Part three

BIOPROCESS INTELLIGENT CONTROL

*In this chapter will be configured the general intelligent structure for bioprocess control. Hence, in **# 3.1** will be shown divers original models of different considered bioprocesses and the corresponding modeling results. The model heterogeneity is due to the microorganism types variability. Following the model types, in **# 3.2** will be established the general control criteria, by tackling the general bioprocess control theory in addition with the conceptual modalities to deal with the model fluctuation. Finally, in **# 3.3** will be configured the general intelligent control structure based on working hypothesis, i.e. bioprocess pattern recognition, analytical optimization and fuzzy control. The process database will be configured on AS400 system.*

The experimental data analysis guides to the impossibility to develop global models, which can formalize (from mathematical point of view) all the culture phases. Hence, distinct limited models have been developed, who are applicable through specific evolution conditions. The control algorithm practice with *a priori* model is limited from this point of view. Moreover, the adaptive control systems offer a sub-optimal global solution (despite the optimization during the time samples).

Therefore, the intelligent control techniques offer the possibilities to substitute the bioprocess information lack (and the general evolution rules) for the subjective principles established by a human expert. So, an intelligent control system can be built in order to manage specific bioprocess types.

3.1. PROPOSED MODELS OF SPECIFIC BIOPROCESSES

3.1.1. Mathematical modeling of enzymatic hydrolysis of wheat straw under the inhibitory effect of the end product

Cellulose is the most abundant organic compound in the world and is constantly replenished by photosynthesis. The conversion of cellulose to soluble sugars is accomplished by cellulolytic enzymes or mineral acids, both methods being characterized by advantages and disadvantages (Marsden, Gray, 1986).

The enzymatic hydrolysis is a process performed in heterogeneous system, involving the action of soluble enzyme (cellulase) on insoluble substrate. Cellulase is not a single enzyme but is, depending on the source, a multicomponent entity with variable composition. In general, cellulases secreted by fungi consist of three major classes of components: endoglucanases, cellobio-hydrolases and beta-glucosidases. The conversion of cellulose to glucose occurred by the synergism between the individual components of the cellulase system. Despite the great number of the studies concerning the mode of cellulase action (Estebauer *et al.*, 1991, Kuhard *et al.*, 1993, Steiner, 1994, Maheswari, 1993, Olama, 1993), the results provide only an approximate representation of the enzyme hydrolysis kinetics of an insoluble cellulose.

The kinetics of the enzymatic hydrolysis of insoluble cellulose primary depends on three groups of factors (David *et al.*, 1986, Morisson, 1988, Ramos *et al.*, Ropars *et al.*, 1992, Zacchi *et al.*, 1988): the structural features of cellulose, the nature of the enzyme system employed and the mode of interaction between cellulose and enzyme. Furthermore, kinetic characteristics of the heterogeneous cellulose-cellulase system, such as mass transfer adsorption and desorption of the enzyme, surface reaction and product inhibition should be examined. A kinetic model, which considers all these factors, has not been proposed.

Experimental conditions
- *Enzyme:* cellulase system was obtained from the culture of the cellulolytic fungus *Aspergillus niger* (Archer, 1994, Orsson, 1993, Szewczyk, 1994; Mandels, 1969). The culture broth was filtered through nylon cloth and filtrate was used as enzyme preparate;
- *Substrate:* milled and alkaline treated wheat straw (NaOH at 120° C, one hour); (Jecu, 1996);
- *Enzymatic hydrolysis:* enzyme digestion of substrate was carried out at 45° C in acetate buffer, pH 4.5, to which sodium azide has been added to prevent microbial contamination. The reaction mixture contained various amount of substrate (12; 20; 40; and 60 g/l). The influence of the

accumulation of end product (glucose) was determined by supplementation of the medium with various amounts of exogene glucose (10; 20 and 40 g/L). After 24 hours of reaction, representative aliquots of each digestion mixture were withdrawn, centrifuged to remove all solid substrate. Reducing sugar content of the supernatant was determined (as glucose) using the dinitrosalycilic method of Miller (1959).

The experimental data have shown the inhibitory effect of the accumulation of end product in the mixture reaction. The yield of substrate conversion has decreased greatly with the increase of the glucose concentration.

In the limited conditions (Caramihai and Jecu, 1995), for a function P=f(inhibitor), taking into account a finite inhibitor concentration ($I=I_{lim}$) the product accumulation being 0, the inhibitory effects were produced only by the substrate concentration. For this case, the proposed model has the following equation:

$$P = P_0(1 - \frac{I}{I_{lim}})e^{-kI} \qquad \textbf{3.1.1.1}$$

where: P_0 = obtained product (without inhibitor effect) [mg/mL];
I = inhibitor concentration [g/L];
I_{lim} = limiting inhibitor concentration (which correspond to zero product) [g/L];
k = constant.

Considering the inhibitory effect of the substrate, the equation (3.1.1.1) becomes more complex:

$$P = \frac{c_1 S}{c_2 + S}(1 - \frac{I}{I_{lim}})e^{-kI} \qquad \textbf{3.1.1.2}$$

In this case, P_o depends on substrate concentration (c_1, c_2 = constants). The enzymatic hydrolysis typical profile, for lower

substrate concentrations, presents a linear dependence between the product formation and substrate concentration (fig. 3.1.1.1.).
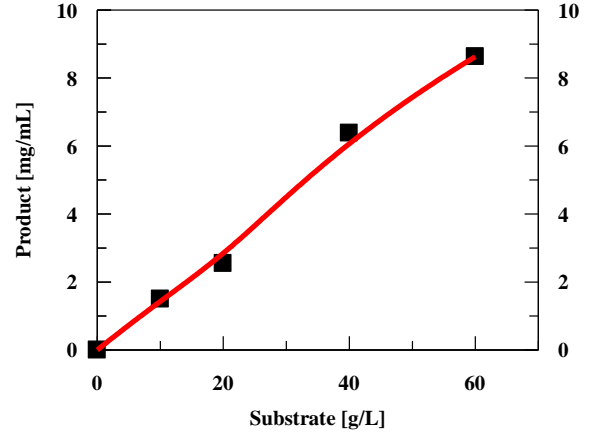

Fig. 3.1.1.1 Product formation vs. substrate concentration (without inhibitor effect); ■ **real data,** ▬ **model**

For higher substrate concentration values (S=100 ÷ 120 g/L) it has obtained a decreasing product formation rate, as consequence of the substrate variations and enzyme adsorption onto substrate (Caramihai, Jecu, 1995).

A previous paper (Caramihai and Jecu, 1995) has confirmed, in a substrate range of 10-60 g/L, the maintenance of a linear dependence between the glucose release and the substrate concentration.

In enzyme reaction, the accumulated end products usually inhibit the rate of the process. In this case, glucose was competing with the substrate cellulose for the active sites on the enzyme. The supplementation of the mixture reaction with different amounts of glucose determined important decreases of the product concentration (Annex 1, Table 1).
As shown from the figures 3.1.1.1, 3.1.1.2 and 3.1.1.3 the most significant decrease of the product release was obtained in the presence of 20 g/L exogene glucose.
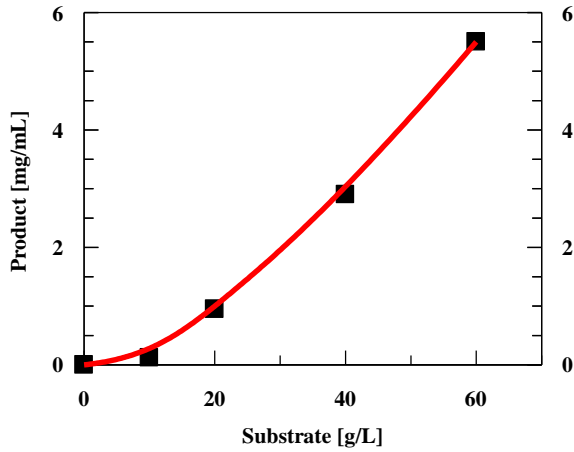
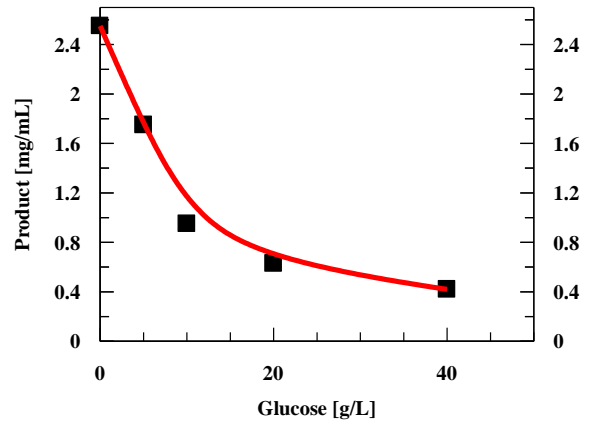**Fig. 3.1.1.2 Product formation vs. substrate concentration (glucose: 10g/L);** ■ **real data,** ━ **model**

Fig. 3.1.1.4 Product formation vs. inhibitor concentration (initial substrate concentration: 20 g/L); ■ **real data,** ━ **model**
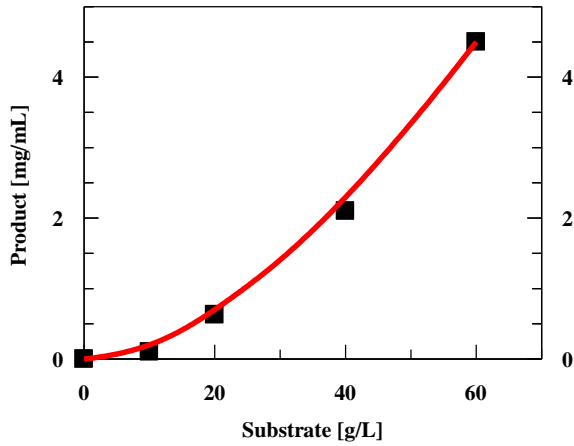

Fig. 3.1.1.3 Product formation vs. substrate concentration (glucose: 20g/L); ■ **real data,** ━ **model**
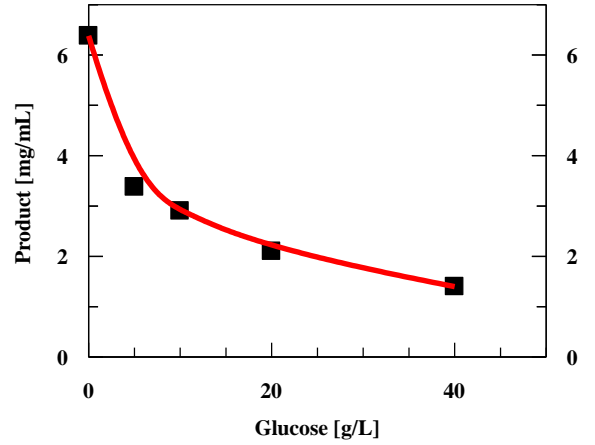

Fig. 3.1.1.5 Product formation vs. inhibitor concentration (initial substrate concentration: 40 g/L); ■ **real data,** ━ **model**

The modeling results were presented in Annex 1, Table 2 (for all experiments I_{lim} = 100 g/L). The experimental data show a good correspondence with the simulated values, i.e. the proposed model is adequate.
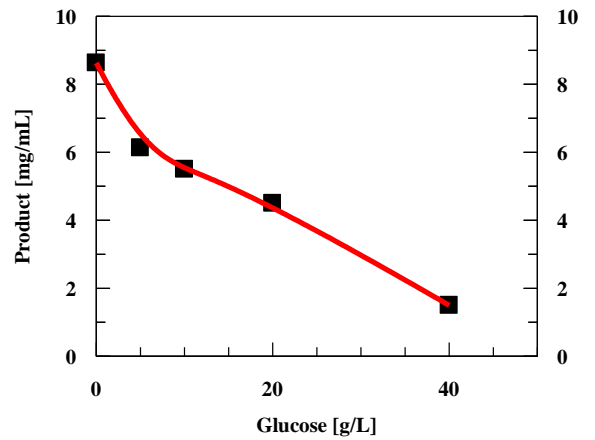

Fig. 3.1.1.6 Product formation vs. inhibitor concentration (initial substrate concentration: 60 g/L); ■ **real data,** ━ **model**

Figures 3.1.1.4, 3.1.1.5 and 3.1.1.6 present the influence of the inhibitor concentration on the product formation, for various substrate levels. Table 3 (Annex 1) indicates the experimental data, and Table 4 (Annex 1) shows the modeling errors.

Finally, it should be point out that the equations 3.1.1.1 and 3.1.1.2 describe enzymatic hydrolysis processes, and can be used for determination of optimal conditions. It would be evaluated if the optimum of the function P=f(S,I), easily (analytical) determined at this moment, can be send up using an intelligent control system.

3.1.2. Kinetic model of catalase accumulation in aerobic bioprocess with *Micrococcus lysodeikticus*

In a discontinuous aerobic bioprocess a high producing bacterium *Micrococcus lysodeikticus* accumulates intracellular catalase for the H_2O_2 decomposing. The toxic H_2O_2 formation is due to cellular respiration, and consequently a high catalase activity is characteristic for the exponential growth phase.

Following **#2.1.1.3** and Gaden`s recommendation, the product accumulation is to be considered conforming to the metabolic route of product formation and related to the culture growth:
a) Product formation kinetic is a function of substrate consumption rate, ipso *facto* of the growth rate (growth associated product accumulation); the total change in free enthalpy is negative ($\Delta G < 0$) - *type I*. The main product appears as a result of primary energy metabolism by oxidation of the substrate. Growth curve, substrate consumption and product formation are very similar.
b) Product formation kinetic is only indirectly dependent on the substrate

consumption, the relationship with the growth curve being complex; the total change in free enthalpy is also negative ($\Delta G < 0$) - *type II*.
c) Product formation kinetic does not depend on the substrate consumption and the change in free enthalpy is positive ($\Delta G > 0$) - *type III*.

Taking into account of these criteria, a *type I* product formation kinetic is a logical answer for the intracellular catalase accumulation.

Experimental conditions

The *Micrococcus lysodeikticus* bacterium was cultivated in a stirred tank lab-bioreactor[1] with 10L medium. The main aerobic batch bioprocess parameters were:
•   *Temperature*: 30 °C
•   *Pressure*: 0.5 atm
•   *Stirrer speed*: 450 - 550 rpm
•   *pH* 9, unnecessary control
•   *Air-flow*: 400 - 800 L/h
•   *Time process*: 20-24h

The bioprocess parameters were monitored and controlled by conventional procedures. Medium composition: $NaHCO_3$ 1,7%, $CaHPO_4$ 0,2 %, yeast extract 2%, molasses 1%/r.s., microelements.

During the exponential growth phase, the C substrate is mainly used for metabolites accumulation, principal product formation and growth energy obtaining; on the other hand in the stationary phase the share for growth metabolites and energy obtaining is much decreasing to the benefit of the substrate used for maintaining energy and product formation.

In these conditions, the following general kinetic model can be considered (Schugerl, 1987, Schugerl, 1993):

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[1] The controlled parameters were speed, temperature, airflow and pressure.

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X \qquad \textbf{3.1.2.1}$$

where α and β can be time dependent (Caramihai *et al*., 1995) related to the batch process evolution and consequently with the growth curve:

$$\alpha = \frac{k_1}{1 + e^{k_2 t}} \qquad \textbf{3.1.2.2}$$
$$\beta = k_3$$

If k_1 is a constant, the value of α parameter can be adjusted by using the coefficient k_2, with a specific rate significance (h^{-1}). Finally, the mathematical form of the parameter expresses that to the final period of the batch bioprocess the product formation rate is lesser and lesser dependent of the growth kinetic (this dependence is decreasing till 0).

Meanwhile, the β parameter has a constant value (linear dependence) expressing the relative contribution of the existing cells to the product formation.

The final form of the equation (3.1.2.1) becomes:

$$\frac{dP}{dt} = \frac{k_1}{1 + e^{k_2 t}} \frac{dX}{dt} + k_3 X \qquad \textbf{3.1.2.3}$$

The experimental data are shown in Table 1, Annex 2. Two experiments are investigated. The experiment duration was 20h. The cell concentration (as dry weight) and the enzymatic activity were checked from 4 to 4 hours.
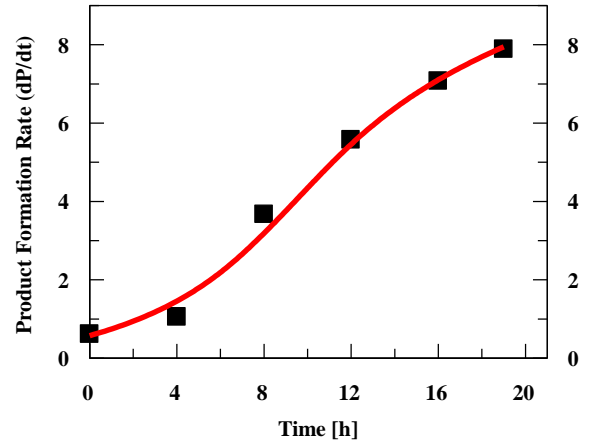

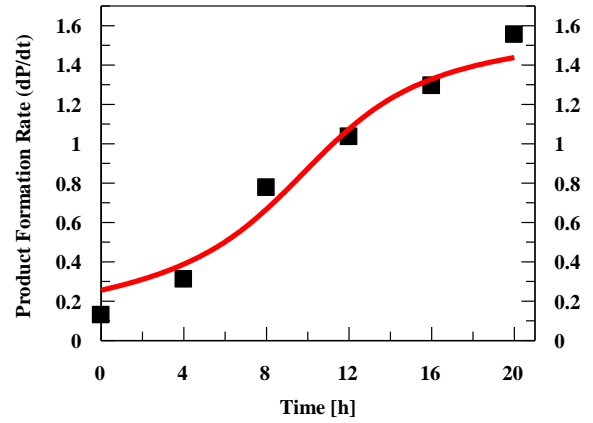**Fig. 3.1.2.1 Product formation rate vs. time (experiment 1);** ■ **real data,** ▬ **model**

Fig. 3.1.2.2 Product formation rate vs. time (experiment 2); ■ **real data,** ▬ **model**

Fig. 3.1.2.1 and 3.1.2.2 show the experimental data in analogy with the model simulated value

The model described by the equation (3.1.2.3), a growth associated product accumulation model, predicted well enough the experimental behavior. The proposed model is not only an extension of the classical model, but expresses better the more complex dependence between growth and product formation, and finally the existing metabolic routes. Furthermore, the modeling error is better that the classic model errors (see Table 2, Annex 2).

3.1.3. Modeling of alcoholoxydase production with *Hansenula polymorpha*

Experimental conditions

A fed-batch bioprocess for alcoholoxydase obtaining with the methylotrophic yeast *Hansenula polymorpha* CBS-4732 was operated in an airlift lab bioreactor (draft concentric airlift with air sparged riser). The enzyme is used for obtaining a high-specialized kit for methanol/ethanol determination.

The yeast was cultivated on a complex medium containing $(NH4)_2SO_4$, KH_2PO_4, Na_2HPO_4, $MgSO_4 \ast 7H_2O$, $CaCl_2$, yeast extract or autolysed residual beer yeast as organic N source and microelements (Fe, B, Cu, I, Mn, Zn, Mo).

The main process parameters were:
- continuous temperature control 37-38°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;
- no foam control, if the main parameters are optimally controlled;
- pH: 4.5-5

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 previous studies (Chirvase and Marica, 1992) for airlift performance characterization and bioprocess optimization demonstrated that on these conditions a high level of alcoholoxydase was accumulated during the first part of the exponential phase growth. So that the assumption of a growth associated product formation model becomes plausible.

Conforming to the studies presented in the experimental part a growth associated product accumulation model (type I) is to be considered (Caramihai *et al.*, 1995):

$$\frac{dP}{dt} = k_1 \frac{dX}{dt} e^{k_2(X_P - X)} \qquad \textbf{3.1.3.1}$$

where: k_1, k_2 = constants;

$e^{k_2(X_P - X)}$ = autocatalysis factor (which expresses the special nature of a growth associated enzyme as bioprocess product);

X = cells concentration expressed as dry weight (g/L);

X_P = cells concentration corresponding to a threshold level (g/L);

P = alcoholoxydase activity (EA/mL of extract);

Note that k_1, k_2 and X_P are *a posteriori* values

Meanwhile the parameter X_P represents a threshold value, which defines the present model behavior in comparison with the classical Gaden model:
- if $X_P = X$, model (3.1.3.1) corresponds to Gaden model;
- if $X_P < X$, product rate is grater than product rate obtained by Gaden model;
- if $X_P > X$, product rate is lesser than Gaden product rate.

The alcoholoxydase is an intracellular enzyme, so there is a complex procedure for its obtaining after its biosynthesis (cells disruption, extraction with adequate solutions, extract concentration, etc.). The product rate evolution can be expressed in a first form as the activity of the enzyme determined by a known biochemical analysis in the volume of extract (mL) obtained after cells' disruption and extraction.

Three experiments were performed (time bioprocess: 40 – 44h). The optical density (as cell concentration indicator) and the enzymatic activity were measured (Table 1, Annex 3). The estimated parameter values are shown in Table 2, Annex 3. By comparison

with the classical form of a growth associated product accumulation model, the introduction of "autocatalysis factor" expresses the enzyme is a special product which is used as catalyst for culture growth sustaining (the modeling error is $\chi^2 < 0.1$).
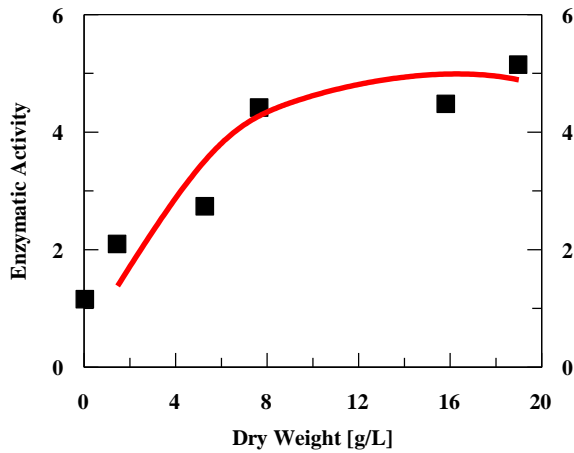

Fig. 3.1.3.1 Product formation rate vs. dry weight (experiment 1); ■ real data (AE [microMol methanol/min/mL of extract], ━━ model [AE [microMol methanol/min/mL of extract])
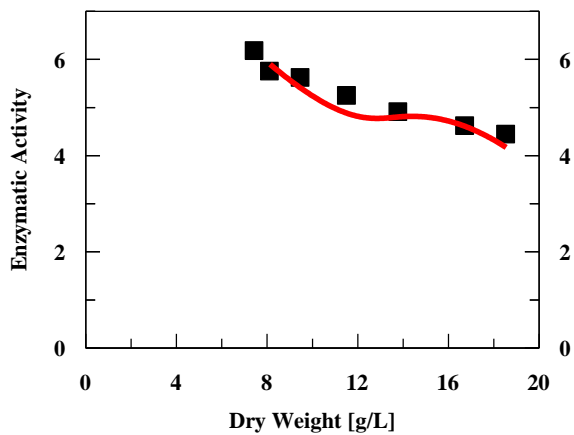

Fig. 3.1.3.2 Product formation rate vs. dry weight (experiment 2); ■ real data (AE [microMol methanol/min/mL of extract], ━━ model [AE [microMol methanol/min/mL of extract])
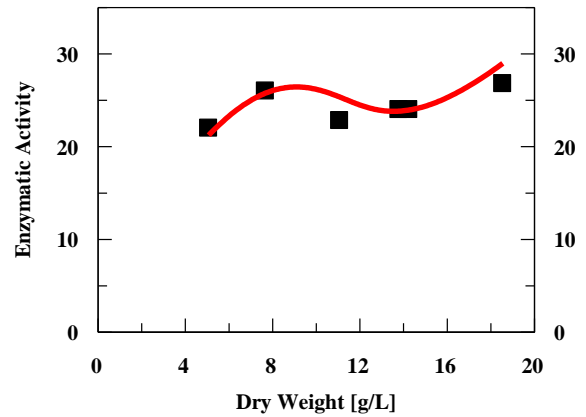

Fig. 3.1.3.3 Product formation rate vs. dry weight (experiment 3); ■ real data (AE [microMol methanol/min/mL of extract], ━━ model [AE [microMol methanol/min/mL of extract])

Fig. 3.1.3.1, 3.1.3.2, 3.1.3.3 represent the product accumulation rate evolution (model curves) by comparison with the experimental data for representative fed - batch runs.

Finally, we can see that the chosen model predicted well enough the experimental behavior and can be considered as a contribution to the product accumulation modeling for the special case, when the bioprocess product is an enzyme. Furthermore, in the three experiments, two levels of alcoholoxydase activity can be reached depending on two cellular metabolism systems for enzyme using as catalyst and probably independent of the cultivation conditions. The idea of two different systems for the enzyme using in the cellular growth is sustained by the good fitting of the same model, but with two levels for the k_1 value (k_1 in experiment 3 $>> k_1$ in experiment 1 and 2).

3.1.4. Mathematical modeling of the enzymatic hydrolysis of wheat straw

Recent interest in renewable resource has prompted many researchers to investigate the enzymatic hydrolysis of cellulose. Despite the efforts of these investigators, much of the mechanism for cellulose hydrolysis is still unclear. The complexity of the problem is, in a large part, due to the significant alteration of

the substrate properties (Gupta, 1992; Wyman *et al*., 1993).

The yield of the enzymatic hydrolysis process is dependent in a large measure of the substrate level. It has been stated that the substrate concentration increase over an optimal value produces the decrease of the rate digestion.

According to our results, we have proposed a mathematical model for describing the enzymatic hydrolysis of pretreated wheat straw using a fungal cellulase complex.

Experimental conditions
- *Microorganism:* cellulolytic fungus *Aspergillus niger,* which belongs to the microbial collection of Research Institute for Chemistry.
- *Growth medium:* Mandels–Weber's medium (1969), using as carbon source, 3.5% wheat straw. The cultures were performed in agitated flasks at 29° C. The culture broth was centrifuged at 4000 rpm, and the supernatant was measured for cellulase activity. The enzymatic activity, as carboxymethylcellulase, was 8.44 IU/ml. One enzyme unit activity was defined as the amount of glucose (micromoles) released in the assay conditions. The amount of reducing sugars was evaluated with dinitrosalicylic reagent, according to Miller (1959).
- *Substrate:* physical and chemical treated wheat straw was used. The milled wheat straw was treated with 1% NaOH, at 120° C, under pressure. After pretreatment, the substrate was washed with water until neutrality and dried at 70° C.
- *Hydrolysis experiments:* the experiments were performed in 25 mL flasks, at 50° C and 4.5 pH. The substrate concentrations were: 0.02; 0.035; 0.04; 0.06; 0.08; 0.1; 0.12; 0.14; 0.16; 0.18; 0.2; 0.21 g/mL. To avoid bacterial contamination, sodium azide was added.
- *Time process*: 24 hours.

Prior to enzymic hydrolysis, the lignocellulosic substrate was treated with sodium hydroxide in order to improve the enzyme accessibility to the substrate. Dilute NaOH treatment causes swelling decrease in the degree of polymerization, separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structure (Table 1. from Annex 4.).

The proposed model for the enzymatic hydrolysis can be considered as an extension of Michaelis–Menten classical kinetic (Michaelis, Menten, 1913), taking into account the inhibition produced by substrate (Caramihai, Jecu, 1995). Based on these premises, the hydrolysis proceeds according to the following equation:

$$\upsilon = \frac{k_1 S}{k_2 + S}(1 - e^{k_3(S - S_{\lim})}) \qquad \textbf{3.1.4.1}$$

where: S = substrate concentration (g/mL);
$S_{\lim}$ = limiting substrate concentration, - the enzymatic hydrolysis rate is zero (g/mL);
k_1, k_2, k_3 = constants.

The modeling parameters and errors are presented in Table 2., Annex 4.

As shown, the modeling error for the untreated substrate is higher as compared with the corresponding value for the treated straw, as a consequence of the off-line measurements and structural modification of the pretreated substrate.

The modeling results vs. real data are presented in fig.3.1.4.1 and 3.1.4.2.

The agreement between real and modeling data is good enough (Table 3., Annex 4.) to allow the obtainment of the optimal substrate concentration for the enzymatic process control.
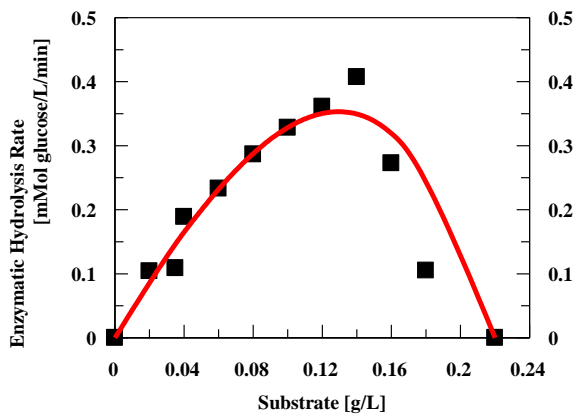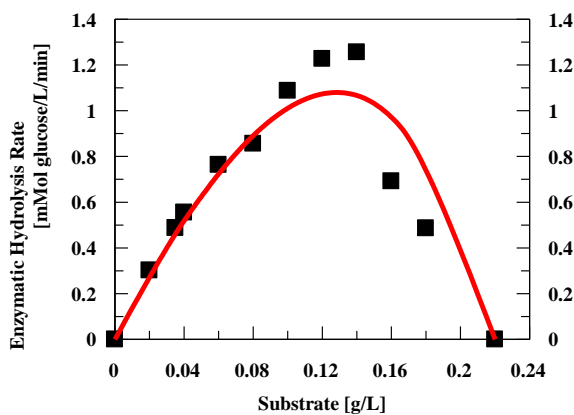
Fig. 3.1.4.1 Untreated wheat straw: ■ **real data,** ▬ **model**

Fig. 3.1.4.2 Treated wheat straw: ■ **real data,** ▬ **model**

Meanwhile, the maximal experimental values of the process rate are higher as those obtained from the model, due to the utilization of the limited substrate concentration, empirical evaluated.

3.1.5. Modeling of the enzymatic hydrolysis in the conditions of product inhibition

In enzyme reactions, the accumulated end products inhibit the rate of the process. Many authors reported that glucose inhibited the hydrolysis of cellulose by filtrates of the majority of the cellulolytic organisms (Wood, 1992; Robson *et al.,* 1989). The degree of inhibition depends on the relative affinities of the inhibitor and substrate for the active sites and on the ratio of the concentration of the inhibitor and substrate.

Experimental conditions

- *Substrate:* alkaline pre-treated wheat straw;
- *Enzyme:* enzymic preparate obtained from *Aspergillus niger* cultures;
- *Enzymatic hydrolysis:* the experiments were performed at 50° C with $10 - 120$ g/L substrate concentrations, in order to avoid the inhibition with substrate excess (cf. #**3.1.4.**). To study the effect of glucose accumulation in the reaction mixture, glucose of different concentrations (10, 20 and 40 g/L) was added. The content of reducing sugars was evaluated with dinitrosalicylic reagent, according to Miller (1959).
- *Hydrolysis time*: 24 hours.

According to experimental data, the product formation (glucose) was found to increase asymptotically to a maximum value. Additionally, high exogene glucose has been shown to be inhibitory in cellulase activity against substrate (Caramihai, Jecu, 1995). In these conditions, the following model (Caramihai, Jecu; 1996) was proposed to describe the process:

$$P = \frac{P_{\max}}{1 + e^{-\frac{k_m - S}{k_i}}} \qquad \textbf{3.1.5.1}$$

where: P = product concentration (g/L)

$P_{\max}$ = maximum product concentration (g/L)

S = substrate concentration (g/L)

k_m = Michaelis & Menten constant type (g/L)

k_i = inhibitory constant

This equation, an extension of the classical enzymatic kinetics, displays a better dependence between the glucose formation and the inhibitory effect of end product in the enzymatic hydrolysis of cellulose.

Fig. 3.1.5.1 presents the modeling data for the hydrolytic processes, with or without various

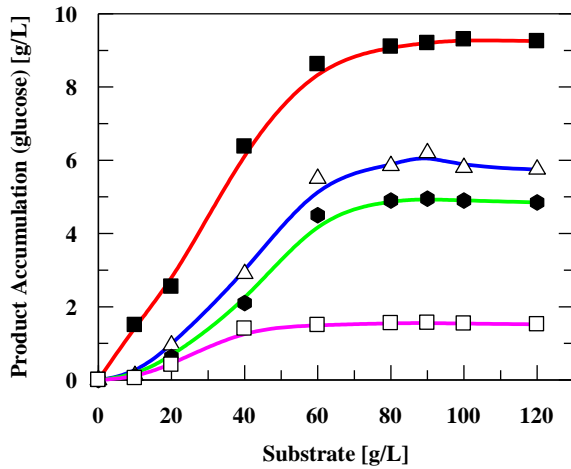exogene glucose concentrations (10, 20 and 40 g/L).

Fig. 3.1.5.1 Product accumulation vs. substrate concentration

■ **real data,** ── **model (0 g/L glucose addition)**

△ **real data,** ── **model (10 g/L glucose addition)**

⬣ **real data,** ── **model (20 g/L glucose addition)**

□ **real data,** ── **model (40 g/L glucose addition)**

The modeling errors are shown in Annex 5, Table 1.

The results can be viewed as a step in the correct control for maximum product formation, in conjunction with the influences of substrate concentration and end product inhibition.

3.1.6. Yeast cultivation on simulated liquor

In order to obtain cellulose, chemical treatment has been considered a viable option for upgrading the huge quantities of waste lignocellulose generated annually through the activities of the agricultural and forest industries (Wenzl, 1970). During the treatment, acetic acid contained in the hemicellulose structure is released and an inhibitory effect on *Candida utilis* growth was observed (Leonard, Hajni, 1945).

Due to assess the influence of acetic acid on the growth of this organism, a study was conducted using a synthetic medium, by simulating the real condition.

Experimental conditions
- *Growth medium:* 20 g/L glucose or 10 g/L xylose; 4 g/L yeast extract, 5 g/L peptone; inhibitory compound, acetic acid (1/100, w/v).
- *Organism: Candida utilis*
- *Agitation:* 180 rpm;
- *Temperature:* 35°C;
- *Process time:* 10 h;
- *Analytical methods:* biomass concentration was evaluated by optical density (DO); dry weight was estimated according to Jeffries and Streenath (1988), where 1 DO=0.19 g/L dry weight. The content of acetic acid was estimated with Perkin Elmer (Model B) gas chromatograph.

In order to develop an original specific growth rate (μ) model, firstly, an equation of inhibitors was elaborated (Caramihai *et al.*, 1995).

$$I = I_0 e^{k(I_0 - I_{\lim})} \qquad \textbf{3.1.6.1}$$

where: I_0 = initial inhibitor concentration (g/L);
$I_{\lim}$ = limiting inhibitor concentration (if $I = I_{\lim}$, $\mu = 0$);
t = time (h);
k = constant

Granted that the specific growth rate is determined by two substrates, of which one is for growth and the second acts as an inhibitor, the following equation can be written:

$$\mu = \frac{k_1 S}{k_2 + S}(1 - e^{k_3(I_0 - I_{\lim})}) \qquad \textbf{3.1.6.2}$$

where: S = non - inhibitory substrate concentration (g/L);

I = inhibitory substrate concentration (g/L)

k_1, k_2, k_3 = constants.

The influence of initial acetic acid concentration on yeast growth is shown in Table 1, Annex 6. Yeast strain assimilated both monosaccharides and acetic acid in cultivation conditions. The yield of biomass/ assimilated substrate was increased when xylose was used as primary substrate - 0.46g biomass dry weight/g assimilated sugar - as compared with 0.40g/g with glucose as primary substrate. When only monosaccharides were used as primary carbon source, without addition of acetic acid, the situation is reversed: 0.47g/g were obtained on glucose and 0.41g/g on xylose. Different metabolic pathways may be involved in contradictory results when acetic acid as inhibitory substrate.
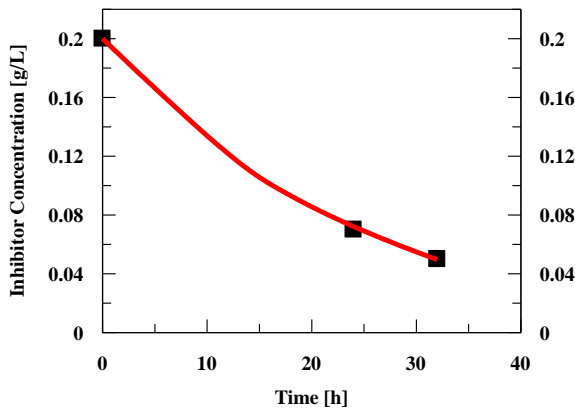

Fig. 3.1.6.1 Inhibitor concentration vs. time (substrate: glucose); ■ **real data,** ── **model**

At the end of fermentation, inhibitor concentrations for both experiments were the same. The above equations were checked with the experimental data presented in the Table 1. Fig. 3.1.6.1 and 3.1.6.2 show the inhibitor concentration vs. time for the both substrate types.
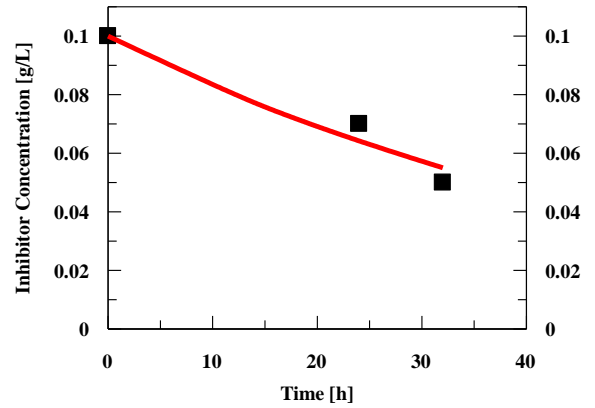

Fig. 3.1.6.2 Inhibitor concentration vs. time (substrate: xylose); ■ **real data,** ── **model**

The k parameter estimated value (from eq. 3.1.6.1) and the corresponding modeling errors are shown in Table 2, Annex 6. Table 3 presents the parameter-estimated values from eq. 3.1.6.2 in comparison with other classical models.
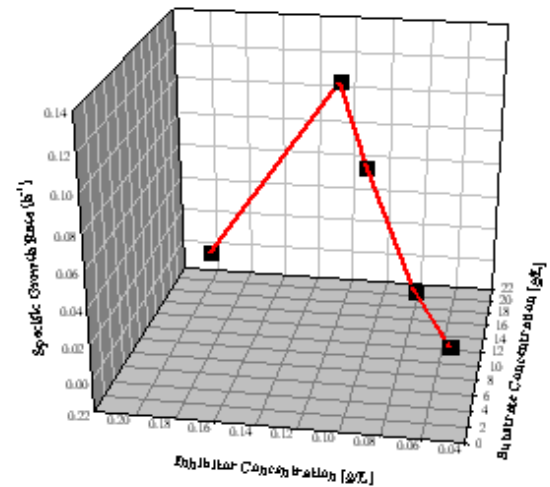

Fig. 3.1.6.3 Specific growth rate vs. substrate and inhibitor concentration (substrate: glucose); ■ **real data,** ── **model**

At last, fig. 3.1.6.3 and 3.1.6.4 present the specific growth rate dependence in connection with the substrate and inhibitor concentrations.
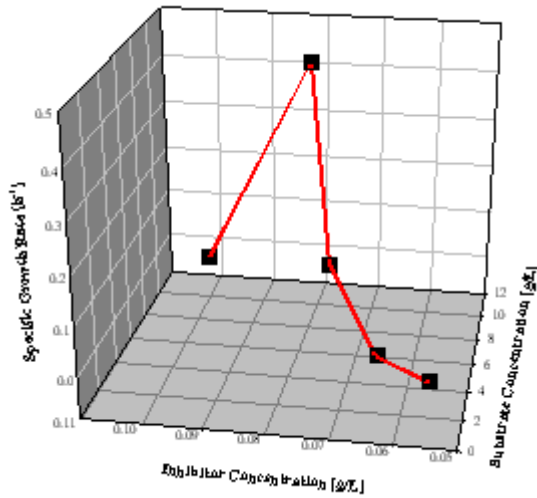
Fig. 3.1.6.4 Specific growth rate vs. substrate and inhibitor concentration (substrate: xylose); ■ **real data,** ▬ **model**

The models described by eq. 3.1.6.1 and 3.1.6.2 predicted well enough the experimental behavior, demonstrating the working hypothesis are valid. A powerful dependence between specific growth rate and inhibitor's evolution is sustained together with the presence of two parameters: S and I.

3.1.7. Modeling of methanol bacteria growth for SCP production

Since yeasts are characterized by low conversion yield of methanol and reduced protein level, it is of interest to study the Single Cell Protein (SCP) obtaining from methylotrophic bacteria. In this circumstance:
- The conversion yield is higher than in the yeast case;
- The protein content is higher (75-80% ref. dry weight, compared to 45-50% for yeast).

Experimental conditions:
- *Culture medium* (g/l)*:* $(NH_4)_2SO_4$ (1.5); KH_2PO_4 (1.5); $NaHPO_4$ (1.5); $MgSO_4$ (0.2); $FeSO_4$ (0.2); $CuSO_4$ (0.005); $CaCl_2$ (0.01). The methanol content varies from 0.1 to 0.2 %;
- *pH:* 6.7-6.9 by the addition of NH_3;
- *Temperature:* 35°C +/-1°C;

- *Pressure:* 0.1 - 0.2 atm;
- *Air flow:* 1vvm;
- *Fermenter:* Biotec LKB of 8.4L working volume;
- *Organism: Methylomonas ICCF-27;*
- *Process time*: 36-40h; 13 experiments.

The bioprocess modeling take into account the inhibitor effect of methanol (Caramihai *et al*, 1992) on the bacteria growth. The experimental data are shown in Annex 7, Table 1-12. The dependence μ = f(S) (Annex 7, Table 13) is presented in Fig. 3.1.7.1. Note that in this case, the specific growth rate is zero for a finite substrate value.

Therefore, a valid hypothesis can be the presence of a Metabolic Reducing Factor, R(S) attached to the classical Haldane (Andrews) model. The metabolic reducing factor is zero when the substrate concentration achieved a limiting value, S = S_{lim}. Meanwhile, R(S) tills to 1 for lower substrate concentration values. Hence, the proposed model is:

$$\mu = \frac{k_1 S}{k_2 + S + k_3 S^2} R(S)$$
$$= \frac{k_1 S}{k_2 + S + k_3 S^2}(1 - e^{k_4(S - S_{lim})})$$

           3.1.7.1

where: R(S) = metabolic reducing factor;
      μ = specific growth rate (h^{-1});
      S = methanol concentration (g/L, g%);
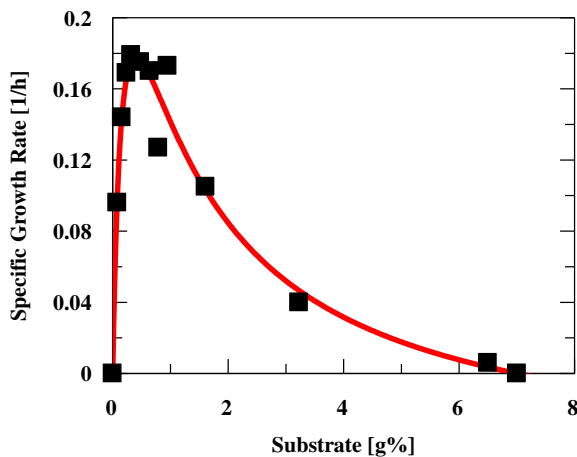 k_1, k_2, k_3, k_4 = constants

13

Fig. 3.1.7.1 Specific growth rate vs. substrate concentration; ■ **real data,** ▬ **model**

Fig. 3.1.7.1 shows a good experimental data fitting. The parameter values and modeling errors are shown in Annex7, Table 14. Moreover, Table 15 exposes comparative results between the proposed model and other literature models.

3.1.8. Modeling of a fungal cellulase process

Microorganisms growing on lignocellulosic substrates are able to produce extracellular enzymes, which degrade cellulose to soluble sugars. These sugars, mainly cellobiose and glucose, represent available carbon source for the secretion of extracellular enzymes, cellulases. Cellulose fermentation thus represents a very interesting example of a fermentation where growth, enzyme production and enzyme reaction are closely interdependent.

Cellulase is a multicomponent enzymatic system, which comprises three main enzymes: endoglucanases, exoglucanases (cellobiohydrolases) and beta-glucosidases. The individual enzymes act synergistic for the complete degradation of insoluble cellulose. The most important cellulolytic fungus is *Trichoderma reesei*, but it is of interest to study other organisms, like *Aspergillus sp.*, which is able to produce a wide range of extracellular enzymes growing on various substrates.

Experimental conditions

- *Organism: Aspergillus niger* strain which belongs to microbial collection of Research Institute for Chemistry;
- *Culture media:* the strain was cultivated on Mandels's medium (1969) with 1% cellulose as sole carbon source. The fungal cultures were performed at 4.5 pH and 28ºC. The culture broth was centrifuged at 3000 rpm and the supernatant was evaluated for cellulase activity;
- *Analytical methods*: the enzymatic activity was measured as endoglucanase activity using 1% carboxymethylcellulose, according to Mandels (1976). One unit of enzyme activity was expressed as the amount of glucose (micromoles) released per minute under the assay conditions. The reducing sugars were evaluated with dinitrosalicylic reagent according to Miller (1959). Soluble protein was determined by the Lowry method (1951). Mycelial protein was extracted with NaOH, the protein was determined on the combined extracts. Cellulose was determined as total carbohydrate in the residue from the NaOH extraction by the sulfuric acid method. Cell mass was calculated as dry weight minus cellulose.
- *Time process:* 120 h.

The relationship between cellulase production and cellulose concentration, based on the assumption of Gaden (1959) describing the growth of the fungus and product release, was given by the following equation (Caramihai *et al.,* 1996):

$$P = \frac{aX}{b + X} e^{-cS^2} \qquad\qquad \textbf{3.1.8.1}$$

where: X = cell concentration, expressed as dry weight (mg/ml);

      S = residual substrate (g/l);
      P = endoglucanase activity (IU/min);
      a, b, c = constants

The experimental results of the fungal cultivation are given in Annex 8, Table 1. The relationship between mycelial protein and enzyme production is shown in figure 3.1.8.1. This indicates an increase in the production of enzyme with increasing growth microorganism, evaluated as mycelial protein.
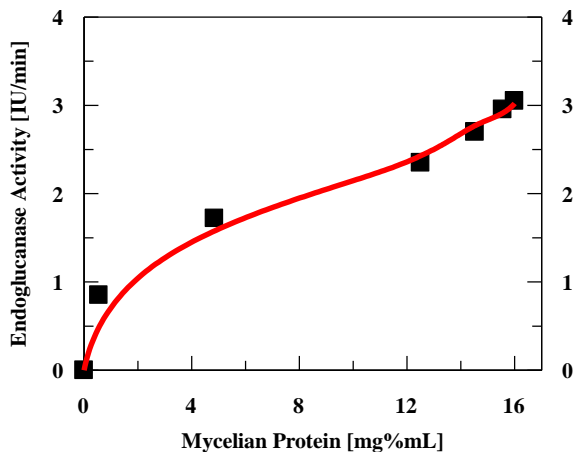

Fig. 3.1.8.1 Endoglucanase production vs. mycelian protein ■ real data; —— model

The response of substrate concentration at the growth and enzyme production is seen in Figure 3.1.8.2. After 120 h of fermentation, the level of residual substrate indicates a carbon source consumption of approx. 30% for maximum cellulase production. The profile indicates a good fitting between the real and modeling data.
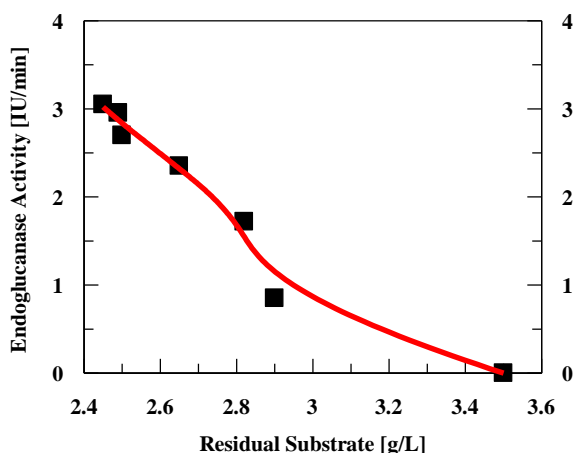

Fig. 3.1.8.2 Endoglucanase production vs. residual substrate; ■ real data, —— model

Hence, fig. 3.1.8.3 shows the dependence of cellulase production vs. cell mass and cellulose utilization in the batch fermentation of cellulolytic fungus *Aspergillus niger*.
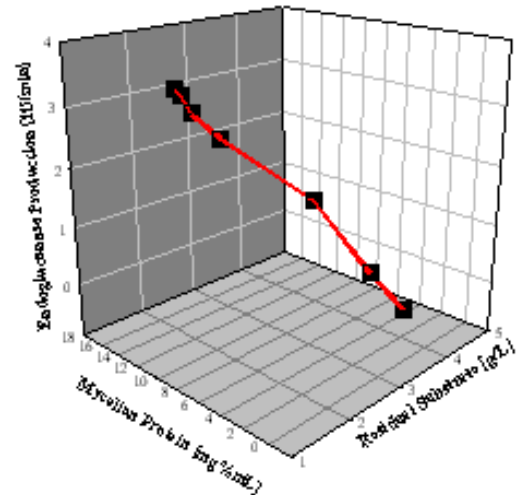

Fig. 3.1.8.3 Endoglucanase production vs. mycelian protein and residual substrate; ■ real data, —— model

The model parameters and modeling errors are given in Table 2, Annex 8.

3.2. OPTIMIZATION STATEMENT

The bioprocess modeling and (intelligent) control imposes the design of a system that can distinguish and develop the corresponding operations in order to control the process (Montague *et al*., 1991). It has already proved (in the above chapters) the multivariability and the nonlinearity of the fermentation processes. In this case, the bioprocess control, based on *a priori* model, is very difficult due to the scarcity of the on line process information and of the deficit concerning the theoretical substantiation regarding the cellular metabolism and its influence on the bioprocess behavior.

In these conditions it is necessary to replace the classical control methods with those based on intelligent techniques. The thorough utilization of one of them guides to a deadlock, either because of the bio-physique-chemical knowledge deficiency regarding the cell population evolution (i.e. the classical control techniques), or the exhaustive utilization of the human expert knowledge, which are subjective and non-standard (i.e. the intelligent control structure) - (Thibault, Najim, 1993).

Hence, 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).

Commonly, a bioprocess control structure departs from its specificity (Chang *et al*., 1988; Lim *et al*., 1986, Semones, Lim, 1989), i.e. the control system is designed for each process type. The bioprocess diversity imposes a corresponding variety of control algorithms.

Consequently, a control structure restriction type can be established through bioprocess

classes substantiation (e.g. cell growth, enzymatic hydrolysis, product obtaining, etc.). Each bioprocess class agrees with a finite number of control algorithms. The intelligent control structure must establish the bioprocess type (from a corresponding class), based on *a priori* information outfitted by the human operator.

Once the bioprocess type established, ICS checks the corresponding model. The main advantage of *a priori* model is the possibility to (analytically) set the global optimum, independently from the subjective human expert information.

The human bioprocess general description is commonly altered by the psychological and perception factors. It has already founded (Garrido-Sanchez *et al*, 1993) that the intelligent control systems founded (only) on the human subjective knowledge is less performant that the control systems who utilize the objective information fitted by a conceptual model. Hence, the literature recommends (Fonteix *et al*., 1994) the intelligent control techniques utilization only if the control structures based on quantitative models fail.

Therefore, the utilization of conceptual models in conjunction with intelligent control techniques appear to be attractive (Chtourou *et al*., 1993; Normandin *et al*., 1994; Willis *et al*., 1992). Through the analytical model the optimum coordinates are determined; the modalities to reach these coordinates are founded by the intelligent structure (Thibault, van Breusegem, 1991). Hence, the subjective conditions (resulted from the human expert) needed to attain the optimality are juxtaposed with the objective elements (outcome from the mathematical formalism) which display the optimum coordinates. Obviously, the biosystems are multivariable. A process

optimum in connection with a variable (or a variables set) is not obligatory the global optimum of the bioprocess (in relation with *all* the variables) - (Luttman *et al*., 1985; Lee *et al*., 1991; Modak *et al*., 1986)). The choice is tacked based on human knowledge and experience in connection with the final objective, related to a specific fermentation process.

CONCLUSIONS

1. The bioprocess evolution analysis has allowed to define models who fit better the process evolution curves. Specific bioprocess classes have been described and some original models have been developed in order to increase the fitting of real data. The estimation errors were better in all cases (see Annexes) than the well-known corresponding literature model errors.
2. The model diversity can be (conceptually) treated through an unique control structure based on analytical model and intelligent techniques. Hence, an intelligent control structure was designed, which is able to detect the bioprocess type, to design the process model and to control the fermentation process based on intelligent techniques, in order to maximize a performance index.
3. The corresponding database was configured through the AS400 system, due to its well-known advantages (security and integrity of data). The database access allows the data monitoring and, on the other hand, the establishing of the bioprocess evolution patterns.