# MONITORING AND CONTROL OF RECOMBINANT PROTEIN PRODUCTION - FROM UP-STREAM TO INTEGRATED DOWN-STREAM PROCESSES

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Abstract: The production of intra cellular heterologous proteins with *Escherichia coli* has a demand of a global monitoring and control system for optimization of complex and uncertain process behavior.
Moreover the expression of APIs - active pharmaceutical ingredients and its secretion by the yeast *Pichia pastoris* enables the development of Integrated Bioprocesses with direct product harvest via cross flow filtration methods.
This paper describes some associated monitoring and control problems.
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## 1. INTRODUCTION

The development of optimized processes for production of heterologous proteins with microorganisms or cell cultures needs well equipped research facilities. In particular the investigation of complex expression behavior requires highly instrumented bioreactors.

However the transfer of such kind of processes in a production scale and the boundary conditions of GMP request a simple and robust technology.

The expression of Green Fluorescent Protein (GFP) with *Escherichia coli* is taken as an intra cellular product example. A sophisticated monitoring and control system enables a global process observation and the control of cell specific reaction rates.

The second example describes the transfer of research work into production environment. The expression of recombinant chemokines with the yeast *Pichia pastoris* and the secretion of this proteins into the media enables the development of Integrated Bioprocesses. In this case up-stream and primary down-stream steps with cross flow filtration are combined to a fully automated process.

## 2. GLOBAL MONITORING AND CONTROL

The research work with *E. coli* is performed in a 15 L BIOSTAT C plant (Sartorius BBI Systems, Germany) schematically shown in fig. 1.

Ambitious analysis techniques, connected to the fully equipped fermenter, allows detailed knowledge about the proceeding within the cultivation broth (Peuker *et al.*, 2004).

Beside classical in-line devices such as pH-, $pO_2$- and turbidity-probes as well as standard off-gas

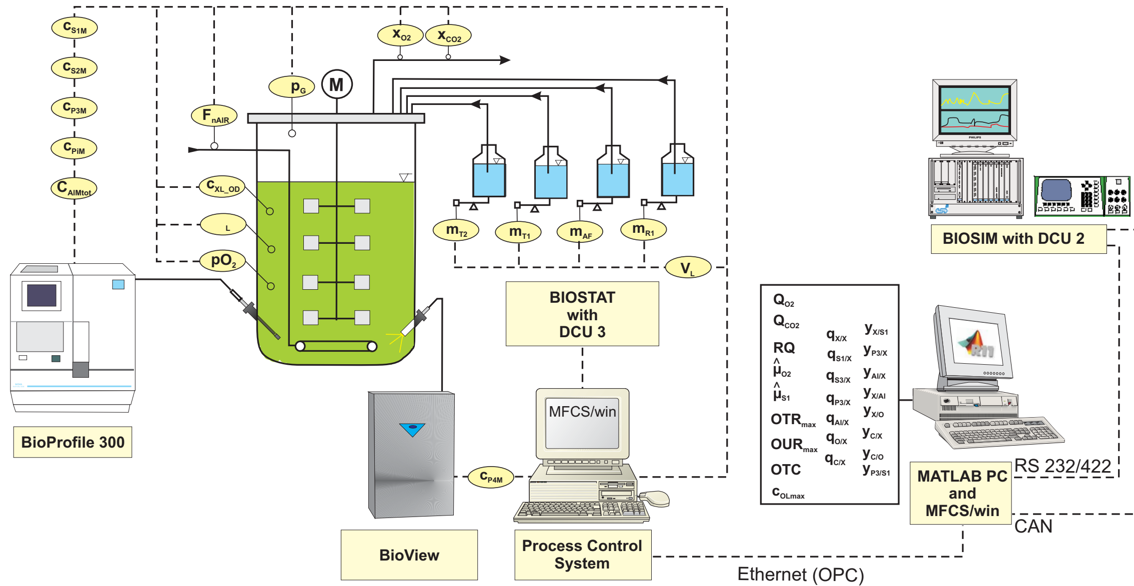


Fig. 1. Enhanced instrumentation of a research bioreactor

analysis, additional at-line measurement devices, namely the multi component analyzer BioProfile 300 (Nova Biomedical, USA), the flow analysis systems ProcessTrace and TAS 2000 (both Trace Analytics, Germany) and an HPLC (VWR International, Germany), are connected to a cell free analysis flow.

Furthermore the integration of a 2D-fluorescence spectrometer BioView (Delta Light & Optics, Denmark) enables a non invasive measurement of the cell internal product GFP. The application of chemometrics allows the detection of several other media and cell components (Boehl *et al.*, 2003).

The bioreactor is operated by a frontend control unit DCU and hosted by the SCADA-system MFCS/win (both BBI).

The integration of a data processing-PC enables the application of MATLAB (Mathworks, USA) for advanced process control.

## 3. SCHEDULE OF AN EXPRESSION PROCESS

### *3.1 Escherichia coli cultivation*

The schedule of a cultivation for expression of GFP with *E. coli* JM105:pGFP is demonstrated in fig. 2.

Starting in a batch phase the cells ($c_{XL}$) adapt to new media conditions, grow exponentially on glucose ($c_{S1M}$) and produce the undesired by-product acetate ($c_{P3M}$). The base ammonia ($c_{AlM}$) is used as a nitrogen source and titrated by the pH-controller.

The protein expression is induced by addition of IsoPropyl-$\beta$-D-ThioGalactopyranosid (IPTG) at t = 53 h. This is observable in the intense increase of soluble GFP ($c_{P1M}$), analyzed off-line by fluorescence reader Genios (TECAN, Austria).

The process is divided into three phases. A batch is followed by a High Cell Density fed batch Cultivation (HCDC). This starts with biomass production and changes in growth associated cell internal protein production.

Heterologous GFP is produced by *E. coli* in soluble form and stored in the cytoplasm. This component is on-line detectable by non invasive fluorescence measuring technique with the BioView analyzer. An over expression leads to an aggregation of the proteins in inclusion bodies (Hoffmann *et al.*, 2001). This part of the product is on-line not observable.

The aim of the following process development is to maximize the soluble fraction, controlled by reaction limiting feeding strategies.

The kernel problem of such processing is an open loop or closed loop control of cell specific growth rate $\mu$.

### *3.2 The $\mu$-stat problem*

A controlled bioreaction process with constant $\mu$ is described as $\mu$-stat. Process control is carried out by limited feed of glucose and unlimited supply of oxygen.

A classical method is an exponential increase of feed rate $F_R$ and a simultaneous $pO_2$-agitation control.

A slightly challenging control procedure is shown in fig. 3. The dissolved oxygen tension $pO_2$ is controlled by glucose feed rate $F_R$. This requires

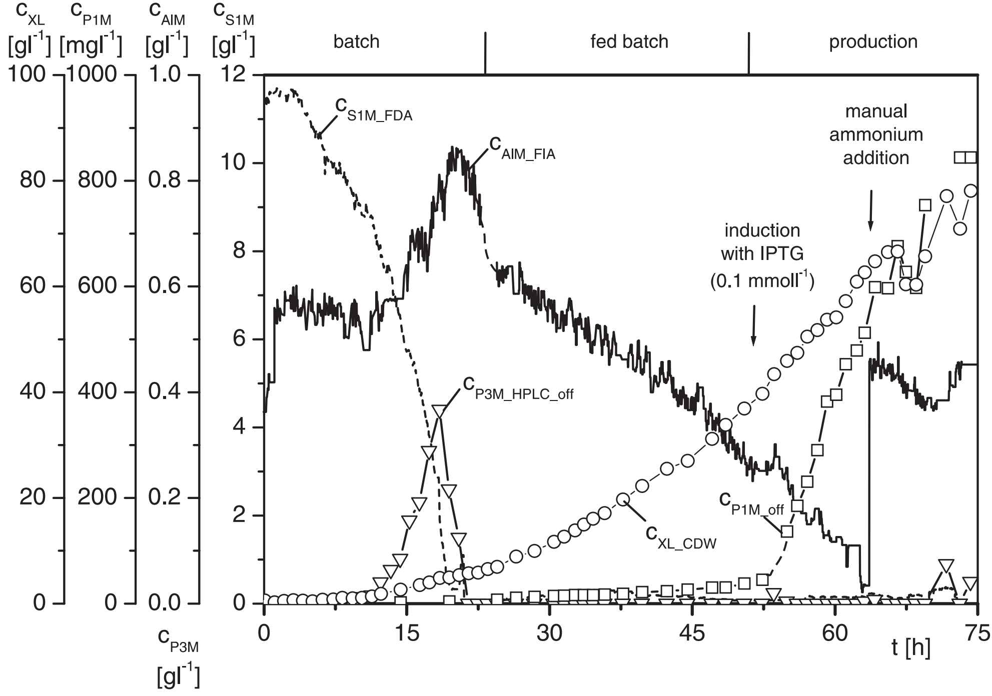


Fig. 2. High Cell Density Cultivation with controlled growth rate and product induction

substrate limited growth. The control of $\mu$ with a set point $\mu_w$ is performed by cascading agitation speed control and needs a $\mu$-observation algorithm.

The cell mass balance,

$$\dot{m}_{XL}(t) = \left[\mu_w - \frac{F_S(t)}{V_L(t)}\right] \cdot m_{XL}(t), \qquad (1)$$

clarifies the unstable exponential increase of control disturbance $m_{XL}$.

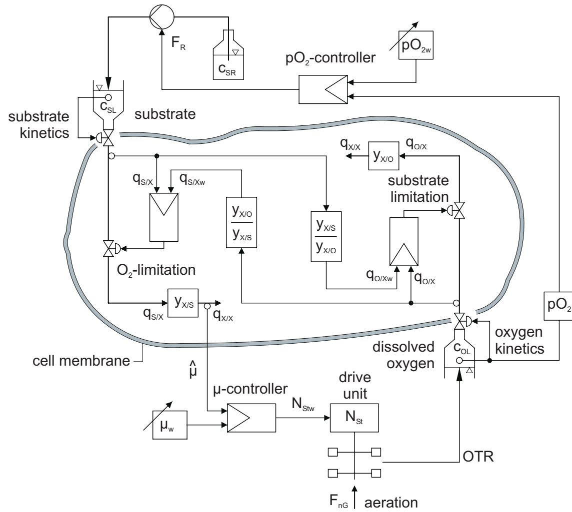


Fig. 3. $\mu$-stat, a servo control problem

A strong nonlinear behavior of cell densities between 10 $gl^{-1}$ and 120 $gl^{-1}$ demands adaptive control procedures. The $pO_2$-feed control is discussed exemplarily.

### 3.3 Adaptive $pO_2$-feed control

A simplified biomass/substrate/oxygen reaction scheme is shown in fig. 4 for explanation of the control concept.

The flow equivalence of observable cell specific reaction rates $q_{I/X}$,

$$\begin{aligned} q_{X/X}(t) &= y_{X/S}(t) \cdot q_{S/X}(t) = y_{X/O}(t) \cdot q_{O/X}(t) \\ &= q_{X/Xgr}(t) - q_{X/Xm} = \mu(t), \end{aligned} \qquad (2)$$

with time variant cell mass yield coefficients $y_{X/I}$, and the balance of nonobservable cell specific growth parts $q_{I/Xgr}$,

$$\begin{aligned} q_{X/Xgr}(t) &= y_{X/Sgr} \cdot q_{S/X}(t) \\ &= y_{X/Ogr} \cdot \left[q_{O/X}(t) - q_{O/Xm}\right], \end{aligned} \qquad (3)$$

with the reaction parameter $y_{X/Igr}$ and $q_{I/Xm}$, are the reaction base of following balances of an ideal fed batch process.

The cell density balance,

$$\dot{c}_{XL}(t) = \left[q_{X/X}(t) - \frac{F_R(t) + F_T(t)}{V_L(t)}\right] \cdot c_{XL}(t), \qquad (4)$$

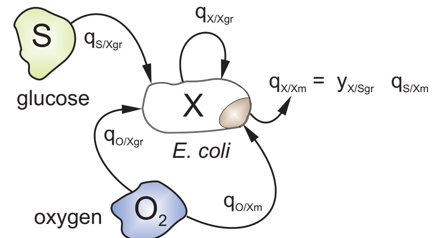


Fig. 4. A simple *E. coli* reaction scheme

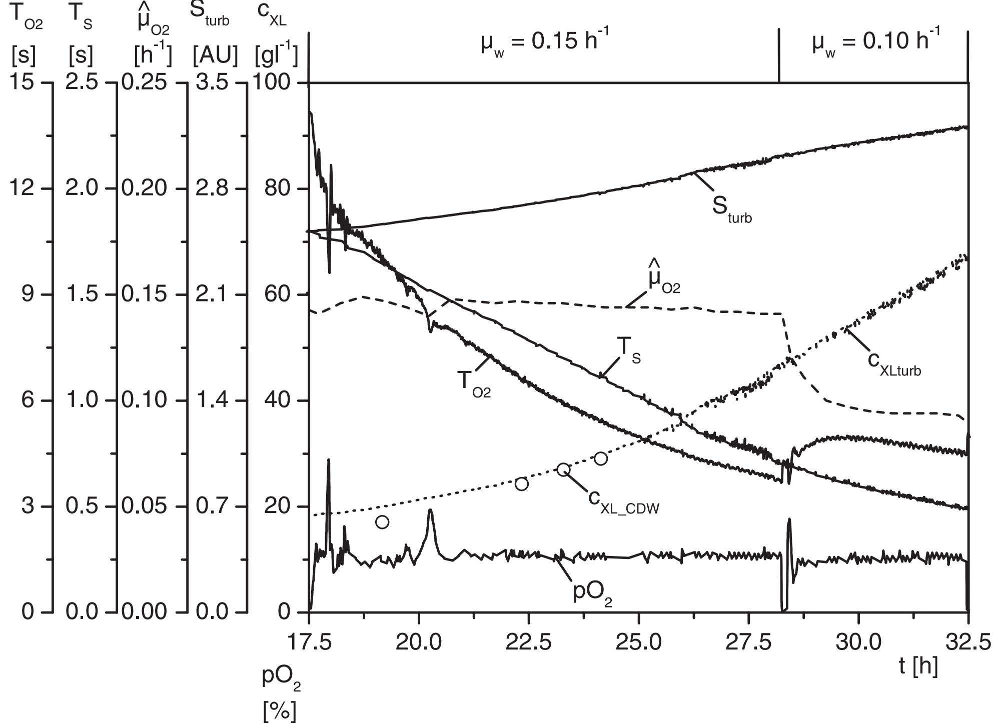


Fig. 5. Time course of an adaptive $\mu$-stat procedure

the glucose balance,

$$\dot{c}_{SL}(t) = \frac{F_R(t)}{V_L(t)} \cdot c_{SR} - q_{S/X}(t) \cdot c_{XL}(t), \qquad (5)$$

under substrate limited conditions $(c_{SR} \gg c_{SL})$, the dissolved oxygen balance,

$$\dot{c}_{OL}(t) = OTR_{opt}(t) \cdot \left[x_{OGin}(t) - \frac{c_{OL}(t)}{c_{OLmax}(t)}\right] - q_{O/X}(t) \cdot c_{XL}(t), \qquad (6)$$

with $RQ \equiv 1$ and omitting convective $O_2$-flow, and the volume balance,

$$\dot{V}_L(t) = F_R(t) + F_T(t) - F_S(t), \qquad (7)$$

describe the controlled process.

In substrate limited conditions cell specific growth rate $q_{X/X}$,

$$q_{X/X}(t) = y_{X/Sgr} \cdot \left[q_{S/Xmax} \cdot \frac{c_{SL}(t)}{k_{Scrit}} - q_{S/Xm}\right], \qquad (8)$$

is described by Blackman kinetics and controlled by $c_{SL}$ via the $pO_2$-controller output $F_R$.

An analysis of the controlled plant with eq. (5) and eq. (6) leads to a time variant second order lag system.

The variable oxygen time constant $T_{O2}$,

$$T_{O2}(t) = \frac{OTR_{opt}(t)}{c_{OLmax}(t)} = \frac{1}{k_La(t)} + \frac{F_{nG}(t) \cdot M_{O2}}{V_L(t) \cdot c_{OLmax}(t) \cdot V_{nM}}, \qquad (9)$$

is on-line obtainable by off-gas balances,

whereas the variable substrate time constant $T_S$,

$$T_S(t) = \frac{k_{Scrit}}{q_{S/Xmax} \cdot c_{XL}(t)}, \qquad (10)$$

includes unknown biomass information.

An adaptive controlled fed batch-phase is shown in fig. 5.

The estimation of cell specific growth rate $\hat{\mu}_{O2}$,

$$\hat{\mu}_{O2}(t) = \frac{y_{X/Ow} \cdot \dot{m}_{OT}(t)}{V_L(t_1) \cdot c_{XL}(t_1) + y_{X/Ow} \cdot \int_{t_1}^{t} \dot{m}_{OT}(\tau) d\tau}, \qquad (11)$$

is calculated from oxygen mass transfer rate $\dot{m}_{OT}$ and controlled by agitation speed $N_{St}$ (Luttmann and Gollmer, 2000).

An exponential increase of $N_{St}$ and $c_{XL}$, observable by turbidity measurement $S_{turb}$ (Optek, Germany),

$$c_{XLturb}(t) = K_1 \cdot e^{K_2 \cdot S_{turb}(t)} \qquad (12)$$

with $K_1 = 0.16\ gl^{-1}$ and $K_2 = 1.88\ AU^{-1}$,

leads to an exponential decrease of $T_{O2}$ and $T_S$.

A stable $\mu$-stat control is the result of adaptive control parameter tuning.

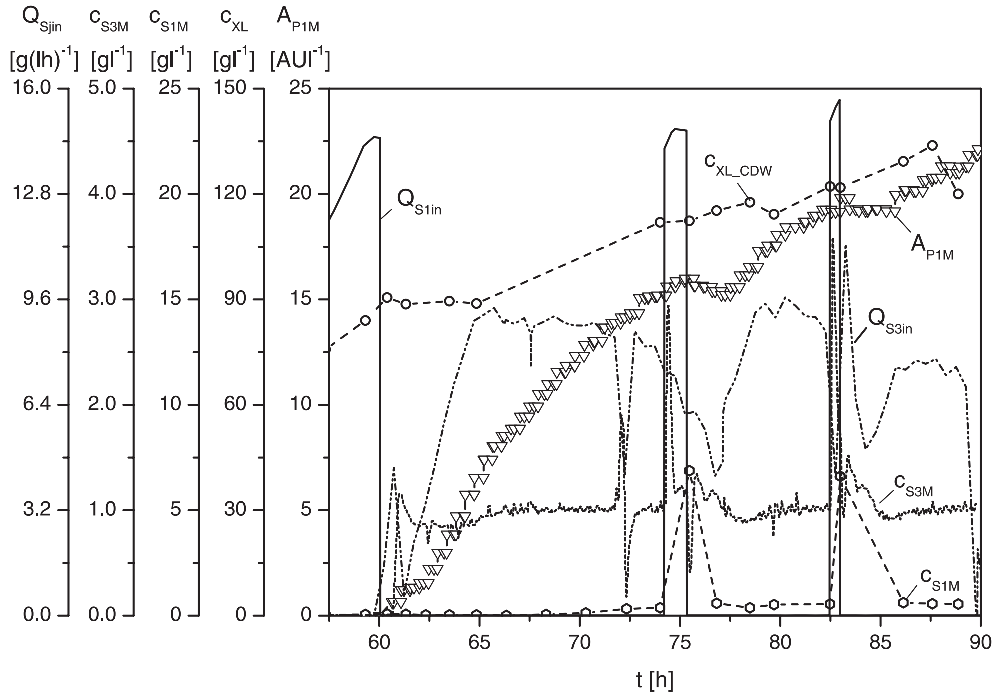


Fig. 6. Induction course of protein expression by *Pichia pastoris*

## 4. PRODUCTION OF EXTRA CELLULAR PHARMACEUTICAL PROTEINS

### *4.1 Pichia pastoris cultivation*

The second example will describe the transfer of process development research to an industrial orientated pilot plant scale.

An insight into the used facultative methylotrophic yeast *Pichia pastoris* delivers fig. 7.

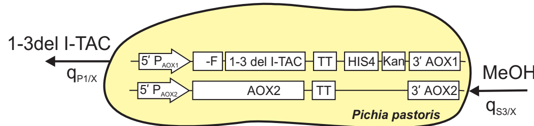


Fig. 7. *Pichia pastoris* and its AOX gene segments

The yeast owns two alcohol oxidase (AOX) gene segments with promotor $P_{AOX1}$ and promotor $P_{AOX2}$ (Cregg *et al.*, 1989). Their activity is inducible by methanol. Genetically manipulation replaces the AOX1 gene by a foreign gene sequence, in this case for chemokine 1-3del I-TAC (Interferon inducible T-cell $\alpha$-Chemoattractant).

During methanol feeding the heterologous protein will be expressed with pass AOX1, whereas processing of enzyme AOX, responsible for utilization of methanol, is controlled by $P_{AOX2}$. Furthermore the target protein is secreted into the culture broth.

### *4.2 Induction of protein expression*

The cultivation of *Pichia pastoris* is carried out similar to the *E. coli* process described before. It takes course in a batch and substrate limited fed batch, both on glycerol. During protein production the feed media is switched to methanol and controlled further on.

An induction course of protein expression is shown in fig. 6. At t = 60 h the process switches from exponential glycerol feeding ($Q_{S1in}$) to methanol ($c_{S3M}$) control. The time course of manipulation variable $Q_{S3in}$ indicates a second or higher order time delay in AOX-processing.

A connection of an HPLC for detection of the secreted product by an adapted purification method offers monitoring of protein expression dynamics.

The strong promotor $P_{AOX1}$ produces a short over-expression, indicated by an exponential increase of protein signal $A_{P1M}$, which is measured by HPLC UV-absorption. Then product time course turns over into a first order delay response and ends in a slight protein increase.

The stagnations in production have to be ceased by stimulations of gene regulation.

Pulsewise glycerol additions in fig. 6 leads to repression of methanol uptake as well as AOX and 1-3del I-TAC production.

AOX dissappears by protein turnover inside the cell, whereas the secreted protein is attacked by proteases in the media.

After reuptake of glycerol ($c_{S1M}$), *Pichia pastoris* starts again with a high transient protein productivity.

Secretion of heterologous proteins by *Pichia pastoris* and pulsewise control actions on production

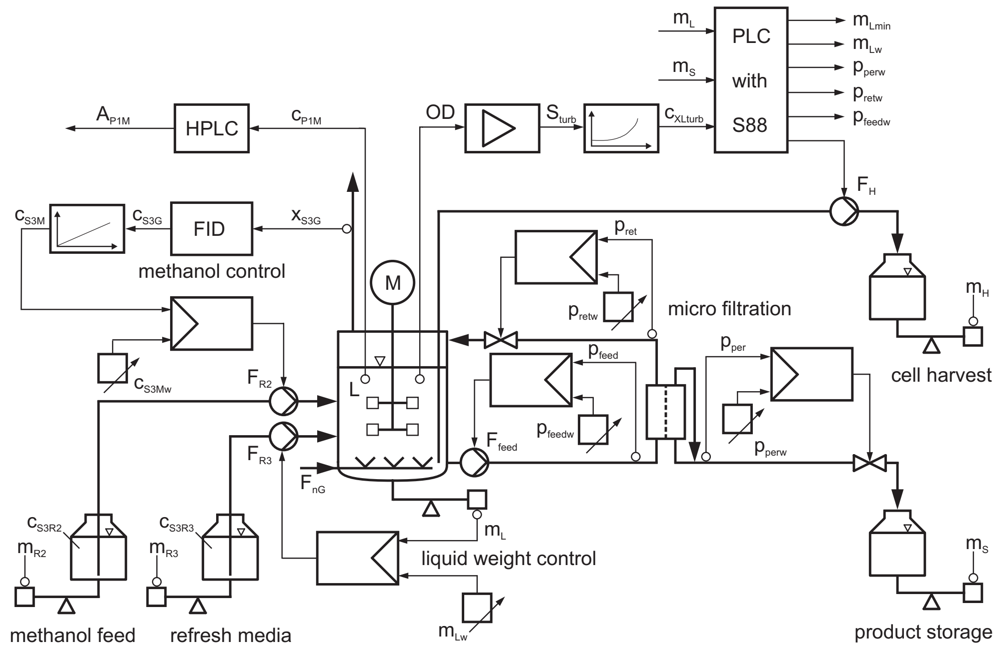


Fig. 8. Control design for combination of protein production and recovery

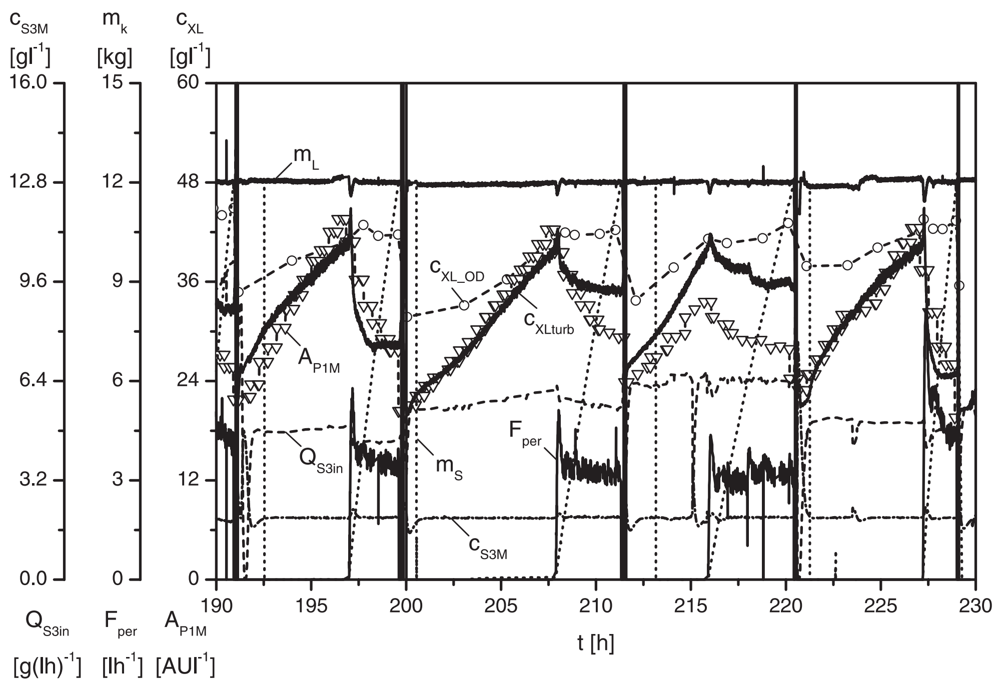


Fig. 9. Recombinant protein production in Sequential Integrated Bioprocessing

behavior recommend to integrate sequential operated primary purification steps into the cultivation procedure.

### *4.3 Integrated Bioprocessing*

A 40 L BIOSTAT C-Xflow plant for Integrated Bioprocessing was developed in cooperation with the company Sartorius BBI Systems to produce and recover pharmaceutical proteins fully automatical (Cornelissen *et al.*, 2003).

The control design in fig. 8 shows the expanded basic automation technique by implementation of the cross flow system Sartocon Slice and the corresponding pressure control loops.

Product separation occurs in diafiltration mode during cultivation. Here permeate including the product is replaced by fresh media from reservoir R3. Process automation is carried out by the sequential control package S88 using turbidity and reaction volume measurements as well as set point control of filtration process.

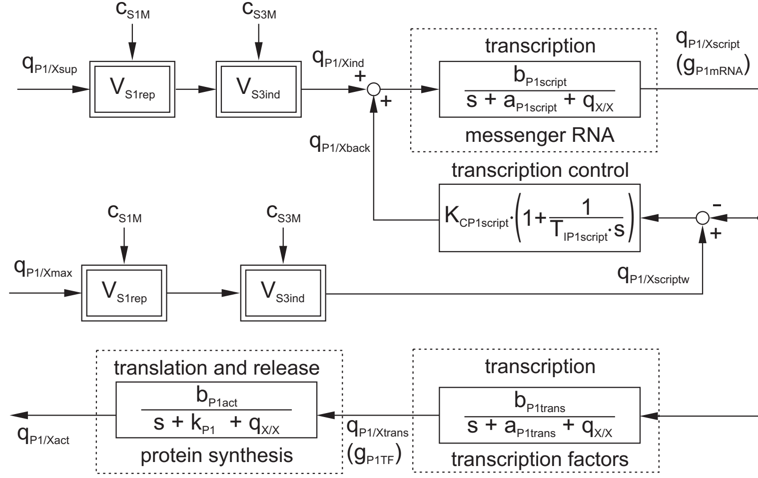


Fig. 10. Simplified regulation network of chemokine production

A typical process schedule is shown in fig. 9. The cyclic process follows the recurrent steps protein production, protein recovery in diafiltration and cell density dilution.

The filtration procedure starts when $c_{XLturb}$ reaches 40 $gl^{-1}$ and ends after one exchange of reaction volume $V_L$, controlled by weight measurements of liquid phase ($m_L$) and storage tank ($m_S$). Cell density is diluted before the next production phase in order to ensure filtration functionality.

A cell harvest and media refresh guarantees stable control conditions.

Nevertheless the filtration process is accompanied by membrane fouling. A Clean In Place procedure and re-sterilization during protein production ensures a long process life cycle longer than 30 days.

### 4.4 *Investigation of induction behavior*

The expression behavior of recombinant *Pichia pastoris* strain is widely unsettled, in particular the interaction of the two AOX regions and the repression dynamics of glycerol feeding.

Attended modelling was carried out for investigation of this reaction behavior. Figure 10 shows a simplified block diagram of the cellular transcription and translation regulation.

The model contains the delay of the involved protein formation as well as the regulation of an over-expression during transcription.

The expression is enabled by methanol with induction gain $V_{S3ind}$,

$$V_{S3ind}(c_{S3M}, t) = \frac{\left(\frac{c_{S3M}(t)}{k_{S3ind}}\right)^{\kappa_{ind}}}{1 + \left(\frac{c_{S3M}(t)}{k_{S3ind}}\right)^{\kappa_{ind}}}, \qquad (13)$$

and disabled by glycerol with repression gain $V_{S1rep}$,

$$V_{S1rep}(c_{S1M}, t) = \frac{1}{1 + \left(\frac{c_{S1M}(t)}{k_{S1rep}}\right)^{\kappa_{rep}}}. \qquad (14)$$

The expression description is part of a general process model which is used for process simulation and identification of unknown reaction parameters.

The regulation mechanism will be clarified by further experiments and a more detailed modelling to attain an expanded optimization potential for production of heterologous proteins with *Pichia pastoris*.

## 5. SUMMARY

The implementation of global monitoring and control systems enables an optimal bioprocessing for production of recombinant proteins.

The control of physiological parameters as well as the integration of primary down-stream equipment into the cultivation procedure opens a wide field of new biochemical engineering strategies.

## 6. ACKNOWLEDGEMENT

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## 7. LIST OF SYMBOLS

| Variable | property | unit |
|---|---|---|
| $a_{P1J}$ | eigenvalue of protein turnover in step J | $[h^{-1}]$ |
| $A_{P1M}$ | UV absorption of recombinant protein | $[AUl^{-1}]$ |
| $b_{P1J}$ | protein expression gain in step J | $[h^{-1}]$ |
| $c_{IK}$ | concentration of component I in subsystem K | $[gl^{-1}]$ |
| $F_K$ | feed or harvest rate from/in subsystem K | $[lh^{-1}]$ |
| $F_{nG}$ | aeration rate in normalized conditions | $[lh^{-1}]$ |
| $k_La$ | volumetric $O_2$-transfer coefficient | $[h^{-1}]$ |
| $k_{P1\alpha}$ | protein secretion coefficient | $[h^{-1}]$ |
| $k_{Scrit}$ | critical glucose concentration | $[gl^{-1}]$ |
| $k_{S1rep}$ | glycerol repression concentration | $[gl^{-1}]$ |
| $k_{S3ind}$ | methanol induction concentration | $[gl^{-1}]$ |
| $K_{CP1script}$ | protein transcription proportional gain | [-] |
| $\dot{m}_{OT}$ | oxygen mass transfer rate | $[gh^{-1}]$ |
| $m_{XL}$ | cell mass in liquid phase | $[g]$ |
| $M_{O2}$ | molecular weight of oxygen | $[gmole^{-1}]$ |
| $N_{St}$ | stirrer agitation speed | $[rpm]$ |
| $OTR_{opt}$ | optimal oxygen transfer rate | $[g(lh)^{-1}]$ |
| $pH$ | pH-value | [-] |
| $pO_2$ | dissolved oxygen tension | [%] |
| $q_{I/X}$ | cell specific reaction rate of component I | $[h^{-1}]$ |
| $Q_{Sjin}$ | volumetric supply rate of substrate j | $[g(lh^{-1})]$ |
| $S_{turb}$ | turbidity measurement signal | $[AU]$ |
| $T_I$ | consumption delay time of component I | $[s]$ |
| $T_{IP1script}$ | protein transcription integral time constant | $[s]$ |
| $V_K$ | volume of subsystem K | $[l]$ |
| $V_{nM}$ | mole volume | $[lmole^{-1}]$ |
| $V_{S1rep}$ | glycerol/protein repression gain | [-] |
| $V_{S3rep}$ | methanol/protein induction gain | [-] |
| $y_{X/I}$ | biomass yield coefficient on component I | $[gg^{-1}]$ |
| $x_{OGin}$ | oxygen mole fraction at aeration inlet | [-] |
| $\mu$ | cell specific growth rate | $[h^{-1}]$ |
| $\kappa$ | exponent in induction/repression function | [-] |

| Index | property |
|---|---|
| $act$ | activity |
| $at$ | at-line |
| $Al$ | alkali |
| $crit$ | critical value |
| $CDW$ | cell dry weight |
| $FDA$ | flow diffusion analysis |
| $FIA$ | flow injection analysis |
| $gr$ | growth |
| $G$ | subsystem gas phase |
| $in$ | input |
| $ind$ | expression induction |
| $L$ | liquid (reaction) phase |
| $m$ | maintenance |
| $max$ | maximum value |
| $M$ | subsystem media |
| $n$ | normalized gas conditions |
| $off$ | off-line |
| $opt$ | optimal value |
| $O, O_2$ | oxygen |
| $OD$ | optical density |
| $per$ | permeate |
| $P1$ | recombinant product |
| $P3$ | product acetate |
| $rep$ | expression repression |
| $R$ | subsystem reservoir |
| $script$ | mRNA-transcription |
| $sup$ | highest value |
| $S$ | subsystem storage |
| $S$ | substrate glucose |
| $S1$ | substrate glycerol |
| $S3$ | substrate methanol |
| $trans$ | transcription factors |
| $turb$ | turbidity |
| $T$ | subsystem base tank |
| $w$ | set point |
| $X$ | bio dry mass |

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