



CHO cell-based production of a secreted model protein using the novel BIOSTAT® A



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Introduction

With the BIOSTAT® A, Sartorius has introduced a new entry level bioreactor for easy control of cell culture and microbial fermentation. The new compact design and intuitive operating interface allows even unexperienced users to achieve fast progress. All gas lines are controlled automatically and continuously for steady and tighter control of pH and DO. Manual adjustments of gas flow meters are no longer necessary to keep up with DO demand. The selection of preconfigured operation modes allows usage of either the UniVessel® Glass or the UniVessel® Single Use. Utilizing modern mobile control devices and running several units in parallel ensures maximum convenience and best use of work area. This application note evaluates a representative mammalian cell cultivation process conducted with a BIOSTAT® A and confirms performance parameters comparable to other bench top systems like BIOSTAT® B's.

In this paper we describe a protocol for the CHO cell-based production of the model protein secreted alkaline phosphatase (SEAP) using a 2 L glass vessel. The model protein SEAP is produced by CHO XM 111-10 suspension cells (Culture Collection of Switzerland) which grow and produce in chemically defined minimal culture media.

At Zurich University of Applied Sciences (ZHAW) cell growth and protein production experiments with this model cell line (originally established by the group of Prof. Dr. Martin Fussenegger, ETHZ) are regularly performed. In this context, many different bioreactor types and scales have already proven their suitability for this cell culture application. The suggested procedure comprises a cell growth and protein production phase with temperature shift, which is also typically the case for industrial protein production processes.

It was our aim to achieve similar results as obtained previously in the BIOSTAT® B systems in the first (non-optimized) runs. We aimed at a viable cell density of $> 6 \times 10^6$ cells and a protein activity of > 8 units/mL. Further we wanted to demonstrate that $k_L a$ values of 10/h could be reached at impeller tip speeds below 1 m/s.

1. Equipment and Material

- BIOSTAT® A
(Sartorius Stedim Biotech GmbH)
- UniVessel® 2 L, glass, single-walled, two 3-blade segment impellers (angle = 30°) mounted in a distance of 70 mm and microsparger 20 µm porosity (Sartorius Stedim Biotech GmbH)
- pH sensor (Endress & Hauser)
- DO sensor (Endress & Hauser)
- ChoMaster® media HP-1/HP-5 (Cell Culture Technologies GmbH)
- Cell line CHO XM 111-10 (Culture Collection of Switzerland, No. 837)
- Cedex HiRes (Roche Diagnostics)
- Bioprofile Analyzer 100 plus (Nova Biomedical)
- Shake-flasks 1000 mL (Corning)
- Shaking incubator (Infors HT)



2. Schedule and methods

Typically, maintenance culture of CHO XM 111-10 suspension cells is realized in T-flasks (75 cm²) using ChoMaster[®] FMX-8 medium. The inoculum for stirred bioreactor production is first prepared in (single-use) shake flasks (1000 mL) using ChoMaster[®] HP-1 medium in order to adapt the cells to the agitation.

2.1 Engineering characterization

Prior SEAP production oxygen transfer and mixing of the BIOSTAT[®] A were studied. $k_L a$ values were determined using the gassing-out method based on DECHEMA guidelines [1]. The measurements were carried out in a 1 % NaCl-solution at the maximum working volume of 2 L and a temperature of 37°C. The aeration rate of 0.03 vvm and a tip speed between 0.43 and 0.63 m/s were investigated. The dissolved oxygen concentration was measured with an Endress and Hauser sensor (Oxymax COS22D) with a response time of 15.5 s (63% of the signal acc. DECHEMA guidelines). All $k_L a$ investigations were repeated five times for each set of operating conditions.

Mixing time was determined using the decolourization method (per DECHEMA guidelines) at the maximum working volume of 2 L and a tip speed between 0.43 and 0.63 m/s [1]. All mixing time investigations were repeated five times for each set of operating conditions.

2.2 Schedule

- Day 1: Establishment of the inoculum culture (seeding density of 0.5×10^6 viable cells/mL) in shake flasks (1000 mL) with CHO XM 111-10 suspension cells characterized by logarithmic growth and doubling times ≤ 24 hours.
- Day 2: Feeding of the inoculum culture with ChoMaster[®] HP-1 growth medium (see section 2.4).
- Day 3: Feeding of the inoculum culture with ChoMaster[®] HP-1 growth medium (see section 2.4).
Preparation of the UniVessel[®] cultivation vessel:
– Calibration of pH and DO sensor(s)
– Connected inlet gas and outlet exhaust gas filters
– Sterilization (121°C/30 minutes)
– Sterility and sensor tests by adding 1 L ChoMaster[®] HP-1 medium and starting all control loops
- Day 4: The BIOSTAT[®] A 2L was inoculated with 0.8 L cell suspension (0.5×10^6 viable cells/mL) using ChoMaster[®] HP-1 growth medium

Day 5 & 6: Sampling (see section 2.6), successive feeding of ChoMaster[®] growth medium (first feeding with 400 mL ChoMaster HP-1, subsequent feeding with 600 mL ChoMaster HP-5 growth medium) and increase of stirrer speed

Day 7: Sampling and subsequent medium exchange (replacement of the growth medium by production medium)

Day 8: Sampling, subsequent temperature shift down to 31°C and stirrer speed increase

Day 9-x: Sampling and cessation of culture after cell viability dropped below 30%

2.3 Media

Chemically defined, minimal ChoMaster[®] HP-1 medium was used for the inoculum production and the start of the cultivation in the UniVessel[®] glass vessel connected to the BIOSTAT[®] A. The ChoMaster[®] HP-1 medium was supplemented with 2.0 g/L Pluronic F-68 and 2.5 mg/L tetracycline. The feeding was realized with ChoMaster[®] HP-5 growth medium (supplemented with Pluronic F-68 and tetracycline). CHO XM 111-10 cell lines include the Tet-Off system for controlled expression of SEAP. SEAP secretion was induced by medium exchange to tetracycline-free ChoMaster[®] HP-5 production medium.

2.4 Preparation of Inoculum

Single-use shake flasks of 1000 mL were used for the inoculum production. The cell propagation in the shake flasks starts with a viable cell density of 0.5×10^6 cells/mL in 100 mL suspension (using ChoMaster[®] HP-1). The cells are incubated in a shaking incubator at 37°C with a shaking frequency of 120 rpm, amplitude of 25 mm, relative humidity of 70 % relH and a CO₂ level of 7.5 %. After 24 and 48 hours, 50 mL and 100 mL of ChoMaster[®] HP-1 are added in order to provide the cells with glucose and dilute the intoxicating metabolites.

Three hours prior to the inoculation, all inoculum cells were pooled and the same amount of fresh ChoMaster[®] HP-1 medium was added, without shaking, to allow the settling down of the cells. After 3 hours, the supernatant was removed and the cells were transferred into the autoclaved glass vessel.

2.5 Setup for cultivation

The pH sensor was calibrated by performing a two-point calibration with buffer pH 4.01 and buffer pH 7.00.

Prior to autoclaving, the glass vessel was filled with 1 L PBS and the pH and DO sensors (both Endress and Hauser, Germany) inserted. After vessel sterilization at 121°C for 30 minutes, PBS was replaced with 1000 mL ChoMaster® HP-1 medium under a safety cabinet hood. The ChoMaster® HP-5 growth and production medium containers (FlexBoy® 3 L, Sartorius Stedim Biotech) were connected to the bioreactor via LuerLock. In addition, antifoam solution (3000 ppm Antifoam, Sigma Aldrich) was connected to the glass vessel via a LuerLock connector. The glass vessel was transferred to the control unit where temperature, agitation and pH conditions were initiated and monitored for confirmation of sterile 24hr hold and trouble-free operation of sensors prior to start of experimental culture.

2.6 Sampling and analysis

Daily one 4 mL sample is taken by connecting a sterile 10 mL syringe via a clave adapter. In-Process-Control was performed by CedexHiRes cell counter (viable cell density, viability) and BioProfile Analyzer 100Plus (metabolites and substrate concentration) once a day (4 mL). In addition, the pH value was determined offline by pH meter (Mettler Toledo). Activity of the expressed SEAP was measured by para-nitrophenol phosphate enzymatic reaction to para-nitrophenol causing a colour change of the prepared supernatant [2].

2.7 Culture conditions

Starting culture volume	0.8 L
Final culture volume	1.8 L
Agitation speed	100–220 rpm (stepwise increase)
DO	30 % controlled by air and O ₂ flow
pH	7.2
Temperature	37°C (growth) 31°C (protein production)
Aeration rate	max. 0.02 vvm (sparge oxygen)
Start cell density	0.5 × 10 ⁶ viable cells/mL
Cultivation time	14 days (3 days growth phase and 11 days production phase)

3. Results

3.1 Engineering characterization

Figure 1 depicts the experimentally obtained mixing time (θ_m) and volumetric mass transfer coefficient ($k_L a$) for the BIOSTAT® A 2 L, which are represented as a function of tip speed (u_{Tip}). Turbulence increases as tip speed increases, therefore this directly leads to a decrease in the mixing time and increases the volumetric mass transfer coefficient. Although about 13 s are required to achieve 95 % homogenization at the lowest tip speed (0.43 m/s), only about 8 s are required at maximum used tip speed (0.63 m/s). In addition, a minimum $k_L a$ of about 8.5 1/h (0.43 m/s) and maximum of 11 1/h (0.63 m/s) were determined.

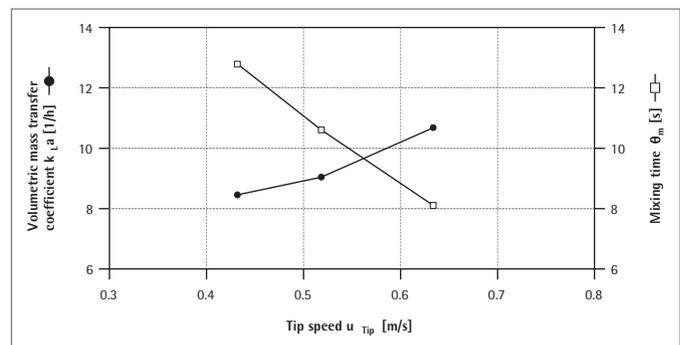


Figure 1: Determined volumetric mass transfer coefficients and mixing times in the reusable UniVessel® 2L controlled with BIOSTAT® A.

3.2 CHO-cell based growth and SEAP production

Good cell growth was obtained during growth phase and in the beginning of the production phase. Starting with an initial cell density of 0.4×10^6 cells/mL, the maximum viable cell density of 7.09×10^6 cells/mL was obtained ~144 hours after inoculation. The average specific growth rate during growth phase was 0.037 ± 0.013 corresponding to a doubling time of 20.4 ± 6.1 hours.

Product formation was increased after induction of SEAP expression. Final and maximum SEAP activity was 17.0 ± 0.13 U/mL. Substrates (glucose and glutamine) decreased as the viable cell density increased. Glucose was consumed completely after ~168 hours of cultivation. In contrast, glutamine was depleted within 24 hours after inoculation, each feeding or medium exchange.

Both metabolites, lactate and ammonium, accumulated as glucose and glutamine were consumed. Ammonium accumulation increased after medium exchange and achieved a maximum concentration of 4.74 mmol/L.

CO₂-based down regulation of pH worked excellent (standard PID parameter) and pH never exceeded the pH setpoint of 7.2 for a long time. Online and offline pH measurements corresponded well. pH recalibration adjustments only took place when the difference between online and offline measurements were larger than 0.1. During the entire process, the pH sensor only needed to be recalibrated three times indicating robust and reliable measurement of the standard pH probe.

Dissolved oxygen initially dropped down during the first 24 hours to 30 % due to cell growth. Afterwards, the DO fluctuated around the setpoint of 30 % for the remaining ~264 hours indicating non-optimized PID parameters. Towards the end of the experiment DO increased - indicating decreasing cell viability and cellular respiration. The break in DO after 72 hours was caused by the decoupling of the vessel from the control unit and sedimentation under laminar flow (medium exchange).

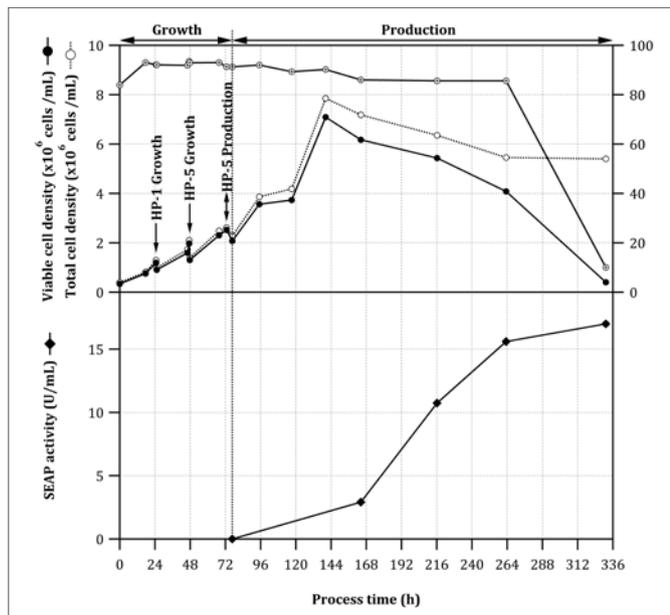


Figure 2: Profiles of viable and total cell density, viability and SEAP activity in the reusable UniVessel[®] 2L controlled with BIOSTAT[®] A.

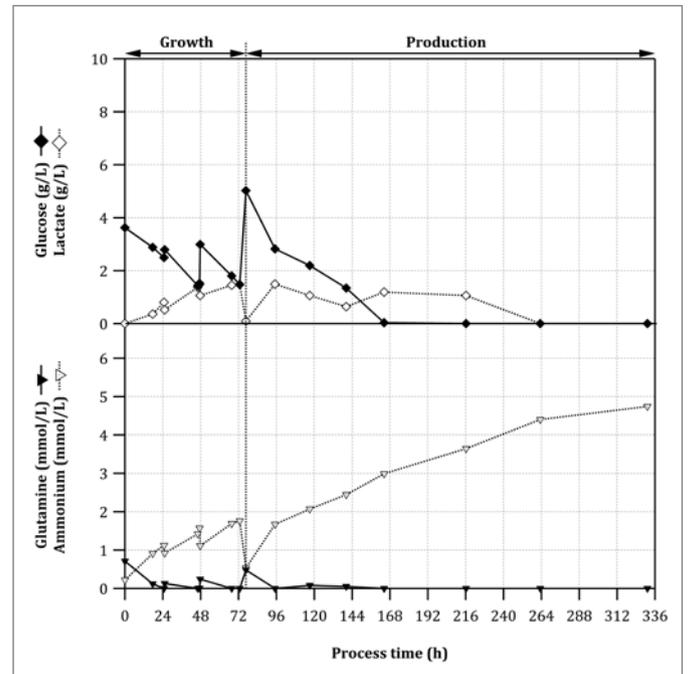


Figure 3: Profiles of substrates and metabolites in the reusable UniVessel[®] 2L controlled with BIOSTAT[®] A.

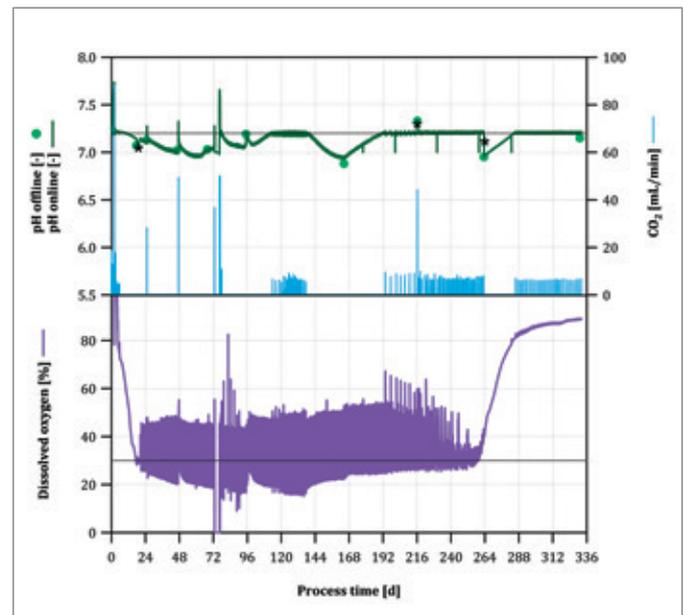


Figure 4: Profiles of online and offline pH measurement, CO₂ flow rate and dissolved oxygen measurement in the BIOSTAT[®] A. * = recalibration required.

4. Conclusion

The BIOSTAT® A provides an easy to use and reliable stand-alone solution for control of mammalian cell cultivations. It could be shown that oxygen transfer being required to reach high cell densities ($k_L a > 10/h$) was already reached at 220 rpm (0.6 m/s tip speed). Due to the short data sampling rate of one second, analysis of DO values for $k_L a$ determination could be done perfectly.

In a cell culture experiment using CHO XM 111-10 suspension cells, data in terms of viability and viable cell density with 7.1×10^6 cells/mL are fully comparable with that of other cultivation systems. In addition, obtained SEAP activity of 17.0 U/mL exceeded expected values for stirred glass vessels and confirms the excellent performance of the BIOSTAT® A for mammalian cell culture processes. These results could also be verified in a second run. Stable control of temperature, pH and DO was already achieved with standard PID settings. Optimizing DO and pH PID control parameters will lead to further process improvements.

The presented protocol may provide a blueprint for cultivation and protein production utilizing other animal cell lines such as HEK- or insect (Sf-9 or High Five) suspension cells using the BIOSTAT® A.

5. References

- [1] Meusel, W., Löffelholz, C., Husemann, U., Dreher, D., Greller, G., Kauling, J., Bauer, I., Eibl, D. (2015) , Empfehlung zur verfahrenstechnischen Charakterisierung von Single-Use Bioreaktoren und Single-Use Mischsystemen mittels experimenteller Methoden, DECHEMA, in press.
- [2] Eibl, R., Löffelholz, C. and Eibl, D., Disposable Bioreactors for Inoculum Production and Protein Expression, in Animal Cell Biotechnology, R. Pörtner, Editor. 2014, Humana Press. p. 265-284.

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