Bioprocess Engineering 13 (1995) 69–78 © Springer-Verlag 1995

Applications of modelling for bioprocess design and control in industrial production

M. Rohner, H.-P. Meyer

Abstract The on-line measurement of the relevant parameters and the control conception for three production processes for fine chemicals by fermentation and biotransformation at the 15 m^3 scale were developed. The models describe the bioprocesses which successfully result in fully automated manufacturing steps. Modelling also proved to be a valuable tool for a better insight into biochemical fundamentals of the processes. Moreover, proper use of data logging, modelling and process control was important for quality, since two processes were controlled on-line and quality relevant deviations were registered early. Finally, combining modelling with simulation, we could drastically reduce both development time and cost.

List of symbols

F	l/h	flux
V	l	volume
u_0	g/l	nicotinonitrile concentration influx
u	g/l	actual nicotinonitrile concentration
q_u	g/gh	specific educt (=nicotinonitrile) transformation rate
x	g/l	biocatalyst concentration
p_0	g/l	nicotinamide concentration influx
p	g/l	actual nicotinamide concentration
q_p	g/gh	specific product (=nicotinamide) formation rate
k		parameter loss of activity
$q_{u,\max}$	g/gh	max. specific educt transformation rate
K_u	g/l	saturation constant for nicotinonitrile
K_i	g/l	inhibition constant for nicotinonitrile
K_{ii}	g/l	inhibition constant for nicotinamide
MW_A	g/mol	molecular weight for nicotinonitrile
MW_B	g/mol	molecular weight for nicotinamide
NS		Nicotinic acid
6-HNS		6-Hydroxynicotinic acid
$r_{NS,6HNS}$	g/lh	6-HNS production rate
$r_{6HNS,X}$	g/lh	biomass production rate
$r_{NS,6HNS,max}$	g/lh	max. 6-HNS production rate
s_{NS}	g/l	actual NS concentration
$K_{s,NS}$	g/l	saturation constant for NS
$K_{i,6HNS}$	g/l	inhibition constant for 6-HNS
K_{O2}	g/l	saturation constant for oxygen
$r_{6HNS,X,max}$	g/lh	max. biomass production rate
s_{6HNS}	g/l	actual 6-HNS concentration
$K_{ii,NS}$	g/l	inhibition constant for NS
RQ	mol/mol	respiration quotient
s_{xyl}	g/l	actual xylene concentration
$K_{i,xyl}$	g/	inhibition constant for xylene
$K_{i,DMPY}$	g/	inhibition constant for 2,5-dimethylpyrazine
r_X	g/lh	biomass production rate
$r_{X,max}$	g/lh	max. biomass production rate
$K_{s,xyl}$	g/l	saturation constant for xylene
s_{DMPY}	g/l	actual concentration of DMPY
$K_{i,MPCA}$	g/	inhibition constant for MPCA
K_{O2}	g/	saturation constant for oxygen
s_{MPCA}	g/l	actual MPCA concentration
s_{O2}	g/l	actual oxygen concentration
r_{MPCA}	g/lh	MPCA production rate
$r_{MPCA,max}$	g/lh	max. MPCA production rate
K_I	gl	inhibition constant for the intermediates
$K_{s,DMPY}$	gl	saturation constant for DMPY

Received 21 July 1994

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1 Introduction

Since a single bacterial cell contains several thousand enzymes catalyzing different biochemical reactions, growth and product formation (biotransformations) are a result of complex interactions. Using modelling as a tool, it is possible to simplify these complex interactions qualitatively and quantitatively. These formulated models can be checked and the behaviour predicted by the model compared with experimental data. Observed differences between model and experimental data are used to further improve the model until good agreement is obtained. Even hidden variables behind parameters can be detected, and performance under differing conditions predicted. However, it is necessary to know the coherence of the variables involved, and the parameters, thus allowing the black box model to become a grey box [1, 2]. In combination with statistical tools, the modelling technique complements purely empirical procedures, accelerates and simplifies process development and opens access to better process understanding. The necessary steps for a useful model are schematically shown in Fig. 1 [3]. The first and most

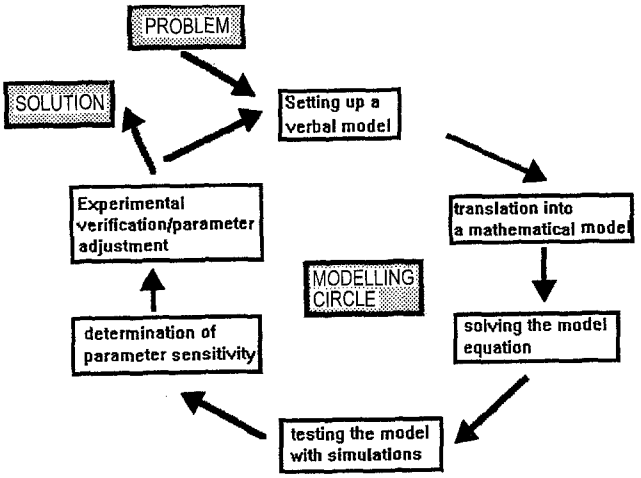

Fig. 1. The modelling circle

difficult step is to define a verbal model of the process, which is translated into a mathematical model and solved numerically. Modelling offers in general the following advantages for (bio)processes:

Design: processes and plant can be designed optimally and a high degree of automation can be reached.
Process control: bioprocesses can be sustained at their economical and ecological optimum. Process diagnosis can be carried out at any time and deviations (problems) from the process optimum are recognised early.
Gateway variables: access to non-measurable variables.
CIM: access for integrated future production systems [4].
Evolutionary operation: optimisation during manufacturing becomes possible.

One differential equation for one process variable is easy to solve, but in a real transformation process the number of variables involved is much higher. If there are more than 2 differential equations involved, it can be very difficult to predict the right solution, and the correct mathematical formulation of the model becomes important. Fortunately, numerical methods help solving mathematical models (differential equations) for the benefit of the process understanding. They can be used routinely for process development. This technology helps to elaborate the important process variables and their effect on the process.

It was not surprising that all the biotransformations for the production of fine chemicals, which we scaled up to production scale [5] exhibited extreme inhibition patterns by educts or products. For this reason, LONZA routinely applies modelling in combination with online analysis for biotransformation proceses on an industrial scale, to overcome inhibition by organic educts. Some of our complex processes are fully automated in production scale [6]. LONZA bioprocesses for the production of fine chemicals are exclusively fed-batch processes, where substrates and educts are added during fermentation and biotransformation according to a process-specific regime. This regime is controlled by a computer based algorithm (model), which was developed individually for each process. The time course of the disappearance of educts and substrates, and product and biomass formation are measured and used for steering the complex production systems. The objective of this work is to demonstrate, how we model and simulate our processes, and how we apply this information in production processes.

2
Material and methods

2.1
Organism and enzymes

The organism used for the biotransformation of nicotinic acid to 6-hydroxy nicotinic acid is Pseudomonas acidovorans, a mutant of a Lonza strain, originally isolated from a culture growing on nicotinic acid solutions. The strain was described in a patent [7]. The catabolic activities are influenced by nicotinic acid concentrations; this regulatory effect is used to prevent the further degradation of 6-hydroxynicotinic acid during the biotransformation step. The same patent describes culture conditions and media composition for fermentation and biotransformation. Previous to biotransformation, the biomass was grown in a fed batch culture using nicotinic acid as carbon and nitrogen source.

The organism used for the biotransformation of 2,5-dimethylpyrazine to 5-methyl pyrazinic acid is a Pseudomonas putida strain (ATCC 33015). The strain is described in a patent [8]. The same patent also describes culture conditions and media compositions for the biotrasformation. The biomass was grown in a fed batch culture and in parallel the product, 5-methyl-2-pyrazinic acid, is formed. The product and its biochemistry is further described by Kiener [9].

The enzyme used for nicotinamide was an immobilized nitrile hydratase of Rhodococcus rhodochrous [10, 11, 12] and transforms nicotinonitrile into nicotinamide.

2.2
Fermentation equipment

The biotransformations for 6-hydroxynicotinic acid production were carried out in 15 m^3 fermentors.

The biotransformations for 5-methyl-2-pyrazinic acid were carried out in 1.5 m^3 fermentors. The biotransformations using nitrile hydratase were carried out in 2 l stirred vessels.

2.3
Analytical methods and on-line control (FIA)

An on-line measurement system was developed to estimate the 6-hydroxynicotinic acid concentrations by a photometric method at 308 nm in the cell-free supernatant (Fig. 2).

The cell free supernatant was diluted in a mixed chamber and continuously measured. The amplitude of the signal was used for the process computer (=6-HNS concentration). Measurements were taken every three minutes. The FIA could be operated with a fixed dilution, since only low values of 6-HNS were of interest for the biomass cultivation. The zero

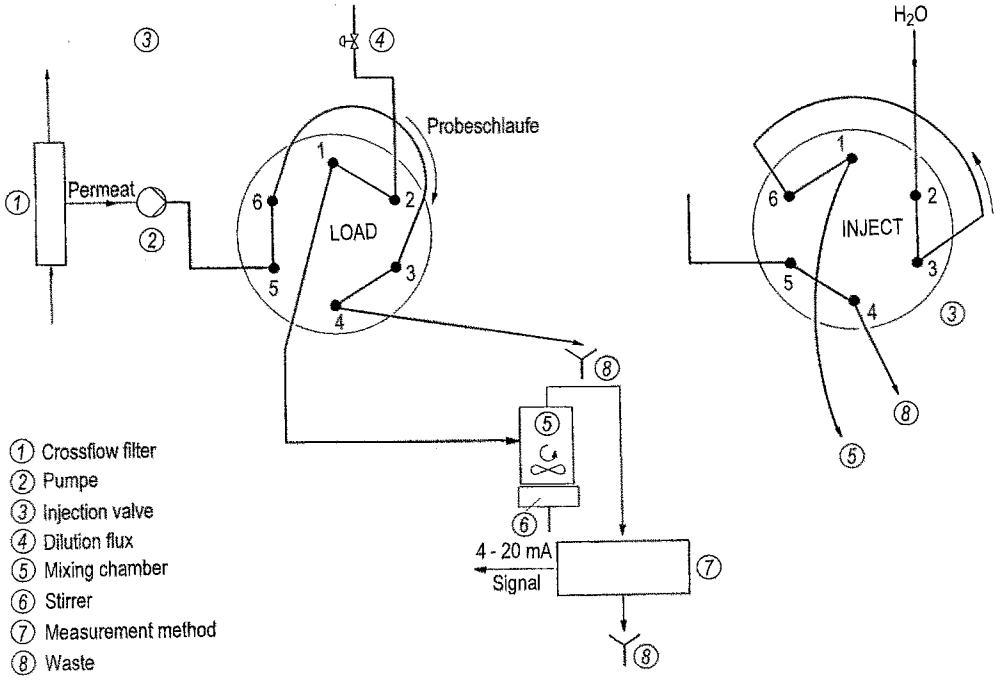

Fig. 2. Flow injection analysis used for the 6-HNS controller. The signal output of 4–20 mA is directed to the process controller

line was always constant and no filter clogging problems occurred (cell separation).

Another on-line system was developed to measure 2,5-dimethylpyrazine by an HPLC method (Fig. 9). The cell-free supernatant was injected without dilution onto an HPLC column using an automatic sample injection system. The FIA could be installed without dilution, since again only low concentrations (0–5 g/l) were of interest. Based on an on-line mass balance, the product titer of 5-methyl-2-pyrazinic carbonic acid was calculated. The HPLC method proved to be extremely stable and less than 1% failure (air bubbles) was observed. Stable operating time without any problems for more than 50 h were established.

Xylene was measured in the off-gas of the fermenter using an on-line photometer (ISCO, USA) at 214 nm [9].

The pH and the partial pressure of oxygen were measured with Ingold pH and pO_2 sensors (Ingold AG, 8902 Urdorf, Switzerland).

Off-gas analysis: Oxygen and carbon dioxide in the exhaust gases were continuously measured with a Servomex paramagnetic oxygen analyser (series 1100) and a Servomex infrared carbon dioxide analyser (2500 IR) (Servomex Ltd., Crowborough, Sussex TNG 3DU England).

All on-line signals were conected with the process-controlling computer system (PLS 80 E from Eckardt, Germany).

2.4
Computational hard- and software

Simulations were carried out using the computer aided software SIMNON for MS-DOS computers (Dept. of Automatic Control, Lund Institute of Technology, PO Box 118, S-22100 Lund Sweden).

3
Models governing equations

3.1
Modelling of the biotransformation of nicotinic acid to 6-hydroxynicotinic acid

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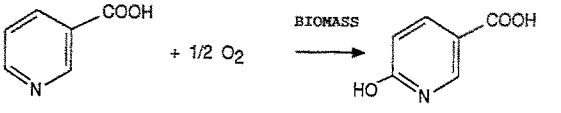


NICOTINIC ACID (NS)

6-HYDROXYNICOTINIC ACID (6-HNS)

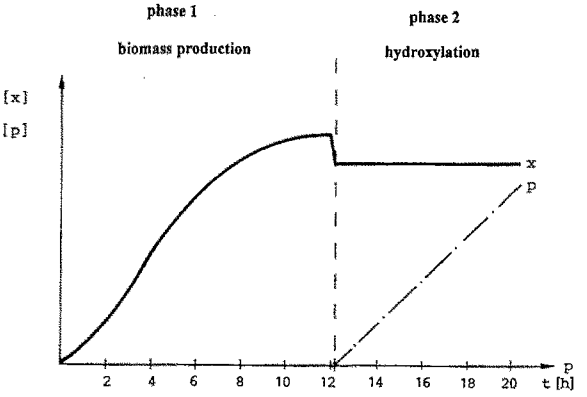


Fig. 3. Two phases of 6-HNS process, first the biomass production step and subsequently the biotransformation step. After approximately 12 h, 5–10% of the working reactor volume are taken as inoculum (cycled fed-batch process) for the next production run

The proces consists of two steps, described in Eq. (1). In the first manufacturing step, the cells are grown and induced on nicotinic acid as a single carbon source (Fig. 3). Nicotinic acid is completely oxidised via the intermediate 6-hydroxynicotinic acid to carbon dioxide, biomass and water, but only if nicotinic acid does not exceed inhibiting concentrations. The formation of the enzymes involved is growth related. Subsequently, in the second manufacturing step, the inhibitory effect of high nicotinic acid concentrations indicated in Eq. (1) is used to block the catabolic step down-stream of 6-hydroxynicotinic acid, resulting in the accumulation of 6-hydroxynicotinic acid. Oxygen in Eq. (1) originates from air, not only for growth, but for hydroxylation as well:

$$\mathrm{NS} + \mathrm{O_2} \rightarrow \text{6-HNS} + \mathrm{O_2} \xrightarrow[\text{NS inhibition}]{} \text{biomass} + \mathrm{CO_2} + \mathrm{H_2O} + \text{energy} + \text{heat}. \quad (1)$$

Both steps have their reaction rates, the total reaction rate being

$$r_{\mathrm{NS},X} = r_{\mathrm{NS,6HNS}} + r_{\mathrm{6HNS},X}. \quad (2)$$

6-hydroxynicotinic acid accumulates in the culture medium, and is transported by an unknown mechanism into the bacterial cell for its complete oxidation to CO_2 and H_2O. Nicotinic acid is transformed to 6-hydroxynicotinic acid at maximal velocity ($r_{\mathrm{NS,6HNS}}$) only if the NS concentration (s_{NS}) is much higher than the saturation constant ($K_{s,\mathrm{NS}}$). We did not observe any inhibition by 6-HNS in the concentration range used (up to 100 g/l). Since oxygen is the main limiting factor [13], the reaction rates of the first reaction can be written as

$$r_{\mathrm{NS,6HNS}} = r_{\mathrm{6NS,6HNS,max}} * \frac{s^{\mathrm{NS}}}{s_{\mathrm{NS}} + K_{s,\mathrm{NS}}} * \frac{K_{i,\mathrm{6HNS}}}{K_{i,\mathrm{6HNS}} + s_{\mathrm{6HNS}}} * \frac{\mathrm{O_2}}{\mathrm{O_2} + \mathrm{K_{O2}}}. \quad (3)$$

Again, the catabolism of 6-HNS is at maximum speed if the 6-HNS concentration ($s_{6\mathrm{HNS}}$) is much higher than the saturation constant for 6-HNS ($K_{s,6\mathrm{HNS}}$). This time, however, the reaction is inhibited due to NS, starting at 0.4 g/l nicotinic acid. Under non-inhibiting conditions, oxygen is the main limiting step of biomass production. Therefore, the second main reaction can be written as

$$r_{\mathrm{6HNS},X} = r_{\mathrm{6HNS},X,\max} * \frac{s_{\mathrm{6HNS}}}{s_{\mathrm{6HNS}} + K_{s,\mathrm{6HNS}}} * \frac{K_{ii,\mathrm{NS}}}{K_{ii,\mathrm{NS}} + s_{\mathrm{NS}}} * \frac{\mathrm{O_2}}{\mathrm{O_2} + K_{2O2}}. \quad (4)$$

The following interactions of the biological variables were determined:

$$K_{i,\mathrm{6HNS}} \gg K_{ii,\mathrm{NS}} \text{ and } K_{s,\mathrm{NS}} \ll K_{s,\mathrm{6HNS}}, \quad (5)$$

and

$$r_{\mathrm{NS,6HNS,max}} \gg r_{\mathrm{6HNS},X,\max}. \quad (6)$$

The biotransformation is further described by five additional variables: Biomass (X), educt concentration (NS), substrate concentration (6-HNS) and the molar gas fractions yO_2 and yCO_2. RQ values, calculated from the molar gas fractions measured on-line, is an important information. We defined three different respiration quotient (RQ) conditions for biomass formation:

$$\mathrm{RQ} > 1.1, \quad (7)$$

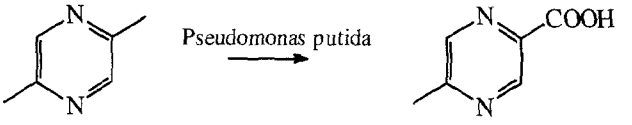
$$\mathrm{RQ} = 1.1, \quad (8)$$

$$\mathrm{RQ} < 1.1. \quad (9)$$

If NS is catabolised completely to biomass without inhibition and accumulation of 6-HNS, the RQ value equals 1.1 (8), and all final products are produced according to the stoichiometric balance equations. In the case of a RQ value below 1.1 (9), there are two possibilities. Firstly 6-hydroxynicotinic acid is accumulated, and O_2 consumption becomes proportionally larger than CO_2 formation (see Eq. (1), because of inhibiting nicotinic acid concentrations (8). Secondly: although the concentration of nicotinic acid is below inhibiting concentrations, 6-hydroxynicotinic acid accumulates because of a high saturation constant for $K_{s,\text{6-HNS}}$ (9). Rapid degradation of accumulated 6-HNS results in RQ values much higher than 1.1 (7), since actual concentrations of 6-hydroxynicotinic acid are much higher than the saturation constant $K_{s,6-\mathrm{HNS}}$. Contrary to 6-HNS, we did not have a rapid on-line NS measurement methodology applicable for this process. The inhibition tolerance ($K_{ii,\mathrm{NS}}$), on the other hand, is low, and any accumulation of NS during the biomass formation should be detected as early as possible. RQ turned out to be a very fast, reliable indirect parameter to monitor any acumulation of NS during biomass production.

3.2
Modelling of the biotransformation of 2,5-dimethylpyrazine to 5-methyl-2-pyrazinic acid

Reaction:



2,5-Dimethylpyrazin — DMPy

5-Methyl-2-pyrazincarbonpyrazinic acid — MPCA

The process is a one step process with biomass growth and product formation in parallel, as described in Eqs. (10) and (11). The cells are grown on *p*-xylene as sole carbon source. The enzyme system for the degradation of *p*-xylene into 5-toluic acid is the same as for the production of 5-methyl-2-pyrazinic acid from 2,5-dimethyl pyrazine via alcohol and aldehyde formation, Eq. (13), and the formation of the enzymes for the biotransformation is growth related. However, for the model, we considered the oxidation of xylene and the oxidation of methylpyrazine as one enzyme process. For the production of biomass, xylene should be not limited, which means that s_{xyl} should be maximized, Eq. (13). On the other hand for production, the enzyme system should be saturated, mainly with 2,5-dimethyl pyrazine, thus s_{xyl} should be minimized. *P*-xylene is completely oxidised only

if *p*-xylene does not form a liquid–liquid phase in the fermenter. Both xylene and 2,5-dimethylpyrazine kill the cells at higher concentrations. As soon as a two phase system is formed, the cells are killed. Oxygen in Eq. (12) originates from air, not only for growth, but for oxidation as well:

$$p\text{-Xylol} + O_2 \xrightarrow[\text{DMPY inhibition}]{} \text{biomass}$$

$$+ CO_2 + H_2O + \text{Energy}, \tag{10}$$

$$\text{DMPY} \rightarrow \text{R–OH} \rightarrow \text{R=O} \rightarrow \text{MPCA}, \tag{11}$$

$$r_X = r_{X,\max} * \frac{s_{xyl}}{s_{xyl} + K_{s,xyl}} * \frac{K_{i,xyl}}{K_{i,xyl} + s_{xyl}} * \frac{K_{i,DMPY}}{K_{i,DMPY} + s_{DMPY}} * \frac{K_{i,MPCA}}{K_{i,MPCA} + s_{MPCA}} * \frac{K_{O2}}{K_{O2} + s_{O2}}, \tag{12}$$

$$r_{MPCA} = r_{MPCA,\max} * \frac{s_{DMPY}}{s_{DMPY} + K_{s,DMPY}} * \frac{K_{i,xyl}}{K_{i,xyl} + s_{xyl}} * K_l * \text{Molfaktor}. \tag{13}$$

The energy balance plays an important role for the living cells. However, the energy mass balance is not known in detail, but we know for example that the first biotransformation step is endergonic (14). Therefore, if too much educt is added at low cell concentrations, too much energy is used for the first intermediate ((11) the energy mass balance is negative), sustainable cell growth is no longer possible and the biotransformation collapses. Therefore, the actual concentration of 2,5-dimethylpyrazine is increased from a suboptimal to an optimal concentration for the reaction rates (14). However, the applicable concentration of 2,5-dimethylpyrazine for a certain amount of cells is so far empirical:

$$\text{dNADH}/\text{d}t = \text{dNADH(biomass)}/\text{d}t - \text{dNADH(reaction)}\text{d}t > 0. \tag{14}$$

The biotransformation is further described by 5 variables, biomass X, educt concentration s_{DMPY}, substrate concentration s_{xyl} and the molar gas fractions y_{O2} and y_{CO2}.

3.3 Modelling the hydratase process

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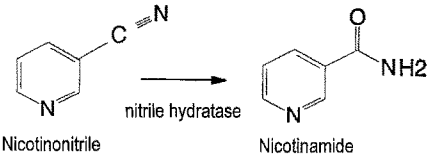

The verbal model of the enzymatic reaction is shown in Fig. 4. Biocatalyst and nicotinonitrile (educt, u) are simultaneously added into the reactor. Nicotinonitrile (u) is transformed into

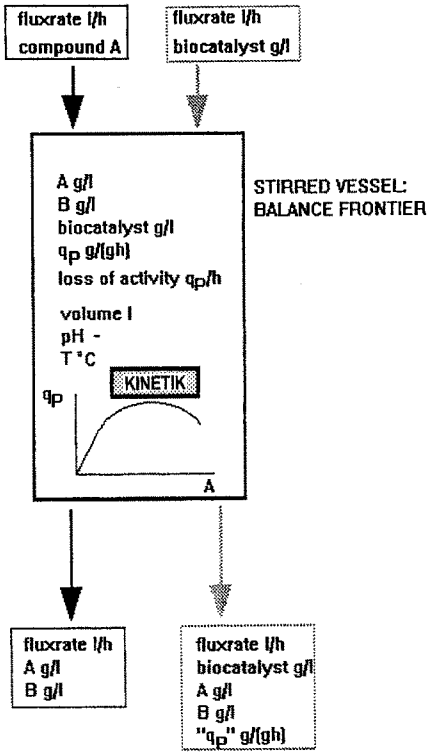

Fig. 4. Verbal model of an enzymatic process for one stage. Nicotinonitrile and biocatalyst is fed. A batch reaction with no biocatalyst and nicotinonitrile feed would be a special case where the transport terms are zero

nicotinamide (product, p), which subsequently is diluted out of the reactor, together with biocatalyst and some remaining nicotinonitrile (u) which has not been completely transformed. The outflux velocity is equal to the incoming flux, and the volume remains constant. For a certain temperature and pH, the reaction rate is given, but the enzyme is not stable and exhibits a loss of activity. The loss of activity is mainly due to the actual nicotinonitrile concentration (u) and the reaction temperature. The nicotinamide (p) causes less loss of activity. The pH had no inhibiting influence on the reaction system within the range we tested. Since the process was well mixed, ideal mixing was assumed, and any diffusion effects were not considered in the model. Michaelis–Menten type kinetics were applied to the enzymatic reaction system. Temperature was considered as a parameter.

The following four differential balance equations, Eqs. (15)–(18), including the kinetic terms describe the verbal model described above for the enzymatic reaction. Eq. (15) describes the change of the nicotinonitrile concentration (u) in the reactor:

$$\text{d}u/\text{d}t = \boldsymbol{F/V}*(\boldsymbol{u_0} - \boldsymbol{u}) - \underline{q_u}*x. \tag{15}$$

The biocatalyst concentration x in the reaction vessel was considered to be feeded with a certain flux velocity (F_x) having a certain specific activity q_u. The bold terms in Eqs. (16) and (15) are balance equation terms, influx and outflux with the respective concentration of nicotinonitrile (u) and nicotinamide (p). Eq. (16) describes the time course of the nicotinamide concentration (p):

$$\text{d}p/\text{d}t = \boldsymbol{F/V}*(\boldsymbol{p_0} - \boldsymbol{p}) + \underline{q_p}*x, \tag{16}$$

and an equation for the loss of activity:

$$dq_{u,max}/dt = -k * q_{u,max} * u, \quad (17)$$

and finally an equation for the biocatalyst concentration:

$$dx/dt = F_x/V * (x_0 - x). \quad (18)$$

For the loss of activity, a logarithmic degradation of the enzyme is assumed. The loss of activity is interpreted as a degradation of the specific activity ($q_{u,max}$) over the time. The underlined terms in Eqs. (15) and (16), the specific educt transformation rate (q_u) and the specific product formation rate (q_p) have now the folowing kinetic terms:

$$q_u = q_{u,max} * u/(u + K_u) * \underline{K_i/(K_i + u)} * \underline{K_{ii}/(K_{ii} + p)}, \quad (19)$$

and

$$q_p = -q_u * MW_A/MW_B. \quad (20)$$

Since the reaction takes place with almost 100% yield, all of the nicotinonitrile is transformed into nicotinamide. This means the rate of degradation of nicotinonitrile is equal to the product formation rate. The only stoichiometric factor is the ratio of the molecular weight for the nicotinonitrile and the nicotinamide. The bold terms are Michaelis–Menten type kinetics, assumed for an enzymatic one substrate, one enzyme reaction.

This model was transformed using a computer simulation program (SIMNON) for solving the differential equation. The solution of the equations became time courses for the variables nicotinamide and nicotinonitrile concentration and the specific rates.

4
Results and discussion

The underlying concept of our models is to achieve and realise such a control strategy, that the manufacturing process can be kept constantly and automatically at its optimum. This implies that educts and substrates are fed in such a manner that their concentrations are maintained precisely at their optimum, between K_i and K_s values.

4.1
1,6-hydroxynicotinic acid, cell production, first reaction step

For both steps, the hydroxylation and the decomposition to biomass (Fig. 3), oxygen from air is used. If the dissolved oxygen concentration in the fermentation broth is high enough (pO_2 high), the feed of nicotinic acid is switched on. If there is not enough oxygen for both steps, nicotinic acid feed has to be stopped. If the process is fed in the right regime (not over- or under-dosed with nicotinic acid), a strong, fast and clear response between dosing of nicotinic acid and use of oxygen (pO_2 response) is observed. The degradation of nicotinic acid from higher concentrations to lower concentrations is autocatalytic and the hydroxylation and subsequent uptake of hydroxynicotinic acid results in cell growth, if not otherwise limited. This means the way back from higher to lower concentrations of nicotinic acid does run autocatalytically, and stops if exhausted. Only the reverse way, the way from lower to higher nicotinic acid concentrations has to be fed (driven), preventing under- and overshooting from the optimum concentration.

This fundamental coherence is used for process control. According to this prescription, the process can be easily driven with a cascade control of the nicotinic acid dose valve connected with a pO_2 controller. However, the enrichment of nicotinic acid up to inhibiting concentrations could not be prevented with this type of controller, and the process proved to be fairly unstable. To gain a stable process, the overdose of nicotinic acid has to be controlled. However, the nicotinic acid concentration cannot be measured fast enough to prevent the enrichment and to keep the process at constant concentrations. To obtain a stable process, gateway sensors and the autocatalytic behaviour of the bacteria was used to solve this problem. As a result, the process is stable, within the optimum conditions for the process and is fully automated.

The partial pressure of oxygen was the leading parameter for process control, with RQ and 6-hydroxynicotinic and concentrations as adjacent parameters for on-line process control. The pO_2 has a similar function as the idle motion of a motor. The speed control is the job of the two additional control cycles, the RQ and 6-hydroxynicotinic controller.

The nicotinic acid concentration at its upper level is controlled by the RQ. The RQ indicates the inhibition due to nicotinic acid (gateway sensor) of the whole pathway if nicotinic acid is enriched (up to the inhibition concentration K_i, RQ < 1.1 see above). In order to prevent the pathway being closed for biomass production, the dose valve of nicotinic acid is closed. Due to the autocatalytic behaviour, the nicotinic acid is metabolized and the concentration is reduced. As soon as the RQ value is again higher than 1.1, the dose valve is opened. How much nicotinic acid is dosed is further controlled by measuring the concentration of the first intermediate, the 6-hydroxynicotinic acid concentration. This regulates the opening and closing frequency of the on/off valve. The RQ allows feeding only if nicotinic acid is lower than the inhibition concentration. A PID controller for the intermediate enables us to drive the process smoothly. The measured value has a low response time, and the system works well.

Two process conditions exist in case of RQ values below 1.1, Eqs. (8) and (9), and in addition the on-line measuring of 6-hydroxynicotinic acid prevents a process standstill if the concentrations of 6-hydroxynicotinic acid are below $K_{s,6\text{-HNS}}$.

This is the description of the principle of the observed oscillation until the process is finished. Keeping the concentration of nicotinic acid below K_i but above K_s of 6-hydroxynicotinic acid results in a transient process (see Fig. 5). The hard- and software, which was used to measure on-line educts and substrates, is described in the materials and methods section, and the configuration of the control cycles for nicotinic acid feed is shown in Fig. 6.

Figure 7 shows the reproducibility of the 6-HNS fed-batch process showing the optical density of several runs. The process time is highly reproducible, as is the enzyme activity produced.

4.1.2
6-HNS production second reaction step

Nicotinic acid is added to the previously produced cells in a fast and uncontrolled way to get high concentrations of nicotinic acid as soon as possible in order to prevent the

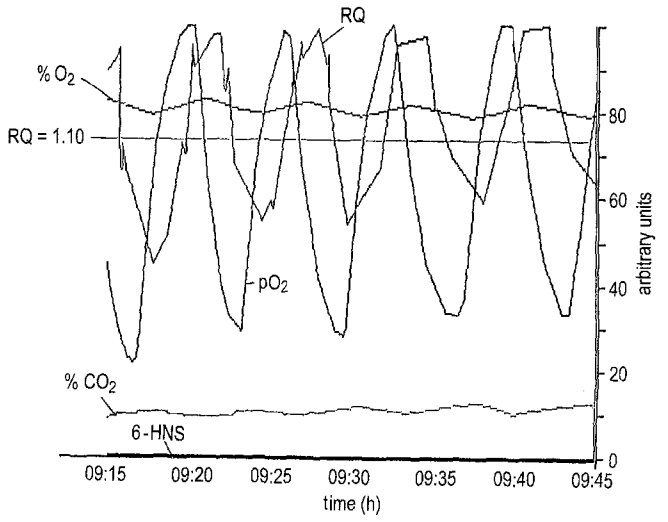

Fig. 5. 6-HNS process computer plot: Growth of Pseudomonas acidovorans in 10 m^3 production scale. The highly inhibiting carbon source is fed under full automation based on three control cycles, namely 6-HNS-, pO_2 and RQ control. Due to the tuning a transient process occurs

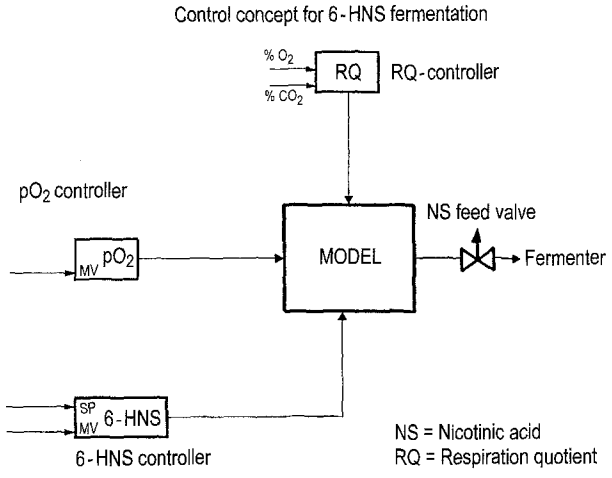

Fig. 6. Control concept for the 6-hydroxy nicotinic acid (6-HNS) fermentation process (only cell growth phase)

biomass production. The RQ falls immediately after adding nicotinic acid, down to values $\ll 1.1$ and only the first reaction step of the whole nicotinic acid degradation pathway can take place. After nicotinic acid is transformed, the increasing RQ values indicate the end of the production step of 6-hydroxynicotinic acid (Fig. 8). Based on the biological parameter which indicates the complete degradation of nicotinic acid if the RQ is again >1.1 (allowing the pathway for 6-HNS degradation to function again), the process can be cooled down early enough to prevent cell growth, which is unwanted in this production step. After crystallisation and drying, the production is finished.

4.2
5-methyl-2-pyrazincarbonic acid production

There are two relevant differences between the 6-HNS and MPCA process. Firstly, biomass production and biotransformation are carried out simultaneously for the

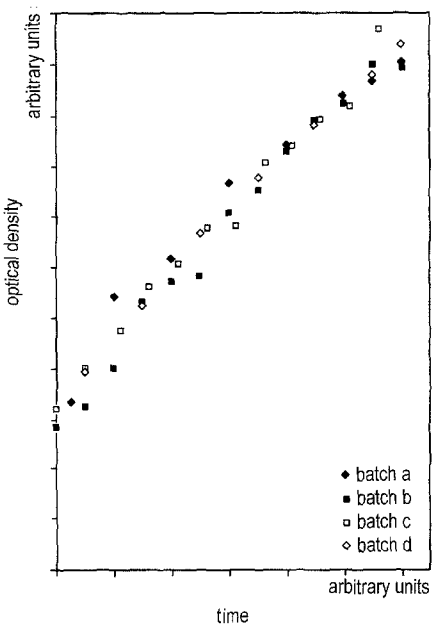

Fig. 7. Time vs. optical density (arbitrary units) of 4 representative batches (only cell growth) in the 10 m^3 scale. The reproducibility is very high and within the error deviation of the off-line optical density measuring method. The biomass is grown at its fastest speed and the enzyme quality is very high and very reproducible

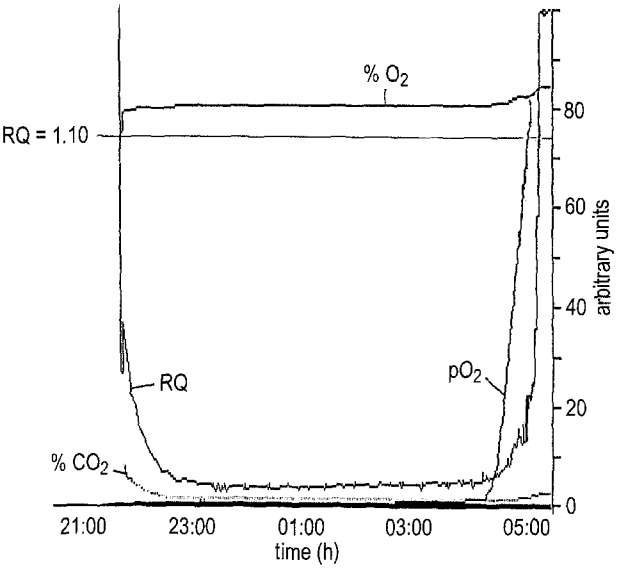

Fig. 8. 6-HNS process computer plot: Time vs. RQ of 1 representative batch in the 10 m^3 scale. Due to the high concentrations of nicotinic acid used in the production step (up to 100 g/l) the RQ is almost zero, indicating complete inhibition of biomass growth. At the end of the biotransformation, the increase of the RQ indicates low concentrations of nicotinic acid and the end of the biotransformation step. Due to the reproducible cell quality, the production time is also very reproducible

production of MPCA. Secondly, the process control is more complex, since two feed streams are used for regulating the process. Both steps, the production of biomass and the parallel production of 5-methyl-2-pyrazincarbonic acid are dependent from oxygen (air). Similar to the 6-hydroxynicotinic acid cell production process, the partial pressure of oxygen (pO_2) is the main parameter for process control, together with *p*-xylene

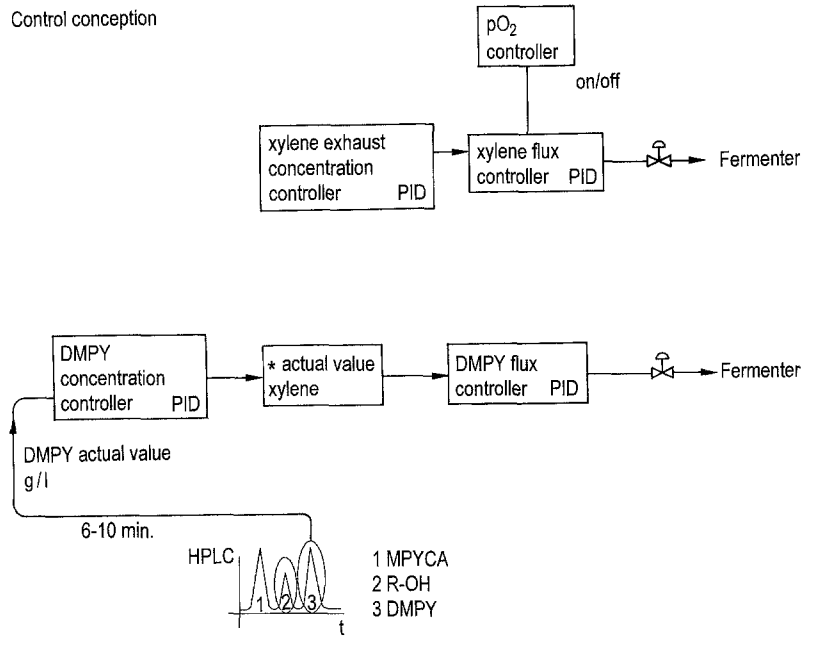

Fig. 9. Control concept for the 5-methyl-2-pyrazinic acid fermentation process. Cell growth and biotransformation in parallel is fully automated

concentration (measurement in the exhaust gas of the fermenter) and 2,5-dimethylpyrazin concentration (detection in the fermenter broth by HPLC, see Chapter 2.) as adjacent parameters for on-line process control (Fig. 9).

Again, the pO_2 is the basic controlling parameter, flanked by two additional control cycles for the *p*-xylene and 2,5-dimethylpyrazine concentrations.

If the dissolved oxygen concentration in the fermentation broth is high enough, feeding of *p*-xylene and 2,5-dimethylpyrazine is started. If there is not enough oxygen for both steps, the feeding is stopped. If the process is fed in the right regime, a strong, fast and clear response between dosing of *p*-xylene and use of oxygen is observed. The degradation of *p*-xylene from higher concentrations to lower concentrations is autocatalytic and results in cell growth if not otherwise limited. Only the reverse way, the way from lower to higher concentrations has to be regulated, avoiding formation of the second phase in the fermentor by the xylene, which has a low solubility in water.

The *p*-xylene concentration at its upper level is controlled by *p*-xylene measurement in the exhaust gas. Extensive studies have been carried out to identify the physical properties and the stable process window. In order to keep the pathway open and the cells growing for biotransformation, the dose valve of *p*-xylene is closed if a too high value for *p*-xylene is reached. The *p*-xylene is metabolized and the concentration is reduced. As soon as the lower level of *p*-xylene is again reached, the dose valve is reopened.

The controller for 2,5-dimethylpyrazin regulates the dose valve for the educt and in parallel to the *p*-xylene valve.

A *P*-controller for the educt regulates the process smoothly. Keeping the concentration of *p*-xylene and 2,5-dimethylpyrazin below K_i but above K_S, results in a transient process (Fig. 10).

The parameter fitting for the first 10 h allows unlimited xylene concentrations for rapid cell growth (exponential). After 10 h, the *p*-xylene feed is regulated to reach limiting

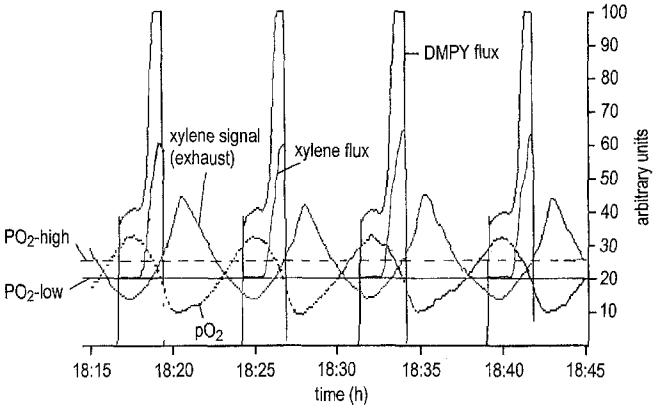

Fig. 10. MPCA process computer plot: Growth and biotransformation of Pseudomonas putida in 1.5 m^3 piloting scale. The feeding of the highly inhibiting carbon source and educt is fully automated based on three control cycles, namely DMPY-, pO_2 and xylene control. Due to the tuning a transient process occurs. The process is a very dynamic one. One oscillation takes only 3 minutes

concentrations of xylene, resulting in slow growth and maximal biotransformation rate (Fig. 11).

4.3 Nicotinamide production

Fig. 12 shows the comparison of the time course of experimental data and simulations of a continuous one step reaction for the hydratase enzyme (with constant concentration of biocatalyst, without feeding of biocatalyst). This figure shows, that both simulated and experimental data fit well. The experimental set of data was used to estimate the hydratase deactivation (parameter k in Eq. (17)). The model fits the process also under complete changed process conditions (data not shown). The parameters are obviously

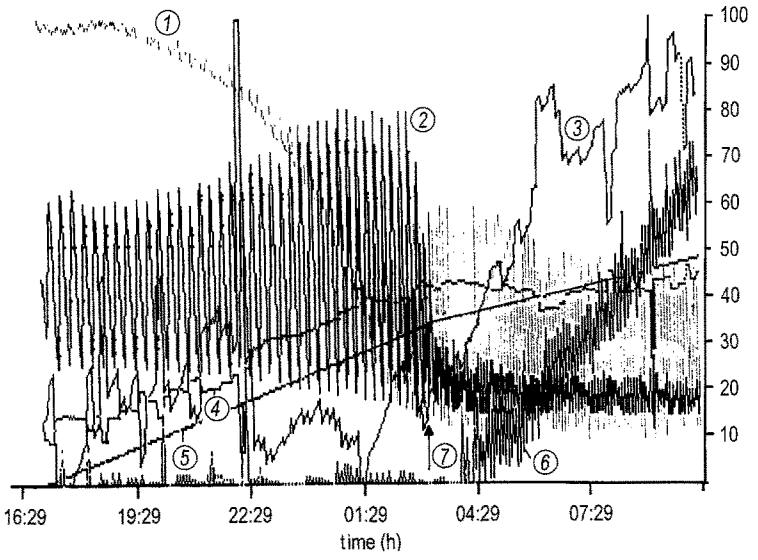

Fig. 11. MPCA process computer plot: The picture shows a batch. After 10 h the process switches from xylene unlimited conditions (for cell growth) to xylene limited conditions (for production). The signals are the following *1* pO_2 (%), *2* *p*-xylene in the exhaust gas (mA), *3* output DMPY dose controller (%) *4* HPLC on-line signal of DMPY (g/l), *5* setpoint ramp DMPY concentration (g/l), *6* MPCA calculated (mass balance, g/l), *7* Change from cell growth to mainly biotransformation

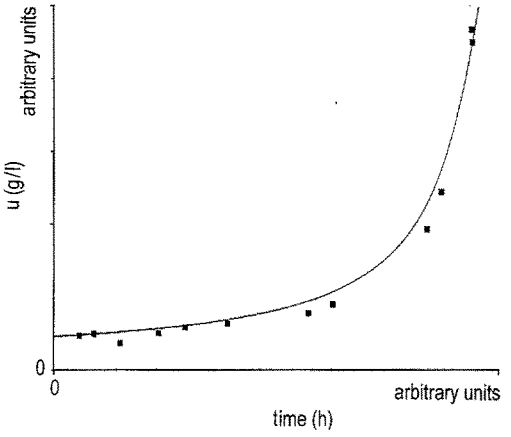

Fig. 12. Simulation of a continuous one stage enzyme reactor without feeding the biocatalyst for the nicotinamide process. The model fits the process also under complete changed process conditions (data not shown). The parameters are well adjusted and no variables are hidden behind the parameters

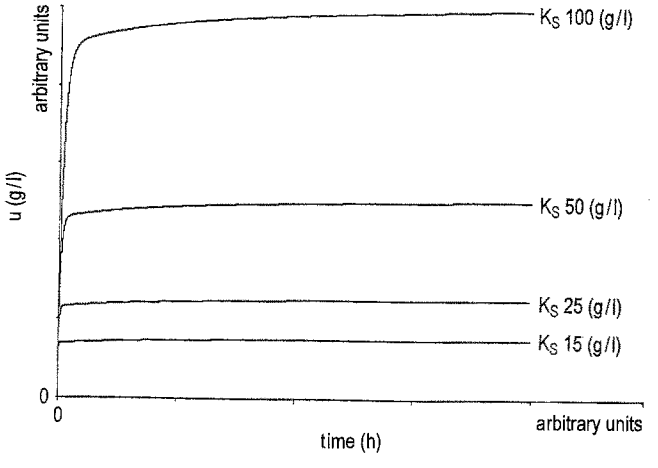

Fig. 13. Parameter sensitivity study: K_s. Changing the saturation constant (K_s) for the nicotinonitrile (u) from 15 to 100 g/l has significant influence on the actual concentration in a continuous operated stage (steady states calculated)

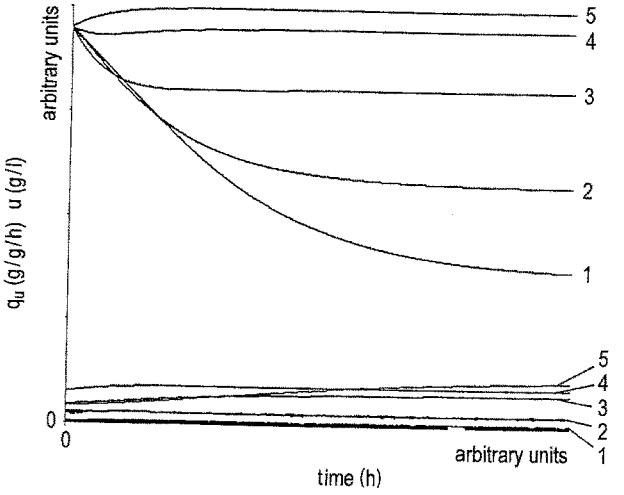

Fig. 14. Process simulation of a 5 stage continuous cascade with the biocatalyst fed in the counter current flow. Tht nicotinonitrile is fed in the current flow direction (stage 1 to 5). The 5 upper curves show the specific educt transformation rate (q_u). Since the biocatalyst and the nicotinonitrile are fed constant steady states process conditions occur. The biocatalyst leaves the reactor system with a final specific rate of 30% of the initial rate. The lower curves show the actual concentration of the nicotinonitrile (u) in each stage. However, the process is not built according to this design, but all options were tested using the simulation tool, thus obtaining the optimal solution

well adjusted and no variables are so far hidden behind the parameters. Based on this kinetic process model, LONZA did further the process design for an industrial production process of 3000 tons nicotinamide per year using extensively simulation methods in combination with experimental results. The process was designed completely on a computer using the simulation language SIMNON. Since experiments were very time and labour consuming, simulation not only speeded up the process design it even reduced costs considerably. An experiment of several hundred hours is easily simulated in several minutes. Fig. 13 shows for example how simulation can be also used for a better process understanding. The figure

shows the influence of K_s onto the enzymatic reaction. The enzyme used has a high K_s in the range of 20–30 g/l (experimental data), which means that the nicotinonitrile concentration should be kept high for maximal volumetric output of the reaction vessel. On the other hand, a high nicotinonitrile concentration destroys the enzyme. However, a compromise between this target conflict was followed in the plant design. Fig. 14 shows one simulated set-up, a cascade of five reaction vessels continuously operated. A continuous five step cascade with the biocatalyst fed in a counter current flow (from stage 5 to 1) and nicotinonitrile fed in a current flow is shown in this graph. The figure shows the specific educt transformation rate and the actual educt concentration at each stage. In stage five, the specifications are reached and the nicotinonitrile (u) is no longer measurable (lower than the detection limits).

5
Conclusions

The successful use of simulation for the nicotinamide process shortened its development time for at least 50%, since the number of actual experimnts could be drastically reduced, thus reducing also costs considerably.

For both processes, 6-hydroxynicotinic acid and 5-methyl-2-pyrazinic acid, measurement and control were one of the keys for production success.

Without strict control of the growth-inhibiting compounds involved, we would not be able to run one batch when using living systems. The complete process automation (fermentation) safes personnel costs, which can then be spent for the down-stream processing where high-level automation is not possible. Since the kinetic and biological models describe the reaction conditions (fermentation) very well, they could also be used as masters in an "expert system". In this case, the model could early find out deviations of the actual parameters from the model.

Automation has also certain quality aspects since processes can be controlled on-line and any quality relevant deviation can be registered early. In other words, the process becomes intrinsically more defined. The possibiliy of virtually unlimited data logging, using on-line measurement of educts and products, should theoretically have a beneficial effect on validation and GMP (Good manufacturing practice). Moreover, appropriate controlling of a bioprocess facilitates the use of defined media. Avoiding complex media is an extremely valuable improvement for GMP products. Capital cost for expensive on-line control equipment, computer hard- and software are rapidly recovered because of reduced labour costs for tedious off-line analytical procedures, and improved reliability of manufacturing processes. We are now working on a concept which integrates process development and transfer from research to development and production using modelling and state of the art electronic data processing. The prerequisite is using compatible hard- and software from research through to production, and using the modelling tool right from the start. Ultimately, process information from research to development and production should be transferred by electronic data storage means or a network. Since simulation methods save costs, it is important that these tools are implemented as early as possible in research.

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