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PROCESS DEVELOPMENT FOR PRODUCTION OF ACTIVE PHARMACEUTICAL INGREDIENTS WITH *PICHIA PASTORIS*

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Abstract: Integrated Bioprocesses as a direct technological link between methods for the cultivation of the yeast *Pichia pastoris* and additional down-stream procedures ranging from cross-flow filtration for product recovery up to purification with combined chromatography systems are developed for a fully automated production of recombinant pharmaceutical proteins. The set-up of an appropriate 40 l pilot plant with an extended Process Analytical Technology (PAT) for on-line monitoring of important process variables, e.g. the recombinant product, as well as the development of complex control strategies by means of Apparent Processing with a real-time simulation system are explained. *Copyright © IFAC 2007*

Keywords: integrated bioprocess; Pichia pastoris; PAT-process analytical technology; modeling of expression kinetics; 1-3del I-TAC; on-line detection of recombinant protein; cross-flow filtration; process development in Apparent Processing

1. INTRODUCTION

The production of secreted recombinant pharma-proteins with the yeast *Pichia pastoris* provides the advantage of sensitive extra cellular product removal during cultivation (Thömmes et al., 2001).

Fig. 1 shows a typical expression course during a high cell density cultivation (HCDC). The process starts with a batch phase (not shown) followed by a substrate limited HCDC fed-batch phase, both on glycerol. During fed-batch cells grow with a controlled specific growth rate accordant to the µ-stat principle (Luttmann et al., 1994).

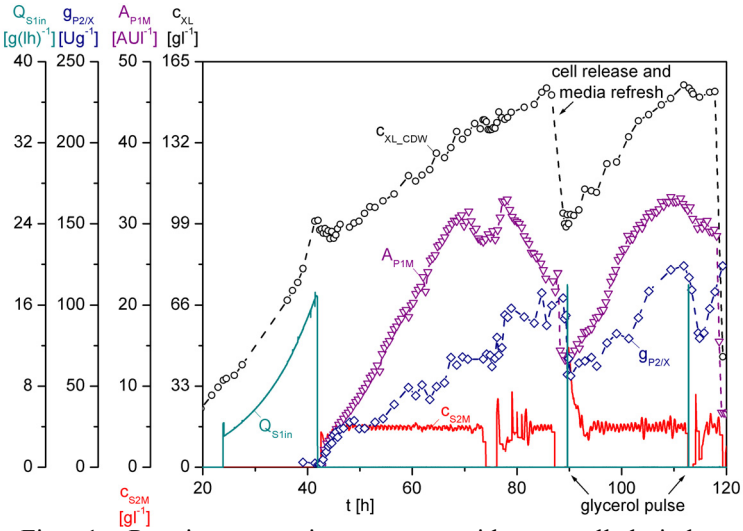

Fig. 1. Protein expression course with controlled inducer concentration in HCDC with P. pastoris c_{XL_CDW} := cell dry weight (○, off-line); A_{P1M} := target protein UV-absorption (▽, at-line); c_{S2M} := methanol (S2) concentration (on-line); $g_{P2/X}$:= specific AOX content (◇, off-line); Q_{S1in} := volumetric glycerol (S1) supply rate (on-line)

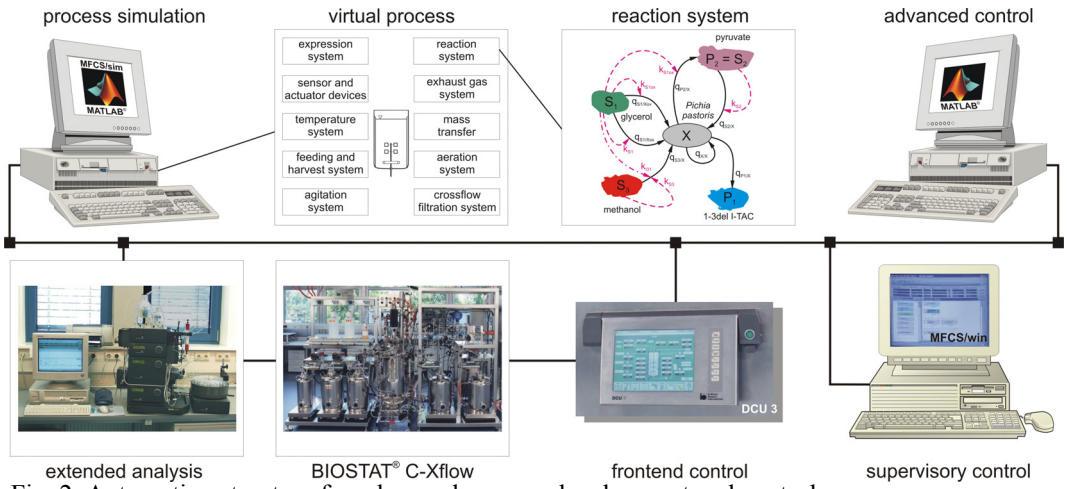

Fig. 2. Automation structure for advanced process development and control

The production of recombinant proteins is induced by methanol addition with a controlled inducer concentration c_{S2M} at 5 gl^{-1}. The expression of the genetically modified chemotactic cytokine *1-3del I-TAC* (interferon-inducible T-cell alpha chemo-attractant) (Cole et al., 1998) is kept under control by the *AOX1*-promotor (Cregg et al., 1993). A linear product increase is observable for over 25 hours. The expression activity stopped at a cell dry density c_{XL} of about 140 gl^{-1} with a high cell specific alcohol oxidase content g_{AOX} and a protein absorption signal of about 33 AUl^{-1} (~130 mgl^{-1}). A long residence time in the fermentation broth results in a decrease of target protein absorption, e.g. caused by product sensivity and/or protease activity in the broth. A new protein formation after stagnating production phases is achieved by concurrent cell release and media refresh at t = 90 h.

Integrated bioprocesses, which combine production and purification steps of secreted recombinant pharmaceutical proteins, result as a logical consequence to achieve permanent expression activity at high expression levels with a product conservation at optimal conditions.

2. PAT - PROCESS ANALYTICAL TECHNOLOGY -

For research and guiding of such processes, an extended bioreactor monitoring with a number of biochemical analysis systems and an advanced data processing and process control is established. Fig. 2 shows the technology applied to the Pichia process. Cultivations are carried out using a highly instrumented novel 40 l bioreactor system BIOSTAT® C-Xflow. For local bioprocess automation the digital fermenter controller DCU3 is used. Supervisory control and data acquisition are carried out by the MFCS/win SCADA software.

Besides classic biotechnological parameters such as pH, pO_2, O_2- and CO_2-off-gas values additional measurement systems have to be integrated for monitoring and control of Sequential Integrated Bioprocesses (SIB). Cell density is reconstructed on-line via turbidity measurement (optek-Danulat), capacitive live cell detection (Aber Instruments) as well as off-gas analysis (BlueSens). The on-line methanol measurement (Kempe Biotechnologie) is carried out via a tubing procedure whereas ammonium and glycerol are detected at-line using flow analysis systems. The recombinant protein is observable at-line with an adapted purification method using HPLC systems (VWR). In addition the computer concept offers an OPC-linked integration of MATLAB® PC's, where scripts are used for process-attendant simulations, parameter identifications and model based on-line estimation of volumetric and cell physiological reaction rates.

3. PLANT DEVELOPMENT FOR INTEGRATED BIOPROCESSES

A combined plant for cell cultivation and direct product recovery with cross-flow membranes was developed in cooperation of Sartorius BBI Systems with Hamburg University of Applied Sciences (Cornelissen et al., 2003)

The control structure in Fig. 3 shows an advanced cultivation technology in combination of a bioreactor BIOSTAT® C with a Sartocon® Slice cross-flow filtration system, the corresponding pressure control devices as well as cell and product harvest facilities. During micro filtration, removed permeate volume is substituted by fresh media through liquid weight control. The in-line measurement system FMC serves as a transmitter for controlling of the methanol concentration in the media phase. Three HPLC systems are installed for at-line observation of target protein in the bioreactor, in the retentate flow and in the product tank. For monitoring of biological activities O_2- and CO_2-off-gas balances are performed. Besides the cell density c_{XLturb} the mass of permeate m_P in the product tank and the liquid phase m_L are used for automated phase transitions during SIB.

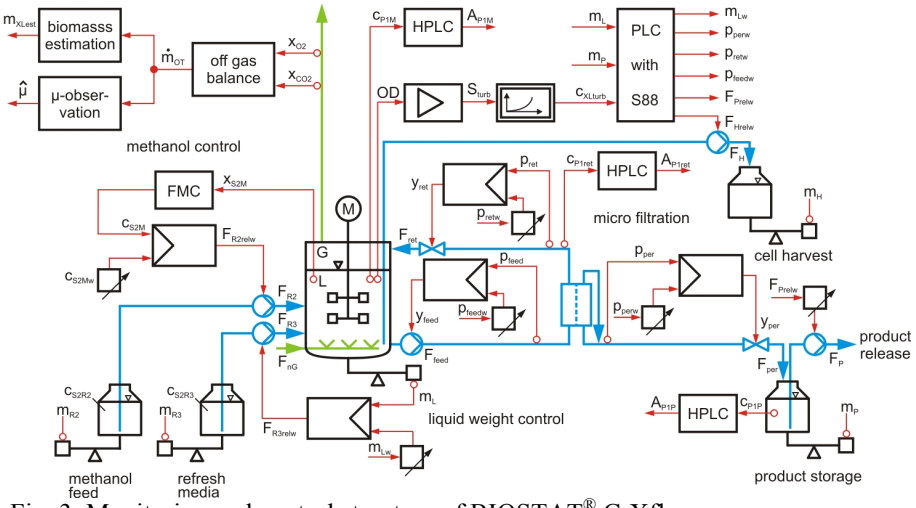

Fig. 3. Monitoring and control structure of BIOSTAT® C-Xflow

4. PROTEIN PRODUCTION IN SEQUENTIAL INTEGRATED BIOPROCESSING

The production of secreted proteins results in a considerable exposure of the product to the media during long term processes. Hence, it is reasonable to perform a cyclic separation via a cross flow membrane parallel to the production process to enable a fast subsequent processing. Fig. 4 demonstrates the process course of recombinant 1-3del ITAC based on at-line measurements carried out by three Elite LaChrom® (VWR International) HPLC systems.

During the production phase the protein signals resulting from both, the measurement in the media contained in the reactor, A_{P1M}, and in the retentate backflow, A_{P1ret}, show an almost linear increase. Since the permeate conduit is closed the protein content in the permeate vessel, A_{P1P}, remains at a constant level. Previous to the subsequent harvest cycle the permeate tank is initially emptied to allow for refilling in the course of one harvest step. During this operation diafiltration leads to one complete media exchange without cell loss and, at the same time, to the removal of approximately 63 % of the target protein. The following refresh cycle is necessary to adjust the cell density for subsequent production by a small discharge of cells and the addition of an equivalent amount of media.

5. PROCESS DEVELOPMENT IN APPARENT PROCESSING

Realization of the desired strategy requires a set of time consuming experiments. A faster, more reliable and cost reducing way for the implementation of process control strategies is the principle of Apparent Processing. In this case the real bioreactor is replaced by a virtual system, which is able to image the whole biotechnological plant in a real-time simulation. The basis of this procedure is a general mathematical model of the whole Integrated Bioplant, as shown in Fig. 2. This requires descriptions of measurement and final control elements, of all biochemical unit

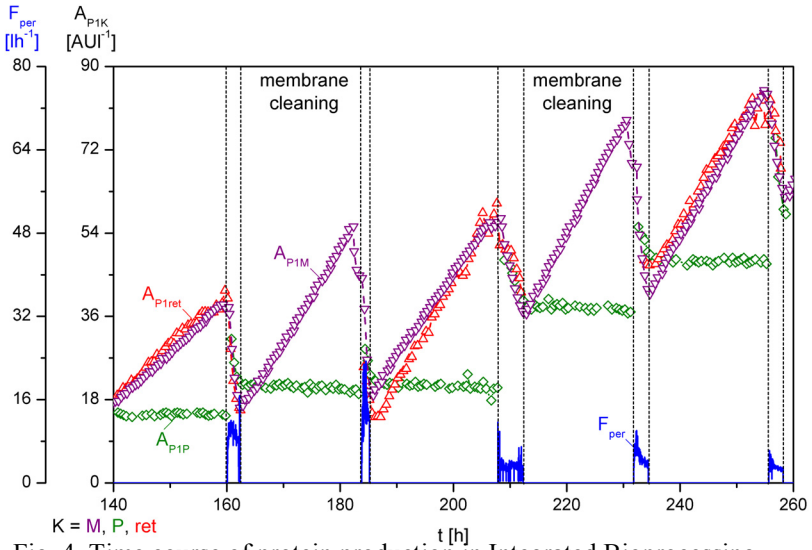

Fig. 4. Time course of protein production in Integrated Bioprocessing
A_{P1K} := target protein UV-absorption in subsystem K
(P: permeate tank ◇, at-line; M: bioreactor media phase ▽, at-line; ret: retentate backflow Δ, at-line) F_{per} := permeate flow rate

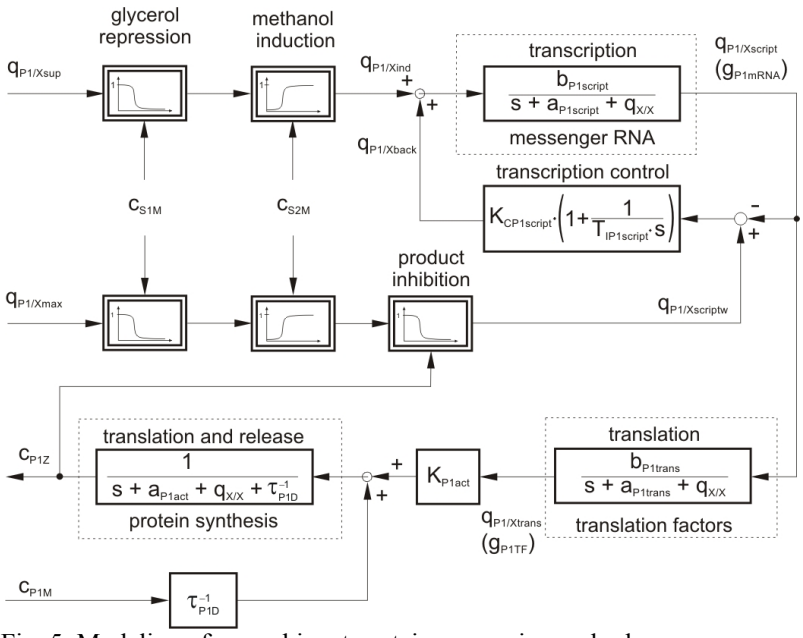

Fig. 5: Modeling of recombinant protein expression and release

operations, of multi-substrate and by-products bio-reaction kinetics and of induction, protein expression and product release dynamics in *Pichia pastoris*. Furthermore a dynamic model for cross-flow operations is developed and included into the bioreactor model in order to simulate the integrated cultivation and harvest procedures.

The kernel of the bioreaction model for HCDC-processes is based on mass balances of all three bioreactor phases. The gas phase G balances describe the oxygen supply rate Q_{O2} and carbon dioxide discharge rate Q_{CO2}, whereas the reaction phase L balances have to be divided into the bio phase (wet cells) Z and the liquid media phase M.

A concentration balance of component I in the media phase M,

$$\dot{c}_{IM}(t) = \frac{-F_{in}(t) + q_{X/X}(t)\cdot V_Z(t)}{V_M(t)}\cdot c_{IM}(t) + \frac{\sum F_K(t)\cdot c_{IK} + \dot{m}_{IT}(t) \pm q_{I/X}(t)\cdot m_{XL}(t)}{V_M(t)} \quad (1)$$

with

c_{IJ} := concentration of component I in subsystem J;
F_{in} := total feed rate;
F_K := rate from feed tank K;
V_J := volume of phase J;
$q_{I/X}$:= cell specific reaction rate of component I;
m_{XL} := cell mass in phase L;
$\dot{m}_{IT}$:= mass transfer of component I from gas phase,

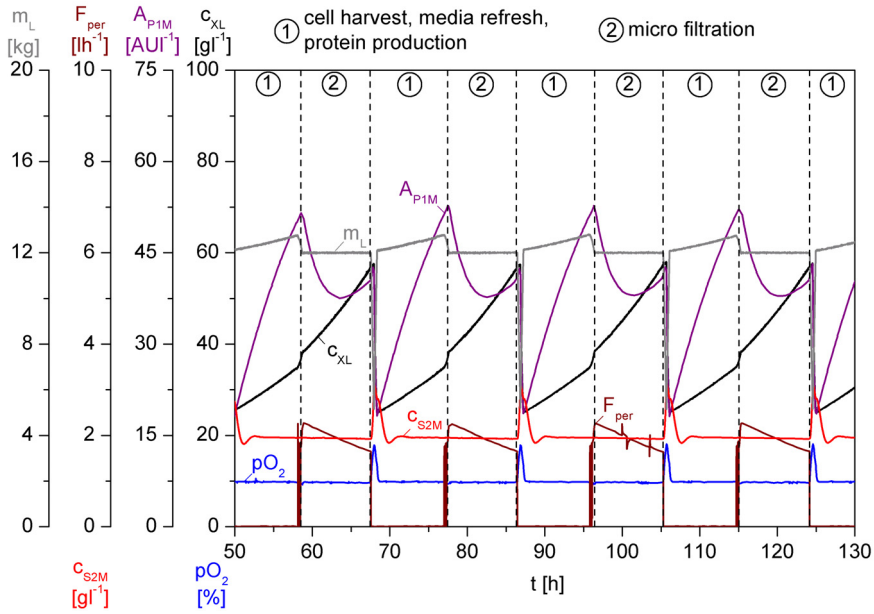

Fig. 6:Simulation of Sequential Integrated Bioprocessing

c_{XL} := cell density in liquid phase; pO_2 := dissolved oxygen tension; c_{S2M} := methanol concentration in media phase; A_{P1M} := target protein absorption; F_{per} := permeate flow rate; m_L := mass of liquid phase.

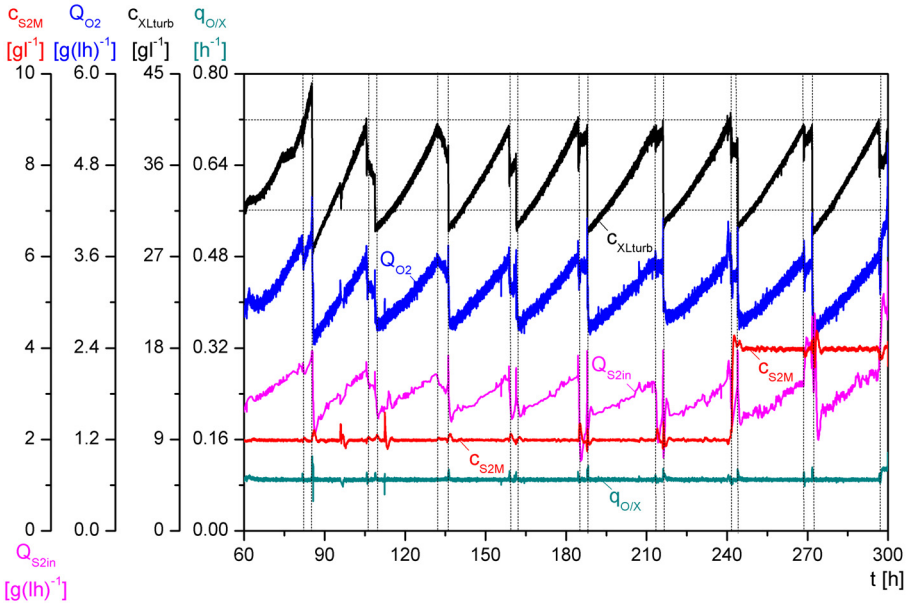

Fig. 7: Time course of a fully automated recombinant protein production
c_{XLturb} := cell density in liquid phase, reconstructed via turbidity measurement
c_{S2M} := methanol concentration in media phase; Q_{S2in} := volumetric methanol supply rate;

is needed for all essential substrate and by-product concentrations of a Pichia process. Moreover, molar concentration balances are required to account for the effects of titrants and other ions on the pH-control.

The balance of the recombinant product *1-3del I-TAC* (P1) in the media phase,

$$\dot{c}_{P1M}(t) = \frac{-F_{in}(t) + [q_{X/X}(t) - \tau_{DP1}^{-1}] \cdot V_Z(t)}{V_M(t)} \cdot c_{P1M}(t) + \frac{\tau_{DP1}^{-1} \cdot V_Z(t)}{V_M(t)} \cdot c_{P1Z}(t) \quad (2)$$

is influenced by protein secretion of the cells, described by a simple diffusion model with a time constant τ_{DP1}.

The solution of Eq. (2) needs a modeling approach for the cell internal expression of the product, shown in Fig. 5. The cell internal product concentration c_{P1Z} is a result of a three step (over-) expression process with an internal feedback regulation structure. Expression is controlled by induction with methanol, by repression due to a high glycerol concentration and the product P_1 itself as a result of the product release.

The developed global process model of the Integrated Bioprocess contains about 40 deqs., 160 algebraic eqs. and approximately 250 parameters, all of them based on essential bioreaction phenomena, process engineering correlations and physical laws.

A detailed description of a similar model, developed for *E.coli* processes without recombinant products, is published in (Luttmann and Gollmer, 2000).

6. DEVELOPMENT OF SEQUENTIAL INTEGRATED BIOPROCESSING

A sequential cultivation and harvest procedure, shown in Fig. 6, was developed in Apparent Processing. After cell growth, induction and cake formation, a SIB is carried out. Crossflow filtration, cell harvest, media refresh and production phases are performed cyclically to increase space-time-yield of protein production. Methanol is controlled at a concentration level of 2 gl^{-1}, whereas pO_2 is controlled sequentially by manipulation of air aeration rate and agitation speed on 10 %.

7. AUTOMATED RECOMBINANT PROTEIN PRODUCTION

Several long term protein production processes, based on strategies similar to Fig. 6, were carried out. Some phases of an automated experiment are shown in Fig. 7. In order to avoid pure oxygen addition in the aeration line, the cell specific oxygen uptake rate $q_{O/X}$ is controlled at an O_2-limiting setpoint of 0.1 h^{-1}. The automation is based on an upper cell density c_{XLturb} of 40 gl^{-1} and one liquid volume exchange per cycle and works reliable for over 720 h (19 cycles).

8. CONCLUSIONS

Advanced monitoring and control with included Apparent Processing offers new ways for the development of complex combined expression and harvest procedures such as in Integrated Bioprocesses for secreted recombinant protein production.

9. ACKNOWLEDGEMENT

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