac{E_S}{\rho_S} + \frac{E_M}{\rho_M}\right)
\end{aligned} \tag{31}$$

where: E_S and E_M are the substrate and medium loss by evaporation [g/h]; ϱ_S and ϱ_M are the substrate and medium densities [g/L]; $Y_{X/S}$ is the substrate conversion yield referred to the biomass [g dry matter/ g substrate]; μ is the specific growth rate [1/h]; V is the volume of the cultivation medium in the bioreactor [L]; X and S are the biomass and substrate concentrations [g/L] and *t* is the time [h], μ_{max} represents the maximum specific growth rate [1/h] and K_S is the saturation constant [g/g]. The main process parameters were: continuous temperature control 37°C; a minimal level of pO_2 - 10% from the saturation concentration was maintained during the exponential growth; continuous pH control between 4.5 - 5.0 by addition of NH_4OH (12.5%); no foam control, if the main parameters are optimally controlled. The unique C source, the methanol was introduced function of the yeast growth rate in connection with the substrate consumption rate for avoiding the growth inhibition by substrate concentration. The developed model (1) is based on the mass-balance principle and on the hypothesis of a non-inhibitive substrate effect (i.e. the specific growth rate is defined by

the Monod equation). In line with the operation mode (fed-batch with discontinuous substrate feeding), there are discontinuous variations of the main variables due to: substrate feeding, medium feeding (to overcome the loss by evaporation or sample collection) or samples withdraws. That is why the following mass-balance equations are to be added to express each discontinuous modification for volume, and substrate or biomass concentrations:

$$\begin{aligned} V_k + A_{Sk} + A_{Mk} &= P_{Mk} + V_{k+1} \\ S_k \rho_M V_k + A_{Sk} \rho_S &= P_{Mk} \rho_M S_k + S_{k+1} \rho_M V_{k+1} \\ X_k V_k &= P_{Mk} X_k + X_{k+1} V_{k+1} \end{aligned} \tag{32}$$

where: V_k, V_{k+1}=volume before / after modification [L]; A_{Sk}, A_{Mk}=substrate volume and respectively medium volume adding [L]; P_{Mk}=sample withdraw [L]. The same notations are used for S_k, S_{k+1} and X_k, X_{k+1}. We use: ϱ_S = 800[g/L]., respectively ϱ_M = 1000[g/L]. The identification of the model parameters was carried out based on measured values in order to minimize the modeling error. The identification procedure (i.e. Nelder-Mead algorithm) determines the optimum values for the following process parameters: E_S, E_M, μ_{max}, K_S and $Y_{X/S}$.

For this bioprocess, the overall control objective is to obtain large biomass quantities, based on the assumption that high biomass concentration will assure the obtaining of important alcoholoxydase-active biomass. In this paper a control system based on fuzzy logic is proposed. It is well known that Fuzzy Control Systems (FCS) can manipulate incomplete and uncertain information about the process assuring high control performances [6-8]. The proposed FCS receives information about the state of the bioprocess expressed by the biomass and substrate concentrations. Based on this information, FCS computes the quantity of substrate to be added into the reactor. According to these observations the inputs of FCS are the biomass (X) and substrate (S) concentrations, and the output is the correction to be applied on the substrate addition. The rules of FCS are presented in Table 1.

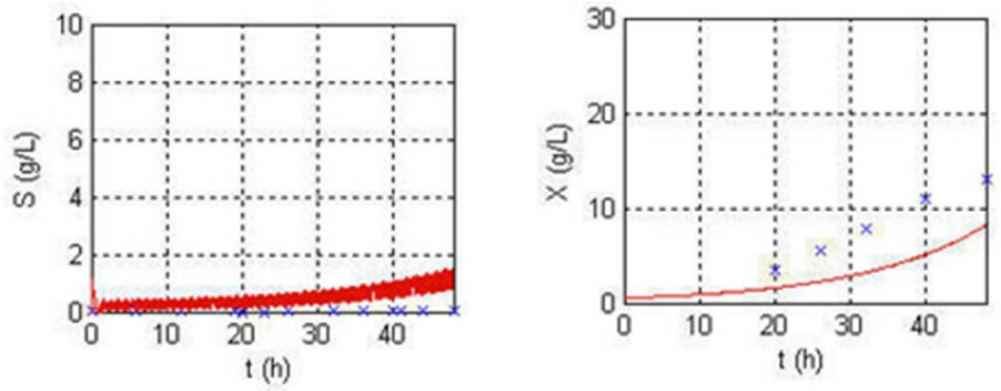
Rules evaluation by the inference engine is made according to the min-max inference rule and the output defuzzyfication is made based on the centroid defuzzyfication method.

Xk Sk	S	M	L
S	Z	PZ	P
M	NZ	Z	PZ
L	N	NZ	Z

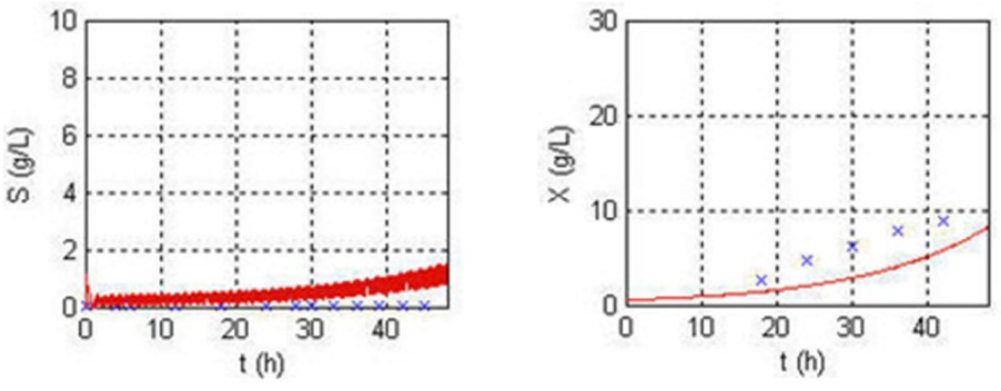
Table 6. The rule base

6. Results & discussions

The control loop was implemented in MATLAB, version 7.5. For control loop simulation the proposed mathematical model was used and the simulation results were compared with the experimental data.



a)



b)

Figure 6. Simulation results of the control loop: a) first experiment; b) second experiment; ('-' – simulation results; 'x' – experimental data)

The simulation results show that the proposed fuzzy control system is capable of computing the substrate feedings needed for cell growth according to the biomass concentration increase. The evolution of the substrate concentration marks the substrate consumption and additions, as well as the increase of the additions along with cell growth. The biomass concentration obtained by simulation follow closely the experimental data. As a conclusion of this case-study, it can be accepted that the success of such a control implementation is critically dependent upon the technical operating conditions of the process.

7. Conclusions

The overview on the current status of bioprocess modeling and control focuses on three main topics: (i) unstructured versus structured and metabolic modeling; (ii) control based on common technique (model based control and adaptive control); (iii) control based on artificial intelligence.

It is finally to underline that the framework of bioprocess modeling & control still offers interesting perspectives to obtain robust control solutions for the aerobic bioprocess. Moreover the future of bioprocesses' optimal control will rely on applying the same concept:

the use of different modeling methods in conjunction with intelligent control techniques. If a simplified representation of the bioprocess exists (i.e. an *a priori* model), this optimal profile can serve as an initial trajectory for intelligent control algorithms when the complexity of the process representation is described in a subjective mode (by human expert).

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