

PAT – PROCESS ANALYTICAL TECHNOLOGY IN CULTIVATION PROCESSES WITH RECOMBINANT *ESCHERICHIA COLI*

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Abstract: An extensive monitoring system is build up to fully automate and scan production processes of recombinant *Escherichia coli* for optimal process performance. It is based on in-line, on-line and at-line measurements for substrates and products. MATLAB® is connected via OPC¹ to the SCADA² system MFCS/win® to realise on-line calculations of cell specific and volumetric reaction rates, based on the applied analytical systems. For on-line determination of intracellular target protein recombinant GFP is expressed either as single or fusion protein. Copyright © 2007 IFAC

Keywords: *Escherichia coli*, GFP, bioprocess automation, on-line protein monitoring, PAT-process analytical technology, MATLAB®

1. INTRODUCTION

Escherichia coli is one of the most common hosts for the production of recombinant proteins in fed-batch high cell density (HDCD) mode. The primary goal of microbial process development is the enhancement of foreign protein production which can be achieved by increasing either the cell specific production ($q_{p1/X}$) or cell productivity (μ). However, product formation depends on various parameters such as cell specific growth rate (Ramirez and Bentley, 1995; Shin, et al., 1998; Sanden, et al., 2002), media composition (Li, et al., 1990; Nancib, et al., 1991; Ramirez and Bentley, 1993) inducer concentration (Kosinski and Bailey, 1991; Wood and Peretti, 1991) etc. and mainly on the used strain, plasmid and target protein. Multiple parameters can be varied to improve a cultivation process. To screen such a complex system and to eliminate unintentional limitations, further knowledge about the process is a necessity. Extensive monitoring using in-line, on-line and at-line measurement systems provide additional information about the process performance. The outcome of this is a better process understanding, the ability to perform on-line calculations for sophisticated process monitoring and identification as well as the aptitude to fully automate the process to achieve better reproducibility.

Since the main goal of bioprocess optimization is the enhancement of heterologous protein productivity the reporter protein GFP is expressed either as single or fusion protein to enable on-line observation of intracellular protein formation. It was shown by different authors (DeLisa, et al., 1999; Cha, et al., 2000) that GFP is capable to be used as a non-invasive quantitative fusion marker of foreign protein production in *Escherichia coli*.

2. MATERIALS AND METHODS

2.1 Strains and Media

E. coli JM105 pUC19-GFP, *E. coli* BL21 star (DE3) p6xHis-GFP as well as *E. coli* DH5alpha pTRCHIS-T-Sapphire were used for recombinant protein expression. Product formation was induced with Isopropyl- β -D-thiogalaktopyranosid (IPTG) at concentrations between 0.1 - 1.0 mmol l⁻¹.

For pre-cultures as well as for cultivation procedure a defined M9 mineral salt media (Riesenberg, et al., 1990) was used with glucose as the main carbon and energy source. Initial glucose concentration for pre-cultures as well as for batch media were 10 g l⁻¹ and 300 g l⁻¹ for feeding during fed-batch mode. Media preparation was done according to DeLisa, et al., 1999. Apicillin (Sigma) concentration was adjusted to 100 μ g ml⁻¹.

¹OPC Object linked and embedded (OLE) for process control

²SCADA Supervisory control and data acquisition

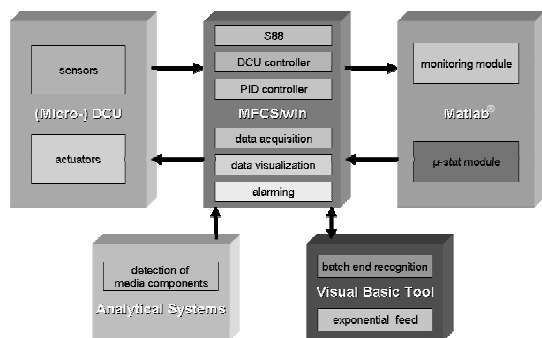


Fig. 1. Schema of process automation

2.2 Process automation and applied strategies

Cultivations are performed in a 15 l BIOSTAT® C (Sartorius BBI Systems, Germany) fermenter with standard instrumentation for pH, DO, temperature and pressure measurement in the head space. All reservoirs, titration tanks, the fermenter as well as all sampling streams are balanced for exact mass balancing. pH is controlled either by adding 3 M phosphoric acid or 25 % aqueous ammonia solution. For supervisory control as well as for data acquisition, visualisation and alarming the SCADA System MFCS/win® is used (Fig.1). It provides an implemented S88 block programming language which is a simple but reliable way to achieve reproducible process conditions. The Micro-DCU³ 2 operates in remote control and the implemented controllers for temperature, pO₂-agitation control, pH and antifoam can be observed and controlled via MFCS. For advanced control strategies PID controllers can be programmed via the SCADA system. For more sophisticated control strategies, process observation and simulation the software packages MATLAB® and Visual Basic® are integrated. Several analytical systems are applied for the detection of media components.

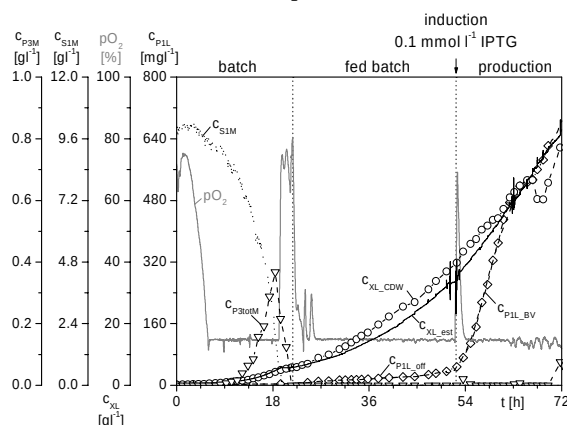


Fig. 2. Typical time course of a HCDC production process with *E. coli*
 c_{P1} : GFP (◇: off-line; dashed line: on-line)
 pO_2 : dissolved oxygen tension;
 c_{XL} : bio dry mass (○: off-line; solid line: on-line)
 c_{SIM} : glucose; c_{P3M} : acetate

³DCU Digital control unit

A typical time course of a HCDC production process is shown in fig. 2. After inoculation with approx. 0.3 g l⁻¹ dissolved oxygen tension (pO_2) decreases while glucose (c_{SIM}) is metabolised. To ensure unlimited growth conditions the pO_2 -agitation controller is initiated by the process control system when dissolved oxygen drops below 15 %.

Glucose depletion is indicated by an increasing pO_2 and a decreasing stirrer speed. Both variables are used as a trigger for process automation. pO_2 -agitation control is switched off and the microorganisms start to utilise the undesired by-product acetate as a C-source. As soon as all carbon sources are exhausted dissolved oxygen tension increases above 80 % and fed-batch phase is initiated by MFCS/win®. pO_2 -agitation control is activated with a set point of 15 %. Substrate limited fed-batch ($\mu_w < \mu_{max}$) is realised by an open loop feed control. For any desired specific growth rate μ_w the relative feeding rate F_{R1relw} is given by

$$F_{R1relw}(t) = \frac{\mu_{wk} \cdot V_{Lk} \cdot c_{XLk}}{Y_{X/S1wk} \cdot c_{S1R1}} \cdot \frac{100\%}{F_{R1max}} \cdot e^{\mu_{wk} \cdot (t-t_k)} \quad (1)$$

where V_L is the liquid volume at t_k , c_{XL} the cell density at t_k , F_{R1max} the maximum feeding rate, c_{S1R1} the glucose reservoir concentration and $Y_{X/S1w}$ the specific biomass yield on glucose at the adjusted growth rate μ_{wk} which has to be determined by preliminary experiments. As soon as a certain cell density is reached, cultivation temperature is reduced to 30 °C and foreign protein production is induced automatically by the addition of IPTG. When the maximum oxygen transfer capacity of the bioreactor is achieved, either cell specific growth rate μ_w is reduced or pO_2 -feed control is executed at maximum stirrer speed and aeration.

2.3 In-line measurements

2D-fluorescence spectroscopy using a BioView®-sensor (DELTA Light & Optics, DK). The BioView® Sensor is optimized for industrial applications of fully automated optical measurements for monitoring of bioprocesses. It uses independent filter wheels for excitation and emission with 15 different filters each. The measurements are performed in steps of 20 nm which is equivalent to the bandwidth of the used filters. Excitation wavelength are $\lambda_{ex} \in [260 - 560]$ nm and emission wavelength are $\lambda_{em} \in [300 - 600]$ nm. Scattered light measurement within the excitation and emission range is performed either. Excitation and emission light are guided via liquid fiber optics towards the head of the probe. This is plugged into an optical adapter (25 mm port) and thus does not stay in contact with the cultivation broth. Light detection is realised at an angle of 180 ° (open end) to excitation light to allow measurements of culture fluorescence even at high optical densities (Marose, et al., 1998). One measurement cycle with 150 measures last approx. 2 minutes.

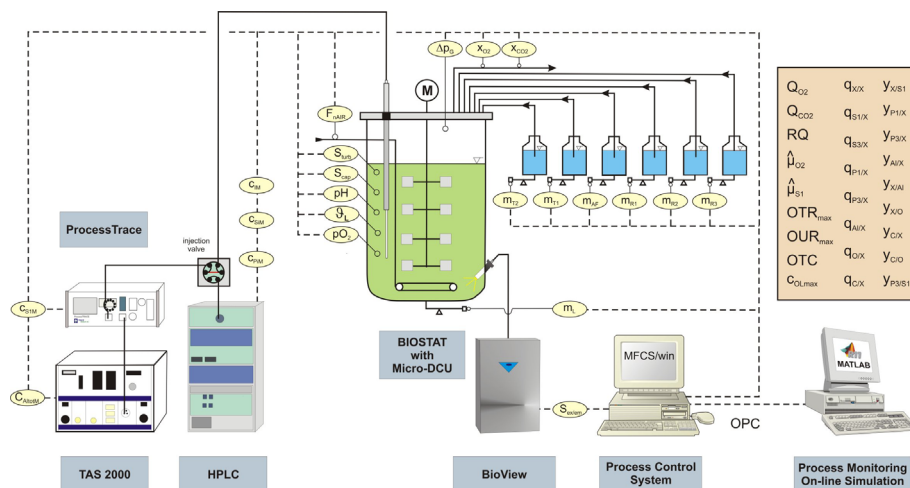


Fig. 3. Set-up of the cultivation plant for advanced process monitoring

Turbidity probe ASD25-BT-N5 (OPTEK Danulat, Germany). The applied turbidity probe uses light in the near infrared range (NIR) from 840 to 910 nm. The precisely defined, constant LED light beam penetrates the process medium with an optical path length of 5 mm. The attenuation of the light intensity caused by absorption and/or scattering by dissolved and undissolved substances is detected by a hermetically sealed photo diode.

Biomassmonitor BM200 (ABER INSTRUMENTS LTD., U.K.). A new BM200 with a ring electrode is applied whilst a couple of cultivations, measuring the capacitance of the cultivation broth at 1.12 MHz and for compensation purposes at 15.65 MHz. Furthermore the conductivity of the cultivation broth is detected using the BM200.

2.4 On-line measurements

Off-gas analyser MULTOR 610 (SICK | MAIHAK, Germany). Off-gas analysis is performed with a MULTOR 610 using a paramagnetic sensor (Maihak Oxor 610) for O_2 and an infrared detector (Maihak Unor 610) for CO_2 .

2.5 At-line measurements

In total three different at-line analysis systems are applied during this work. A peristaltic pump conveys cell free media sample via an ESIP⁴ from the fermenter to the different measuring devices.

Flow diffusion analyser (FDA) ProcessTrace® (TRACE Analytics GmbH, Germany). Cell free media (donor) on one side and a carrying buffer solution (acceptor) on the other side of a cellulose acetate membrane allows diffusion of glucose across the membrane. Enriched with glucose, the acceptor solution passes an electrode with immobilised

glucose oxidase (GOD) which causes H_2O_2 formation that is evaluated by an ammeter. The analyser works in stop-flow mode to generate a baseline signal and a measurement peak. Glucose can be detected in a range of $0.5 - 40 \text{ g l}^{-1}$.

Flow injection analysis (FIA) TAS 2000 (Jüke GmbH, Germany). The FIA system TAS 2000 allows at-line determination of ammonium within the sample broth during cultivation of *E. coli*. A defined sample segment (15 μl) is injected into a reagent flow. This particular reagent flow is diluted with an H_2O carrier. Later on, two more reagents are added to the liquid stream that passes a tempered water bath. A blue-green indophenol dye is formed and finally detected at a wavelength of 605 nm.

HPLC system ELITE LaChrom® (VWR International, Germany). The HPLC system is in use to detect either acetate and glucose, using an AMINEX HPX-87H (Biorad, Germany) column as stationary phase and 25 mmol l^{-1} sulphuric acid as mobile phase, or for the determination of the unknown inducer concentration using a MetaCarb 87P column (VARIAN, USA) and pure water as mobile phase. Determination of the certain substances is performed by using an UV-detector (190 nm) and an RI-detector. The set-up of the cultivation plant for advanced monitoring is shown in fig. 3.

3. SYSTEM INTEGRATION

Figure 4 shows the integration of the different systems into MFCS/win®. Micro-DCU as well as ProcessTrace® and BM200 are connected via serial interfaces (RS422 and RS232 respectively) to the process control PC. Data transfer is realised by a substation driver for the Micro-DCU developed by Sartorius BBI Systems and self developed Visual Basic® (VB) drivers (ProcessTrace®, BM200, TAS2000; HPLC). Communication between MFCS/win® and MATLAB® is realized by TCP/IP

⁴ESIP Eppendorf sterilizable in-line probe

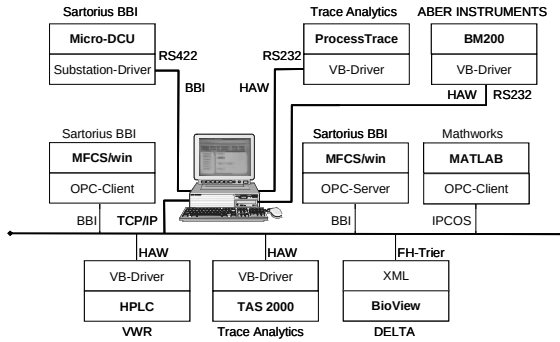


Fig. 4. System Integration

and OPC Client/Server-applications using MATLAB®'s OPC Client 3.0 (IPCOS, Belgium) and the MFCs/win Server 3.0 (Sartorius BBI Systems). The BioView® sensor, TAS2000 and the HPLC system are linked via TCP/IP using the local network. For data transfer between the BioView® sensor and MFCs® XML is used.

4. RESULTS

4.1 Monitoring of cell density and cell growth

Cell density is a key variable in bioprocess engineering especially for process automation and on-line calculations. Different systems are applied and compared (fig. 5). The cell density determined using capacitance measurement with the BM200 (C_{XLcap}) is in excellent accordance with the off-line determined cell dry weight (C_{XLCDW}) for approx. 30 h. The estimated cell density (C_{XLeSt}) applying off-gas balancing on-line shows almost the same trajectory.

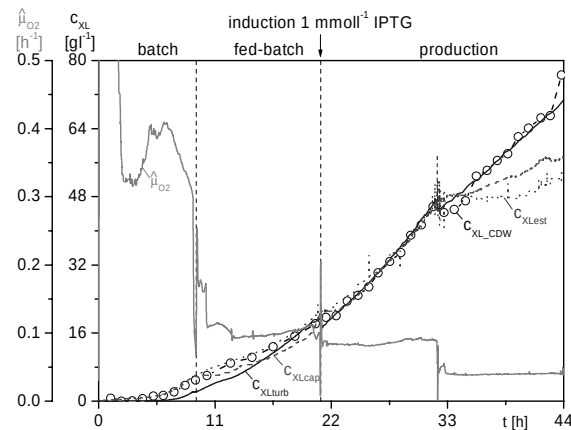


Fig. 5. Comparison of different systems for on-line cell density determination; C_{XLCDW} : off-line determined cell dry weight (o); C_{XLeSt} : via off-gas balancing determined CDW (dotted line); C_{XLcap} : via capacitance measurement determined CDW (dashed line); C_{XL_turb} : via turbidity measurement determined CDW (solid line); μ_{O_2} : reconstructed cell specific growth rate.

For cell mass estimation the following equation is used,

$$c_{XL}(t_j) = \frac{c_{XL}(t_{j-1}) + y_{X/Ogr} \cdot Q_{O_2}(t_j) \cdot [t_j - t_{j-1}]}{V_L(t_j) + [y_{X/Ogr} \cdot Q_{O_2}(t_j) + y_{X/S1gr} \cdot Q_{S1/Xm}] \cdot [t_j - t_{j-1}]} \quad (2)$$

where Q_{O_2} is the volumetric oxygen supply rate, $y_{X/Igr}$ the growth parts of the biomass/component I yield coefficients ($S1 = \text{glucose}$; $O = \text{oxygen}$) and $q_{I/Xm}$ the maintenance parts of the cell specific reaction rate for component I. The yield coefficients and reaction rates have to be determined by preliminary experiments with different stationary phases. Once the cell density is known, it's easy to reconstruct the cell specific growth rate during the cultivation process,

$$\hat{\mu}(t_j) = \frac{m_{XL}(t_j) - m_{XL}(t_{j-1})}{m_{XL}(t_j) \cdot [t_j - t_{j-1}]} + \frac{F_S(t_j)}{V_L(t_j)} \quad (3)$$

where m_{XL} is the cell mass and F_S the discontinuous sampling rate. The parameters for cell density calculation using turbidity measurement are adjusted for higher densities. The turbidity signal shows proper values for cell densities higher than 15 g l^{-1} . After the reduction of the cell specific growth rate at 31 h, the estimated cell mass as well as the capacity measurement differ from the off-line signal and the turbidity signal. This is probably caused by cell death and due to the fact that even cell debris are detected by cell dry weight and turbidity measurements.

4.2 At-line monitoring of substrates and products

An important issue in bioprocesses monitoring, automation and optimisation is to get reliable infor-

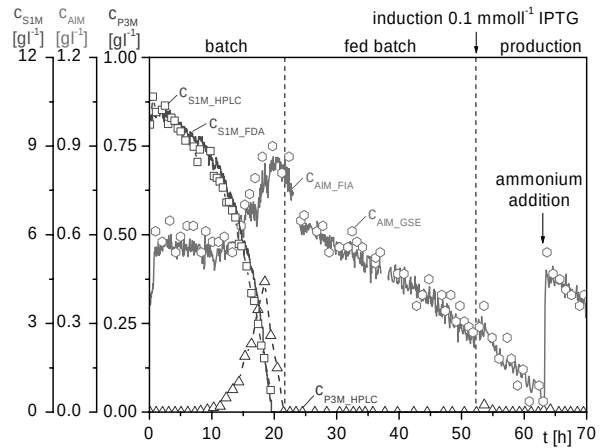


Fig. 6. Comparison of different analytical systems for on-line determination of substrates and products; C_{S1M_HPLC} : at-line via HPLC determined glucose concentration ($\square$); C_{S1M_FDA} : at-line via FDA determined glucose concentration (solid line); C_{AIM_FIA} : at-line via FIA determined ammonium concentration (solid line); C_{AIM_GSE} : off-line via a gas sensitive electrode determined ammonium concentration ($\circ$); C_{P3M_HPLC} : at-line via HPLC determined acetate concentration (Δ)

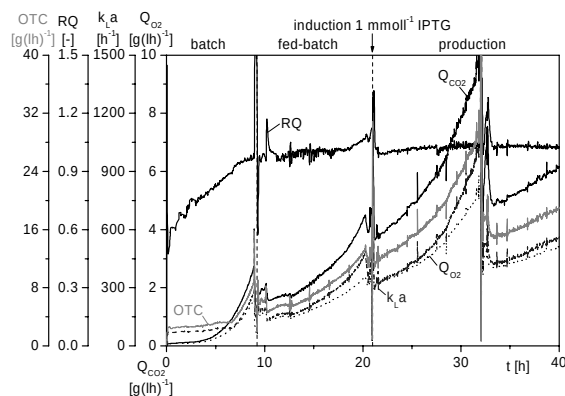


Fig. 7. Volumetric rates during an HCDC;

Q_{O_2} : volumetric oxygen supply rate (solid line); Q_{CO_2} : volumetric carbon dioxide discharge rate (dotted line); k_La : volumetric oxygen transfer coefficient (dashed line); RQ: molar respiratory quotient (solid line); OTC: oxygen transfer capacity (solid line)

mation about substrate and product concentrations within the fermenter. Figure 6 shows the at-line measures for glucose (C_{SIM}), the undesired by-product acetate (C_{PIM}) and the nitrogen source ammonium (C_{AIM}). All measurements are in good accordance with each other. The increase of the ammonium concentration during batch phase is caused by acetate formation and pH correction using ammonia. During fed batch and production phase ammonium was consumed by the microorganisms building amino acids. At approx. 63 h ammonium stock was added to increase the concentration in the cultivation broth about 0.4 g l^{-1} which is indicated by the measurement systems. Ammonium limitation is avoided in subsequent experiments by feeding exponentially di-ammonium hydrogen phosphate solution during fed batch and production phase.

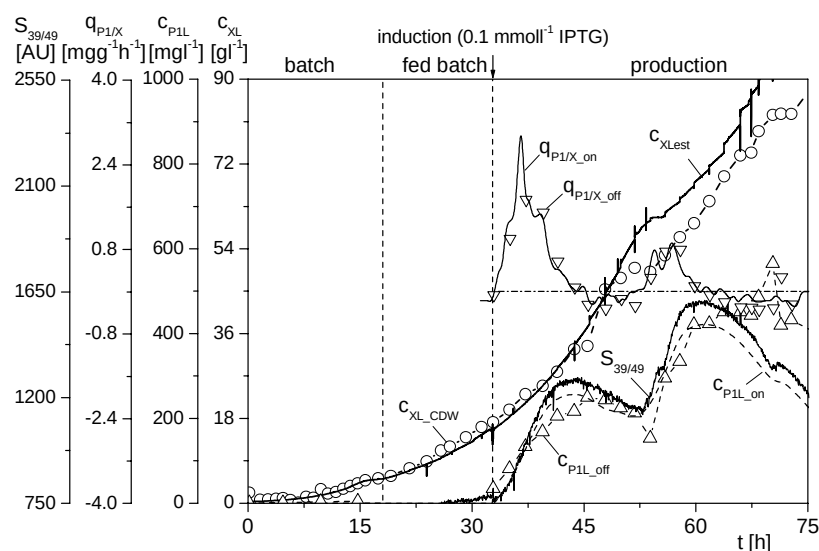


Fig. 8. On-line monitoring of GFP formation during an HCDC cultivation;

C_{XL_CDW} : off-line determined CDW($\circ$); C_{XL_est} : estimated CDW (solid line); C_{P1L_on} : on-line calculated GFP concentration (dashed line); C_{P1L_off} : offline determined GFP concentration (Δ); $S_{39/49}$: fluorescence signal λ_{ex} 390 nm, λ_{em} 490 nm (solid line); $q_{P1/Xon}$: on-line calculated cell specific GFP production rate; $q_{P1/Xoff}$: off-line calculated cell specific GFP production rate

4.3 Volumetric rates during an HCDC

On-line monitoring of volumetric rates enables the estimation of the procedural circumstances within the bioreactor. Figure 7 shows a process course of an HCDC. The volumetric oxygen transfer coefficient k_La is of utmost importance in aerobic cultivations to calculate the oxygen transfer capacity OTC, the actual upper limit of oxygen transfer into the liquid phase. The oxygen demand of the cells (identifiable with Q_{O_2}) increases rapidly exponential in the batch phase and slowly exponential in the fed-batch due to the glucose limited growth ($\mu_w < \mu_{1max}$). As a result of pO_2 /agitation-control, the OTC-value follows this demand.

4.4 On-line observation of foreign protein formation

Figure 8 shows the on-line and off-line determined GFP concentrations during a cultivation process. Gene expression was induced during fed-batch at a cell density of 17 g l^{-1} with 0.1 mmol l^{-1} IPTG. Until process time $t = 60 \text{ h}$ the on-line determined soluble protein concentration (C_{P1L}) shows a proper accordance with the off-line detected value. Cell specific protein expression ($q_{P1/X}$) was observed on-line and correlates with the off-line calculated values. The strongest expression was observed during the first 5 h after induction. This phenomenon was observable in all performed cultivations.

The main problem in on-line quantification of recombinant GFP using the BioView[®] sensor is the background fluorescence in the cultivation broth. NAD(P)H and FAD (Marose, et al., 1998) interfere with the GFP signal as well as products of decomposition of the used antibiotic ampicillin

(Barbhaiya and Turner, 1977) and the vitamin thiamine (Ichinose and Mitsui, 1988) show an increasing fluorescence during the cultivation run. Therefore appropriate calibration models have to be established to realise quantitative GFP observation.

5. CONCLUSION

Global monitoring of HCDC with *E. coli* using different measurement systems and software tools is an important challenge in production of recombinant proteins. Using this information fully automated processes can be established and process strategies can be developed to achieve higher productivity. However, the complex measurement system, and hence the information sources, have to be reduced. By cognition of key quantities model based monitoring and control will be arranged.

6. ACKNOWLEDGEMENT

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