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Cell Culture
Parallel CHO Cell Cultivation in Eppendorf BioBLU® c Single-Use Bioreactors

Katharina Blaschczok1, Sebastian Kleebank2, Ulrike Beeken2, and Dieter Eibl1
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Abstract

In industrial bioprocess development time and cost pressure is high. A promising strategy to reduce the development time is to combine parallel experimentation with the use of single-use equipment. In this study we cooperated with Dieter Eibl and his team at Zurich University of Applied Sciences (ZHAW) to assess the reproducibility of parallel cell culture process runs in a DASbox® Mini Bioreactor System equipped with BioBLU Single-Use Vessels. We compared cell growth, viability, and the metabolic profile in four parallel bioprocesses and found that the processes developed highly similar. These results confirm the excellent suitability of this bioprocess system for efficient process development.

Introduction

Bioprocess development aims at maximizing the yield and the quality of the desired end product while minimizing the costs. Numerous process variables exist, including the producer strain, the medium composition, the process duration, the feeding strategy, and process parameters like temperature, pH, and DO. Researchers usually optimize many of them by empirical experimentation, which requires many process runs.

Parallel bioprocess systems have the great advantage that multiple experimental parameters can be tested simultaneously in one run, which ensures maximal comparability between runs and saves precious time. The development time can be further reduced by the use of single-use bioreactors. With single-use equipment lengthy cleaning and sterilization procedures are omitted and the time between runs can be reduced.

The aim of this study was to test whether cell culture processes run in a parallel DASbox Mini Bioreactor System equipped with BioBLU Single-Use Vessels perform comparably. To assess this, the Eibl team analyzed cell growth and viability, and the metabolic profile in four parallel CHO cell processes.

Fig. 1: DASbox Mini Bioreactor System equipped with BioBLU 0.3c Single-Use Vessels.
Materials and Methods

Bioprocess system and vessel
The researchers carried out the processes in BioBLU 0.3c Single-Use Vessels with a maximum working volume of 250 mL. They controlled four vessels in parallel using a DASbox Mini Bioreactor System. The processes were controlled using DASGIP® Control software*.

Cell line and inoculation
The researchers used the suspension cell line CHO XM 111-10 (CCOS no. 837). To prepare the bioreactor inoculum the cells were expanded in single-use shake flasks in chemically defined ChoMaster® HP-1 medium (Cell Culture Technologies, Switzerland) at a shake rate of 120 rpm and an amplitude of 25 mm. The researchers inoculated all bioreactors to an initial viable cell density of 0.5 x 10⁶ cells/mL.

Process parameters and cultivation procedure
Figure 2 illustrates the cultivation procedure and Table 1 summarizes the cultivation parameters.

Table 1: Parameters for the cultivation of CHO suspensions cells in BioBLU 0.3c Single-Use Vessels

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<th>Production phase</th>
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<td>Initial viable cell density</td>
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<td>ChoMaster HP-5 medium (+ 0.2% Pluronic F-68)</td>
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<tr>
<td>Culture medium</td>
<td>ChoMaster HP-1 and HP-5 medium (+ 0.2% Pluronic F-68)</td>
<td></td>
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<tr>
<td>Culture volume</td>
<td>170 - 240 mL</td>
<td>250 mL</td>
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<tr>
<td>Temperature</td>
<td>37°C</td>
<td>31°C*</td>
</tr>
<tr>
<td>Stirrer speed</td>
<td>180 - 290 rpm</td>
<td>340 rpm*</td>
</tr>
<tr>
<td>Mixing time</td>
<td>5 s</td>
<td>4 s</td>
</tr>
<tr>
<td>Dissolved oxygen</td>
<td>30 %</td>
<td>30 %</td>
</tr>
<tr>
<td>pH</td>
<td>7.2</td>
<td>7.2</td>
</tr>
<tr>
<td>Gasflow</td>
<td>0.05vvm</td>
<td>0.05vvm</td>
</tr>
<tr>
<td>Gas destination (air, O₂, N₂, and CO₂)</td>
<td>submerge</td>
<td>submerge</td>
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* Temperature and stirrer speed were adjusted 24 hours after the medium exchange.

To mimic a typical protein production process, the process was carried out in two phases. During the initial phase the researchers cultivated the cells at 37°C to provide optimal conditions for growth. Subsequently they reduced the temperature to 31°C, as it is typically done in the protein production phase of a bioprocess. During the initial growth phase the researchers cultivated the cells in 170 mL ChoMaster HP-1 medium. Two days after inoculation they fed the culture by addition of 70 mL ChoMaster HP-5 medium.

Three days after inoculation the medium was exchanged with ChoMaster HP-5 medium. For the removal of the growth medium the bioreactor control was switched off so that the cells settled down. After medium removal the bioreactors were re-filled with ChoMaster HP-5 medium to the maximum working volume of 250 mL. All media contained 0.2% Pluronic® F-68 BioChemica (PanReac AppliChem, USA) as the protective agent against mechanical stress.

The cultures in the BioBLU 0.3c vessels were fed and harvested using the system’s integrated pumps. Anti-foam agent was added by pumping.

The temperature was measured using Pt100 sensors and controlled by the heater integrated into the DASbox Mini Bioreactor System. The BioBLU 0.3c vessels were equipped
with DASGIP DO sensors and potentiometric pH probes. The pH was controlled at 7.2 by addition of CO₂. DO was controlled at 30 % by addition of pure oxygen. All gasses were introduced through a submerged sparger. To minimize foaming the researchers set the total gas flow (flow air + flow O₂ + flow CO₂) to a low rate of 0.05 vessel volumes per minute (vvm).

In the course of the process the researchers adjusted the agitation speeds to achieve mixing times of 5 seconds during the growth and of 4 seconds during the production phase. The researchers terminated the cultivation processes as soon as the cell viability decreased to less than 40 %.

Analytics
The researchers analyzed samples of each culture daily. During the growth phase they determined total and viable cell densities, cell viability, pH, as well as substrate and metabolite concentrations offline.

Cell density and viability were determined using a NucleoCounter® NC-200™ (ChemoMetec A/S, Denmark). The specific growth rate and the doubling time were calculated using the following equations:

Specific growth rate: \[ \mu = \frac{\ln(x_{t+1}) - \ln(x_{t})}{t_{t+1} - t_{t}} \]

Doubling time: \[ t_{d} = \frac{\ln 2}{\mu} \]

The concentrations of glutamine, lactate, and ammonium were measured with a BioProfile® 100 Plus analyzer (Nova Biomedical®, USA). Offline pH was determined with a standard laboratory pH meter (Mettler Toledo®, Switzerland).
Results and Discussion

The Eibl team compared four processes operated in parallel in BioBLU 0.3c bioreactors (Unit 1-4).

Viable cell densities

The viable cell densities developed comparably in all four bioprocesses (Fig. 3).

During the first two days the cells proliferated exponentially and reached viable cell densities of about $2.7 \times 10^6$ cells/mL. 48 hours after inoculation the cell densities dropped slightly because of the addition of feed medium. After medium addition the cultures reached viable cell densities between about $2.7 \times 10^6$ and $3.1 \times 10^6$ cells/mL. In the growth phase the cells multiplied with average specific growth rates of 0.030 h$^{-1}$, which corresponds to a doubling time of 24 hours. Some cells were lost when the growth medium was exchanged for production medium three days after inoculation. During the first 24 h after medium exchange, the cells grew with average specific growth rates of 0.024 h$^{-1}$. The cell densities reached their maximum six days after inoculation.

During the growth phase more than 98.6 % of the cells were viable in all bioreactors, and the cell viabilities remained nearly constant until the cultures reached their peak densities. The viabilities started to decrease nine days after inoculation.

Substrates and metabolites

Figure 4 shows the concentration profiles of the substrates glucose and glutamine, as well as their metabolites lactate and ammonium. The concentration of both substrates decreased equally in all units during the first 47 hours. The initial concentration of 3.64 g/L glucose dropped to 0.52 g/L glucose. Glutamine was already consumed after one day. As a consequence of medium addition on the second day, the glucose concentrations increased again. The medium exchange at day 3 led to similar glucose concentrations of 4.32 g/L in all four bioreactors. During the production phase glucose and glutamine concentrations dropped fast within the first 24 h. After the temperature shift from 37°C to 31°C on the fourth day after inoculation, glucose consumption decelerated. By then the glutamine was already depleted. The formation of the metabolites lactate and ammonium correlated with the uptake of glucose and glutamine. The processes started with lactate concentrations of 0.11 g/L and ammonium levels of 0.9 mmol/L. During the growth phase, the rates of lactate and ammonium production were comparable between all four units. Before the medium exchange after day three, the lactate concentration reached 2.22 g/L and the ammonium level was 2.35 mmol/L. During the first 24 h after the temperature shift to 31°C the lactate concentration increase from 0.62 g/L to 2.09 g/L. After the temperature shift on day 4, the glucose concentrations fell below 2 g/L while lactate was consumed continuously until the end of the bioprocess. The increase of ammonium concentration during the production phase was also comparable among the vessels. Final ammonium concentrations were about 6.0 to 6.5 mmol/L.
Conclusion

The team at ZHAW analysed four CHO cell culture processes, which they carried out in parallel. The aim of the study was to assess the reproducibility of process performance within the DASbox Mini Bioreactor System equipped with BioBLU 0.3c Single-Use Vessels. The standard deviations of peak viable cell densities, and growth rates were only 7% between the units 1-4. The metabolic profiles were almost identical.

In summary, this case study demonstrates that the system used is suitable for cell culture process development. Parallel operation of multiple bioreactors and the use of single-use equipment can help reducing development times and ultimately costs.

Fig. 4: Metabolic profiles. The concentrations of glucose, lactate, glutamine, and ammonium were determined offline. The one-sided arrow indicates the time of the growth medium addition. The two-sided arrow marks the medium exchange.
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<td>4-fold system for single-use vessels</td>
<td>76DX08CC</td>
</tr>
<tr>
<td>8-fold system for single-use vessels</td>
<td>76DX16CC</td>
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<td>16-fold system for single-use vessels</td>
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Leachable Studies on Mammalian Cell Culture in BioBLU® Single-Use Vessels

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Contact: becken.u@eppendorf.com

Abstract

There is a growing awareness regarding the potential leaching of toxic or inhibitory chemicals from the plastic material of single-use bioreactors into cell culture medium. Based on a standardized cell culture test recommended by the German society for chemical engineering and biotechnology, DECHEMA®, we determined if there were no leachable chemicals from the Eppendorf BioBLU Single-Use Vessel material that affect cell culture performance. We did not observe any effect of leachables on CHO and Vero cell growth and viability, and the metabolic profile. The results suggest that the BioBLU Single-Use Vessels are safe for mammalian cell culture.

Introduction

Single-use bioreactors are routinely used in biopharmaceutical research and development and biologics manufacturing. While reducing turn-around times and contamination risk, one potential problem of single-use systems is the release of chemical compounds into the culture medium. These so-called leachables originate in the raw materials used for bioreactor fabrication, or are produced during irradiation and storage. They could potentially affect cell behavior, growth, and viability. Examples of the chemicals often used in plastic manufacturing are heavy metals (as catalysts for the polymerization process), UV-light stabilizers, antioxidants (to preserve the integrity of the consumable), plasticizers (to alter the mechanical properties), and slip or release agents (for easier and faster removal from the mold during manufacturing).

Some leachables have no effect on cells, but some negatively affect culture performance. One example is bis(2,4-di-tert-butylphenyl) phosphate (bDtBPP). It can derive from the breakdown of trisarylphosphite (Irgafos® 168), which is used in the fabrication of multilayer polyethylene bags, and inhibits CHO cell growth at concentrations as low as 0.1 mg/L [1].

Eppendorf BioBLU Single-Use Vessels are rigid-wall, stirred-tank bioreactors, which are devoid of Irgafos. The scope of this study was to test whether leaching of any other chemicals from the Eppendorf BioBLU Single-Use Vessel material into the culture medium affects cell culture performance. Based on the standardized cell culture test developed by the DEHEMA working group, “Single-Use Technology in Biopharmaceutical Manufacturing” [1], we tested for effects of leachables from the BioBLU 0.3c, 1c, 5c, and 14c Single-Use Vessels (Fig. 1) on CHO and Vero cell growth, viability, and metabolic profile.
Material and Methods

Cell lines
We used suspension FreeStyle™ CHO-S cells (Thermo Fisher Scientific®, USA) and Vero cells (ATCC®, CCL-81). The CHO cells were cultivated in CD CHO media (Gibco®, USA) supplemented with 8 mM L-glutamine (Gibco). Vero cells were cultured in VeroPlus SFM medium (ATCC, ACS-4001) supplemented with 4 mM L-glutamine.

Extraction
To extract potential leachables, we filled BioBLU 0.3c, 1c, 5c, and 14c Single-Use Vessels to 50% of their maximum working volume with serum-free cell culture medium and incubated the vessels for three days at 37°C without agitation. As a control for the experiments with Vero cells we incubated the medium under the same conditions in a borosilicate-glass flask (Pyrex®; Corning®, USA), as recommended in the DEHEMA protocol. The control for the CHO cell experiments was incubated in a polycarbonate shake-flask for cell culture (VWR®, USA). Such flasks are widely used for the cultivation of suspension cell lines (Fig. 2A).

Toxicity study – CHO cells
We precultured CHO cells in CD CHO Medium in a shake flask in a New Brunswick™ S41i Incubator Shaker (Eppendorf) at 37°C, 125 rpm, and 5% CO₂. We transferred 30 mL of the extraction and control media to separate shake flasks, inoculated the cultures with 0.25 x 10⁶ cells/mL, and cultured the cells for two days at 37°C, 125 rpm, and 5% CO₂. The cultivations were performed in triplicates (Fig. 2B). We took a sample from each culture twice daily, determined cell number and viability using a Vi-Cell® XR Cell Viability Analyzer (Beckman Coulter®, USA), and measured the concentrations of glucose, lactate and NH₃ using a Cedex® Bio Analyzer (Roche Diagnostics®, Switzerland) (Fig. 2D).

Toxicity study – Vero cells
We inoculated nine 6-well plates with the same concentration of Vero cells from the same pool and let

Fig. 1: The BioBLU Single-Use Vessels used in this study

Fig. 2: Experimental setup. A: Extraction with medium. B: CHO-cell culture in extraction media. C: Vero cell culture in extraction media. D: Analysis of cell density, viability, and metabolism.
Results

To evaluate the cytotoxicity of leachables, the DECHEMA recommends to evaluate three criteria, namely cell density, viability, and metabolism. We extracted BioBLU 0.3c, 1c, 5c, and 14c Single-Use Vessels and used the extraction medium for the cultivation of Vero cells and FreeStyle CHO-S cells.

CHO cell culture

![Graph](image1)

**Fig. 3:** Viable cell density. CHO cells were cultivated in extraction media from BioBLU Single-Use Vessels or a shake flask (control). Sampling was done twice daily.

![Graph](image2)

**Fig. 4:** Viability. CHO cells were cultivated in extraction media from BioBLU Single-Use Vessels or a shake flask (control). Sampling was done twice daily.

![Graph](image3)

**Fig. 5:** Cell metabolism. CHO cells were cultivated in extraction media from BioBLU Single-Use Vessels or a shake flask (control) and substrate (glucose) and metabolite (lactate, NH₃) concentrations were measured twice daily.
For both cell lines, cell growth in the extraction media and the control medium was comparable (Fig. 3, 6). Under all conditions close to 100 % of the CHO and Vero cells were viable (Fig. 4, 7). The concentrations of glucose, lactate, and ammonium developed very similar in the different cultures (Fig. 5, 8).

Vero cell culture

**Fig. 6:** The viable cell density was determined daily. Vero cells were cultivated in extraction media from BioBLU Single-Use Vessels or a shake flask (control).

**Fig. 7:** Viability was analyzed daily. Vero cells were cultivated in extraction media from BioBLU Single-Use Vessels or a shake flask (control).

**Fig. 8:** Cell metabolism. Vero cells were cultivated in extraction media from BioBLU Single-Use Vessels or a shake flask (control) and substrate (glucose) and metabolite (lactate, NH₃) concentrations were measured daily.
Conclusion

The materials used for the fabrication of BioBLU Single-Use Vessels were chosen to mitigate issues with leachables. The vessel body and head plate of Eppendorf BioBLU Single-Use Vessels are made of single-layer injection-molded plastic. No additives such as softeners are used for its fabrication. Only virgin raw materials are used to eliminate uncertainties arising from the use of recycled materials.

While it should be noted that the sensitivity to leachables can vary between cell lines, we did not observe any negative effects caused by leachables from the BioBLU vessel material on the growth, viability, and metabolic profile of the two cell lines tested. Hence, the vessel material of the BioBLU vessels can be considered “non-critical” with regard to cytotoxic leachables.

Literature

### Ordering information

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Development of a Scale-Down Model for rAAV Viral Vector Production Using a Sf9/BEV System

Simon Fradin and Ulrike Becken
1 Généthon, Evry, France; 2 Eppendorf AG Bioprocess Center, Juelich, Germany
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Abstract

Single-gene disorders originate in the absence or loss of function of a protein due to a genetic mutation. Gene therapy is a promising therapeutic approach that delivers a normal version of the gene to affected cells to compensate for its missing or defective counterpart. It often employs viral vectors, such as recombinant Adeno Associated Viruses (rAAVs), to insert the genes. The insect cell line Sf9 provides a suitable host for virus production. Sf9 cells are cultured in suspension, and hence working volumes can be adapted to changing needs during process development and manufacturing much more easily than for adherent cell cultures. In this study, researchers at Généthon® developed a scale-down model for rAAV viral vector production in Sf9 cells using an Eppendorf DASbox® Mini Bioreactor System. Parallel experimentation in small working volumes allowed time- and cost-efficient evaluation of process performance.

Introduction

Development and use of gene therapy for single-gene disorders requires optimization of the process for large-scale manufacturing of gene therapy vectors. Recombinant Adeno Associated Viruses (rAAV) are promising vector candidates. They are not known to be pathogenic, their DNA integrates into the human genome only very inefficiently, and the virus particles are very robust, to name only a few advantageous attributes. One big challenge on the road to therapeutic use is producing a sufficient amount of the virus for experimentation in large animal models, clinical trials, and actual treatment. A widely used host for rAAV production is the insect cell line Sf9. Cells are transfected with the genes needed to produce functional virus particles using baculovirus expression vector (BEV) systems. A major advantage of the Sf9/BEV system for large-scale virus production is the possibility of growing Sf9 cells in suspension culture. Higher cell densities can be reached than in adherent cell cultures, and the inherent scalability of the system allows for much easier adaption of production volumes during process development and manufacturing. Process development is usually carried out in small culture sizes, and subsequently scaled up to larger production volumes. It is highly desirable to also establish scale-down models that will mimic the production process in smaller volumes, and which can be used to optimize the process, troubleshoot, and implement changes.

Généthon is a non-profit biotherapy R&D organization created and funded by the Association Française contre les Myopathies, a French organization that supports patients and their families. Its mission is to design gene therapy products for rare diseases, to ensure their pre-clinical and clinical development, as well as the production in order to provide patients with access to these innovative treatments. Généthon is currently sponsoring two gene therapy projects that have reached clinical trial phase, and is involved in several other projects in preclinical or research stages.

This application note describes the successful development of a scale-down model for rAAV production using a Sf9/BEV system. The aim was to reproduce the production performance obtained in a 2 L bioreactor in an Eppendorf DASbox Mini Bioreactor System. It is optimized for parallel process development, meaning that
Material and Methods

**Cell culture**
The Généthon research team cultivated Sf9 cells in Sf-900™ III SFM culture medium (Thermo Fisher Scientific® Inc., USA) at 27°C and dissolved oxygen set to 50 %. The reference production system used a glass bioreactor with a working volume of 2 L, and scale-down experiments used an Eppendorf DASbox Mini Bioreactor System (Eppendorf AG, Germany, Fig. 1), with a working volume of 250 mL. Both systems used marine impellers. The agitation speed of the 2 L production system was 180 rpm. Proper culture mixing and oxygen transfer have to be ensured during development of scale-down models. Agitation speeds of 300 rpm, 375 rpm, and 450 rpm were tested in three vessels simultaneously (Table 1).

Cell density, cell viability, and cell diameter were determined offline, using the Cell Viability Analyzer Vi-CELL® XR (Beckman Coulter®, Inc., USA).

**Virus production and quantification of production yield**
The researchers at Généthon used Sf9 cells as hosts for the production of recombinant Adeno Associated Virus serotype 8 (rAAV8). 72 hours after inoculation they co-infected the Sf9 cells with two distinct baculovirus clones, one carrying the genes for the production of the virus capsid and the other carrying the gene of interest. Through their lytic life cycle, the baculoviruses replicate using the Sf9 cell machinery, infect other cells in the culture, and produce the rAAV8 vectors. The culture was harvested 96 hours after infection, and the vectors were released using a detergent treatment. The team determined the rAAV8 vector titer by measuring viral DNA using real-time quantitative PCR. This analysis leads to a viral genome titer (VG), which is interpreted as the number of viral vector particles carrying the gene of interest. Specific productivity (VG/cell) was calculated by normalizing the viral genome titer to the viable cell density at the time of infection.

In parallel processes production yields were compared for cultures agitated at 300 rpm and 400 rpm, respectively. To test for reproducibility, three bioreactor runs were performed for each agitation speed (Table 1).

**Results**
The study aimed to scale-down rAAV production from a 2 L working volume to a 250 mL working volume, while reproducing production performance. The Généthon research team first compared growth of non-virus infected Sf9 cells in the 2 L glass vessel and in the 250 mL working volume vessels of the DASbox Mini Bioreactor System.
The results demonstrate the successful scale-down of rAAV production using a Sf9/BEV system. The cell growth and production performance obtained in a 2 L glass vessel were reproduced in an Eppendorf DASbox Mini Bioreactor System with a working volume of 250 mL. This study exemplifies the value of the Eppendorf DASbox Mini Bioreactor System for process scale-down. In the course of scale-down model development, multiple experiments must be performed to test a variety of experimental conditions and to ensure the reproducibility of process performance. The use of a parallel bioreactor system saves time, and ensures maximum comparability of experimental runs. By using the DASbox, researchers at Généthon shortened development timelines. They state: “The biological timing is impossible to shorten due to the rAAV production kinetics. But the DASbox system shortens the development timelines, by being able to run the same amount of bioreactors in one week whereas it takes two weeks in 2 L glass bioreactors.” Using the DASbox system also helped reduce costs, by facilitating the performance of studies in small-scale bioreactors.

Conclusion

The results demonstrate the successful scale-down of rAAV production using a Sf9/BEV system. The cell growth and production performance obtained in a 2 L glass vessel were reproduced in an Eppendorf DASbox Mini Bioreactor System with a working volume of 250 mL. This study exemplifies the value of the Eppendorf DASbox Mini Bioreactor System for process scale-down. In the course of scale-down model development, multiple experiments must be performed to test a variety of experimental conditions and to ensure the reproducibility of process performance. The use of a parallel bioreactor system saves time, and ensures maximum comparability of experimental runs. By using the DASbox, researchers at Généthon shortened development timelines. They state: “The biological timing is impossible to shorten due to the rAAV production kinetics. But the DASbox system shortens the development timelines, by being able to run the same amount of bioreactors in one week whereas it takes two weeks in 2 L glass bioreactors.” Using the DASbox system also helped reduce costs, by facilitating the performance of studies in small-scale bioreactors.
### Ordering Information

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Intelligent Control of Chinese Hamster Ovary (CHO) Cell Culture Using the BioFlo® 320 Bioprocess Control Station

Nick Kohlstrom, Stacey Willard, and Ma Sha
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Corresponding author: sha.m@eppendorf.com

Abstract

The recently released BioFlo 320 bioprocess control station offers some of the most intelligent cell culture control mechanisms on the market today. The innovative intelligent software automatically recognizes different type of sensors, from conventional analog sensors to proprietary sensors equipped with Intelligent Sensor Management (ISM®) technology by Mettler-Toledo®, from traditional polarographic sensors to advanced digital and optical sensors. The BioFlo 320 also offers flexible connections to either traditional glass vessels or various BioBLU® single-use vessels without relying on external adaptors. Combined with the seamless integration of biomass sensors from FOGALE nanotech®, the BioFlo 320 presents an intelligent setup with which to conduct and monitor mammalian cell culture. In this application note, Chinese Hamster Ovary (CHO) batch cell culture runs were conducted to highlight the versatility of this new control station, and as such, various sensors and control strategies were employed. First, the new capability of the control station to automatically detect and integrate sensors with ISM technology was utilized. Sensor health and maintenance was monitored using iSense software (Mettler-Toledo). In addition, the evo 200 (FOGALE nanotech) capacitance-based biomass sensor was also included for in-line growth monitoring. The ease of sensor detection and calibration combined with the elimination of the need for offline cell counting elevates this experiment to “intelligent” cell culture.

Introduction

The BioFlo 320 combines features and benefits from the New Brunswick™ BioFlo/CelliGen® 310 benchtop, autoclavable bioreactor system and the New Brunswick CelliGen BLU bioreactor to create an all-in-one bioprocess system with unique capabilities for intelligent cell culture (Figure 1). The BioFlo 320 can interchangeably control industry-standard autoclavable glass vessels or BioBLU single-use vessels. In addition to increased versatility with respect to vessels, the BioFlo 320 offers the ability to seamlessly connect a wide variety of Mettler-Toledo ISM sensors including dissolved oxygen (DO) and carbon dioxide (DCO2), pH, and redox. As with previous models, the BioFlo 320 supports 4 – 20 mA input/output connection with a multitude of ancillary devices including auxiliary pumps, turbidity sensors, capacitance

Figure 1: The left and right-handed BioFlo 320 bioprocess control stations with magnetic drive glass water-jacketed vessel (left) and BioBLU single-use vessel (right)
sensors, extra scales, automatic samplers, and biochemical analyzers, which can be recorded and/or controlled within the software.

In this work, the BioFlo 320 was used to control two batch suspension CHO cultures in a 3 L glass water-jacketed vessel. The runs differed in the automatic gassing strategy employed: one run used the 3-Gas algorithm and the other run used the 4-Gas option. In addition, to highlight the ability to integrate many different sensor types, three different DO sensors were used to monitor the DO levels in both cultures: (1) an ISM polarographic DO sensor, (2) an ISM optical DO sensor and (3) an analog polarographic DO sensor. Using the ISM-compatible BioFlo 320 software paired with the Mettler-Toledo iSense software, users can monitor sensor health, lifetime, calibration data, and autoclave/sterilization times, among other parameters. During these runs, both gassing strategies resulted in an average peak density of $9 \times 10^6$ cells/mL before nutrient depletion occurred.

Materials and Methods

Tables 1 and 2 outline the hardware and consumable reagents used in this study.

Vessel preparation

A 3 L glass water-jacketed vessel with magnetic drive and pitched blade impeller was outfitted with 3 DO sensors (see Table 1), an ISM pH sensor and an evo 200 biomass sensor. All 3 DO sensors were placed directly next to one another at the same height in the vessel. The headplate was also fitted with an exhaust condenser, thermowell, ring sparger (macroparage), harvest dip tube, sampling dip tube, and 2 liquid addition ports (one for media addition and the other for base addition). The vessel was autoclaved with 2 L of phosphate buffered saline (PBS) and the water jacket half filled with water.

Sensor calibration, monitoring, and troubleshooting

Prior to autoclaving the vessel, an ISM gel-filled pH sensor (see Table 1) was connected to the BioFlo 320 control station where it was automatically detected by the control station software. Calibration was performed according to the operating manual using buffers of pH 7 and pH 4 for “zero” and “span,” respectively. Unlike an analog sensor which stores calibration data only in the control software, the calibration data is stored in the ISM pH sensor itself, allowing it to be recalled at any time. In addition, the sensor can be connected to the optional Mettler-Toledo iSense software via the iSense USB adapter. Using this software, a wide range of data is available including the calibration data performed in preparation for this experiment as well as intelligent monitoring of a sensor’s remaining shelf life (sensor “health”). Figure 2 illustrates the “ISM Monitor” screen available in the iSense software. The indicators on this and other screens within use green, yellow, or red icons to show sensor status.

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Table 1: BioFlo 320 hardware configuration and setpoints

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Table 2: Reagents used in this study
Results and Discussion

As seen in Figure 3, both the 3-Gas and 4-Gas automatic DO control algorithms allowed the culture to reach similarly high viable cell densities. The 4-Gas experiment reached its peak cell density ($8.89 \times 10^6$ cells/mL) sooner than the 3-Gas run ($9.54 \times 10^6$ cells/mL). In addition, Figure 4 shows that glucose consumption and lactate and ammonia accumulation were comparable between the two cultures. Consistent with the cell density trend, the 3-Gas culture consumed glucose slightly slower than the 4-Gas culture. When the glucose was exhausted, the cell growth and viability began to drop. Higher peak densities would have been possible if glucose and other necessary nutrients had been supplemented using a fed-batch protocol.

The two gassing control algorithms produced comparably healthy cultures, and showed some notable gas consumption differences. The 4-Gas culture consumed more gas overall, as illustrated in Figure 4D. Since the 3-Gas algorithm does not utilize N₂ for DO control, there is a possibility for the DO to climb above setpoint at the beginning and end of the run when O₂ demand is low. Using 4-Gas control, N₂ is available to keep DO at setpoint, which may be beneficial for some sensitive cell types, and for anaerobic cultures. Whether a culture will be healthier with 3-Gas or 4-Gas automatic gassing control will have to be determined empirically for each cell strain.

The evo 200 capacitance biomass sensor was a valuable in-line measure of cell growth during the runs. Figure 5 shows a comparison between the offline viable cell density measurement and the in-line evo 200 capacitance measurement for one run. After calibrating this sensor for a particular cell line and specific culture process, it can be used in place of sampling the bioreactor which would avoid lost volume and reduce the risk of contamination.

Three DO sensors were incorporated into these experiments. The two ISM sensors were automatically detected by the control station, and including the traditional polarographic sensor, all three were able to accurately track and trend DO levels throughout the run. Figure 6 illustrates an example of the DO sensor trends for the 3-Gas experiment. No significant differences were seen between DO measurement by the three sensors.
Figure 4: Comparing (A) glucose, (B) lactate, (C) ammonia concentrations, and (D) the total gas consumption between 3-Gas and 4-Gas during the bioreactor runs.

Figure 5: Comparing viable cell concentration between an offline cell count and readings taken from the evo 200 biomass sensor at each sample point.
Conclusion

With the intelligent upgrades to the BioFlo 320 software and the utilization of intelligent pH/DO sensors, the BioFlo 320 provides advanced process control for CHO cell culture. This method provided similar results using either the 3-Gas or 4-Gas automatic gassing cascades. The setup can be used to meet a host of culture requirements and the upfront knowledge of an ISM sensor’s “health” dramatically reduces operational risk due to potential sensor failure during a cell culture run. In these experiments, the ability to customize the configuration by adding an evo 200 biomass sensor and multiple ISM DO sensors elevated these runs to “intelligent” CHO cell culture. With the addition of an in-line bioanalyzer, sampling of the bioreactor could be eliminated to reduce the risk of sampling-associated contamination, making the BioFlo 320 a superior setup for cell culture and an intelligent choice for bioprocess laboratories worldwide.

Figure 6: This trend was generated by BioCommand® Batch Control which was used to collect data from all control loops through the runs. In this example, the three DO sensors (red, blue, and pink), sparge air (green) and sparge O₂ (maroon) trends are shown during the 3-Gas run. Note that the three DO trends are superimposed on one another.
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www.eppendorf.com
Perfusion CHO Cell Culture in a BioBLU® 5p Single-Use Packed-Bed Vessel

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Abstract
The market for humanized monoclonal antibodies (hmAbs), has become a multi-billion dollar industry with the expectation of continued growth. One of the most cost-effective methods for the production of secreted proteins is the packed-bed vessel operated under perfusion conditions. The maximum cell density achieved in a packed-bed vessel is typically much higher than suspension cell culture or microcarrier-based adherent cell culture. The protein harvest can be carried out continuously, providing unparalleled product yield. This poster provides an example of using a BioBLU 5p packed-bed single-use vessel to conduct Chinese hamster ovary cell (CHO) perfusion culture producing a secreted hmAb.

The BioBLU 5p vessel (pre-loaded with Fibra-Cel® disks) was controlled by a New Brunswick™ CelliGen® BLU benchtop bioreactor. The BioBLU 5p vessel was inoculated at an initial cell density of 0.3 x 10⁶ cells/mL. Fourteen days of perfusion cell culture were conducted with a working volume of 3.75 L. Glucose, lactate, and hmAb concentrations were monitored daily. The glucose consumption rate was used to estimate the cell density in the packed-bed vessel. After 12 days, the culture reached a peak cell density of approximately 10 x 10⁶ cells/mL.

Introduction
The New Brunswick CelliGen BLU benchtop bioreactor is a versatile, easy-to-use system with built-in controls and monitoring for agitation, temperature, pH, dissolved oxygen (DO), gassing (with air, oxygen, nitrogen and carbon dioxide), and automatic pump control. In addition, the control station can be connected to many other auxiliary devices. The New Brunswick CelliGen BLU benchtop bioreactor is used in conjunction with BioBLU Single-Use Vessels (Eppendorf) allowing for easy scalability while operating in single-use format. Although the single-use bioreactor market has experienced rapid growth in recent years, packed-bed perfusion bioreactor technology has remained predominantly in the traditional glass and stainless steel formats. The single-use packed-bed vessel BioBLU 5p contains Fibra-Cel® which is a solid support growth matrix that is predominantly used for the production of secreted products from cell culture. Since the cells are attached to the Fibra-Cel, it allows for continuous harvest of secreted products without losing cells over an extended period of time. This makes BioBLU 5p Single-Use Vessels an ideal platform for research and production of secreted proteins or virus from mammalian and insect cell culture.

CHO is a robust cell line that can be cultured to very high cell densities in a packed-bed bioreactor. Using CHO cells to produce recombinant proteins allows proper protein folding and correct post-translational modifications so that the proteins remain biologically active once injected into humans. The cell line has a proven track record in the biopharmaceutical industry [1]. The global market for monoclonal antibodies (mAbs) is expected to reach US $58 billion in 2016 with a variety of new mAbs in the pipeline [2]. In this experiment, an attachment CHO cell line expressing a hmAb was grown using a New Brunswick CelliGen BLU benchtop bioreactor with a BioBLU 5p single-use packed-bed vessel.
Materials and Methods

The B13-24 CHO cell line (ATCC®, CRL-11397™) was adapted to CD CHO media (Life Technologies®, 10743) supplemented with 8 mM L-glutamine (Life Technologies, 25030), 0.125 % heat-inactivated fetal bovine serum (Life Technologies, 10438-034) and 1X penicillin/streptomycin (Life Technologies, 15140-122). The initial culture was conducted on BioCoat™ collagen-coated T-flasks (Corning®, 354485). Cells were inoculated into the BioBLU 5p single-use packed-bed vessel at 0.3 x 10⁶ cells/mL to a total working volume of 3.75 L with the previously described media.

The hardware setup and control loop setpoints used in this study are shown in Table 1. Fresh media was perfused into the vessel as needed to keep the glucose concentration between 1 and 2 g/L. Additional D-(+)-glucose (Sigma-Aldrich®, G5146) was added to the perfusion media as needed to keep the glucose concentration at the desired level without increasing the perfusion rate to an unmanageable level.

The culture’s pH was controlled using automatic CO₂ sparging for acid addition and an automatic pump cascade of 1 M sodium bicarbonate (Fisher Scientific®, S631-3) for base addition. Since the cells were attached to the Fibra-Cel packed-bed, bubbles do not interact with the cells which prevents bubble shear. The layer of medium above the packed-bed allows the dilution and mixing of acid or base before allowing them to come in contact with the cells; therefore higher concentrations of acid or base can be used for pH adjustments without adverse effects. Glucose, lactate, and hmAb concentrations were monitored using a Cedex® Bio Analyzer (Roche®).

The approximate amount of glucose consumption per liter per day was calculated by first calculating the average glucose consumption between samples per hour:

\[
R = \frac{V(S_1 - S_2) + \Delta V(P_G - \frac{S_1 + S_2}{2})}{\Delta T}
\]

(g/h)

> \( S_1 \) = Glucose concentration in media sample 1 (g/L)
> \( S_2 \) = Glucose concentration in media sample 2 (g/L)
> \( V \) = Vessel working volume (L)
> \( P_G \) = Glucose concentration of fresh perfusate (g/L)
> \( \Delta V \) = Perfusion volume between samples (L)
> \( \Delta T \) = Change in time between samples (h)

\( R \) was used to calculate the grams of glucose consumption per day by adding the glucose consumed per hour over the 24 hour period. This was then divided by the working volume (3.75 L) to obtain the normalized glucose consumption (g/L/day).

The approximate cell concentration was determined by correlating \( R \) with cell growth to obtain a glucose consumption per cell conversion factor. To obtain the conversion factor, CHO cells were cultured in a T-75 flask until 100 % confluence. A precise amount of fresh medium (8 mL) was then added to the flask, the glucose concentration was measured and the cells were incubated for 7.25 h. After the incubation period, the glucose concentration was measured again, the cells were trypsinized from the T-flask and counted on a Vi-Cell® XR automated cell counter (Beckman Coulter®). This information was used to calculate the amount of glucose each cell consumed per hour (~3.92 x 10⁻² ng/cell/h) which was then used to calculate the number of cells in the bioreactor based on the glucose consumption rate. Please note that the conversion factor may be cell line-dependent and may not be applicable to other CHO cells.

Table 1: Parameters and setpoints used for CHO cell growth in the CelliGen BLU bioreactor
Results and Discussion

Continuous perfusion was used during this experiment to keep glucose levels within a narrow range (Figure 1). The alternative method of fed-batch style non-continuous perfusion can cause large fluctuations in glucose and lactate concentrations which may have an effect on cellular metabolism. Glucose and lactate concentrations were measured multiple times per day during the run. The data were used to adjust the perfusion rate as well as glucose addition rate to keep the glucose level between 1 and 2 g/L where possible.

The cells were attached to the Fibra-Cel and could not be counted directly. Assuming that glucose consumption is proportional to cell growth, glucose consumption was used to calculate the approximate cell number. At the start of the run, glucose consumption steadily increased until day 6 where it began to plateau (Figure 2). Using the conversion factor described above, approximate cell concentrations were determined throughout the run (Figure 3).

Samples were taken throughout the bioreactor run and the IgG concentrations were determined by Cedex Bio Analyzer (Figure 4). These concentrations were measured from samples taken from the vessel and do not include IgG harvested during perfusion.

The cell line used was the only healthy CHO cell line available from ATCC expressing an hmAb. Although this cell line is useful as a model system, the antibody yield is very low. Given a different cell line, much higher cell numbers and antibody production yields are possible.

Conclusion

The New Brunswick CelliGen BLU benchtop bioreactor with BioBLU 5p single-use packed-bed vessel provided precise control and good cell growth throughout the culture period. This combination presents an excellent package for those seeking to produce hmAbs using an attachment CHO cell line. The ability for continuous harvest of secreted products over an extended period of time while maintaining optimal control of cell growth provides great prospects for the antibody market. The cell line and experiments shown in this poster were not optimized and should only be used as an example of the product’s capabilities.
### Ordering information

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<tr>
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<td>Pack of 4 vessels</td>
<td>M1363-0120</td>
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<td>M1363-0133</td>
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<td>Pack of 4 vessels</td>
<td>M1363-0134</td>
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<td>BioBLU™ Packed-Bed Vessel Kit, includes heat blanket, RTD, DO probe, optical pH transmitter, needle-free syringes</td>
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### References


Abstract

In the following application note, the pitched-blade impeller, the spin filter impeller and the packed-bed basket impeller are discussed, highlighting the uses and advantages for each type. Then examples of actual CHO cell cultures are given for each impeller type; showing the perfusion capability when using the spin filter or packed-bed basket impeller and the resulting higher cell densities over the pitched-blade impeller.

Introduction

In the world of bioprocess, there are many tools and methods that can be used to culture mammalian cells, each with their own strengths, weaknesses and purposes. One of the most critical decisions that is made before a bioprocess system purchase is which impeller type is ideal for a particular cell culture. In this application note, three impeller types were compared using CHO cell culture: The pitched-blade impeller, the spin filter with marine impeller and the packed-bed basket impeller.

The pitched-blade impeller has three flat blades set at approximately a 45° angle which produces both axial and radial flow (figure 1). Right handed or left handed blades are options that can be considered depending on which direction you would like your axial flow. Pitched-blade impellers are low shear impellers, designed to gently mix both suspension cells and cells attached to a microcarrier. Typically, these impellers are used for mammalian, insect or other shear-sensitive cell lines, but have also been used in highly viscous fermentation cultures with bacteria and fungi, as well as some biofuel processes. When using a pitched-blade impeller, a culture is typically grown in a batch-style run (no media is added or removed) or fed-batch-style run (a culture is started at a lower working volume and more media is added later during the run). A perfusion-style run (fresh media is continuously added and old media is removed) is possible, however, unless a filtering device is attached with this system to prevent the cells from being removed, cells will be depleted with the harvested (“waste”) media.

A spin filter is a cylinder-shaped cage that spins with the impeller shaft and is covered with a screen designed to prevent cells from being collected with the waste media. Typically, underneath the spin filter, a marine impeller is attached to the impeller shaft (figure 1). When attached to the vessel, media is added so it covers the spin filter almost to its top, with a specially designed harvest tube that can reach the media inside the spin filter. When used, this device can keep cells in the vessel while old media is perfused out from inside of the spin filter. The spin filter is offered with two screen sizes, 10 µm openings for suspension cultures and 75 µm openings for microcarrier cultures. The marine impeller attached underneath the spin filter provides gentle
mixing but, due to its unidirectional flow, its use usually results in a lower $k_La$ than use of the pitched-blade. The spin filter is perfect for cultures that secrete proteins or compounds of interest since the desired product can be collected with the media while the cells are left to continue to produce. This also helps with downstream processing as cells will not have to be removed with centrifugation or filtration. It should be noted that at very high density cultures the spin filter may eventually get clogged with cell debris and require cleaning, which can limit run time.

The packed-bed basket impeller, combined with Fibra-Cel disks, is a system perfect for manufacturing high-yield secreted products from both attachment and suspension cultures with perfusion (figure 2). Fibra-Cel is a solid supported fiber-mesh matrix used predominantly for secreted products with perfusion. Fibra-Cel allows for long-term, high-density cultures without the risk of clogging. Fibra-Cel can be used for both anchorage-dependent cultures and suspension cultures due to its electrostatically-treated material and woven nature that traps the cells in a single step within 15 to 60 minutes (no need to stop agitation). The basket consists of two horizontally positioned, perforated metal screens that isolate a section in the interior of the vessel that is filled with Fibra-Cel. The impeller consists of a hollow tube (draft tube) with three smaller discharge tubes radiating from the top. When media is filled over the three tubes at the top of the impeller and it is spun, the centrifugal force exerted on the media forces out the liquid, causing a gentle suction at the bottom of the impeller, which brings media from the bottom of the vessel to the top. The media then gently flows through the Fibra-Cel packed-bed from the top to the bottom. Gases are sparged into the vessel through the central draft tube; this method oxygenates the media but prevents bubbles from interacting with the cells growing inside the Fibra-Cel packed-bed, thus, preventing bubble shear.

Eppendorf also offers other impellers for various bioprocess needs. Some impellers offered but not explored in this application note include the Rushton-type impellers; which are ideal for fermentation cultures with bacteria, yeast and fungi that require higher dissolved oxygen level (oxygen transfer rate) but are not sensitive to mechanical shearing damage; and the cell-lift impeller; which is an ultra-low-shear impeller that provides uniform circulation for microcarrier cultures and a bubble free environment for the cells.

* The New Brunswick CelliGen 310 bioprocess control station has been discontinued. Its successor is the BioFlo 320 bioprocess control station.
Materials and Methods

All process runs were controlled using a CelliGen 310 bioprocess control station (figure 3). The cell line, medium, and material used are listed in table 1.

<table>
<thead>
<tr>
<th>Table 1: Materials, media and cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Material</td>
</tr>
<tr>
<td>CelliGen 310 control station</td>
</tr>
<tr>
<td>4 TMFC (0 - 1 SLPM)</td>
</tr>
<tr>
<td>2.5 L water jacketed vessel (with motor)</td>
</tr>
<tr>
<td>2.5 L pH/DO sensor kit (with cables)</td>
</tr>
<tr>
<td>2.5 L pitched-blade impeller kit</td>
</tr>
<tr>
<td>2.5 L spin filter impeller kit (10 µm)</td>
</tr>
<tr>
<td>2.5 L basket impeller kit</td>
</tr>
<tr>
<td>YSI 2700 Select™ analyzer</td>
</tr>
<tr>
<td>Vi-CELL® XR</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Media and cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibra-Cel disks</td>
</tr>
<tr>
<td>Freestyle® CHO-S</td>
</tr>
<tr>
<td>CD CHO media</td>
</tr>
<tr>
<td>L-glutamine</td>
</tr>
<tr>
<td>Penicillin/streptomycin 100x</td>
</tr>
<tr>
<td>D-(+)-glucose</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
</tr>
</tbody>
</table>

Bioreactor conditions

During all three of the following CHO bioprocess examples, a CelliGen 310 bioreactor with four 0-1 standard liters per minute (SLPM) thermal mass flow controllers (TMFC) were used. A TMFC is a device that monitors specific gas flow and is used by the cabinet to automatically control the gases flowing into the vessel. The vessel was a 2.5 L glass, water-jacketed vessel with a magnetic drive motor. The water jacket provides uniform temperature distribution with gentle heating and cooling for the culture while the magnetic drive motor provides a sterile vessel environment. All three culture types utilized 3 gas mixing (air, O2, and CO2) for DO and pH control with a base addition (pump 2, 0.3 M sodium bicarbonate solution). Table 2 shows all of the settings for each loop used during all three runs. Both the DO and pH were controlled using the cascade parameters seen in tables 3 and 4.

Cells were grown in CD CHO media supplemented with 8 mM of L-glutamine and 1 % penicillin/streptomycin and kept at a total working volume of ~1.6 L. Each vessel was inoculated at identical densities of ~0.3 x 10^6 cells/mL. Glucose was added to the perfusion media as needed. Cell counts were performed on the pitched-blade and spin filter reactors using a Vi-CELL analyzer. A YSI 2700 Biochemical Analyzer was used to determine glucose and lactate concentrations for all three reactors.

Table 2: Loop settings

<table>
<thead>
<tr>
<th>Loop</th>
<th>Setpoint</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agitation</td>
<td>See each example</td>
</tr>
<tr>
<td>Temperature</td>
<td>37 °C</td>
</tr>
<tr>
<td>pH-1</td>
<td>7.20 (deadband 0.05)</td>
</tr>
<tr>
<td>pH-2</td>
<td>Off</td>
</tr>
<tr>
<td>DO-1</td>
<td>50</td>
</tr>
<tr>
<td>DO-2</td>
<td>Off</td>
</tr>
<tr>
<td>Air</td>
<td>Auto</td>
</tr>
<tr>
<td>O2</td>
<td>Auto</td>
</tr>
<tr>
<td>Gas flow</td>
<td>Off</td>
</tr>
<tr>
<td>CO2</td>
<td>Auto</td>
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</table>

Table 3: DO-1 cascade

<table>
<thead>
<tr>
<th>Start setpoint</th>
<th>@ DO start output %</th>
<th>End setpoint</th>
<th>@ DO end output %</th>
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<tbody>
<tr>
<td>Air</td>
<td>0.0</td>
<td>0.0</td>
<td>0.5</td>
</tr>
<tr>
<td>O2</td>
<td>0.0</td>
<td>10</td>
<td>1</td>
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</table>

Table 4: pH-1 cascade

<table>
<thead>
<tr>
<th>Start setpoint</th>
<th>@ pH start output %</th>
<th>End setpoint</th>
<th>@ pH end output %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pump 2</td>
<td>0.0</td>
<td>0.0</td>
<td>0.3</td>
</tr>
<tr>
<td>CO2</td>
<td>0.0</td>
<td>0.0</td>
<td>0.3</td>
</tr>
</tbody>
</table>
Culture with pitched-blade impeller
The bioprocess with the pitched-blade impeller was run at an agitation speed of 80 rpm. It was cultured as a batch-style process so no media was added or removed throughout the process run. As you can see from figure 4, viable cell concentration continued to rise until all of the glucose was consumed from the media at which point the cell viability began to drop. Lactate levels increased until the drop in glucose concentrations caused a shift in cellular metabolism which caused the cells to consume lactate.

![Figure 4](image)

**Fig. 4:** Viable cell concentration and glucose and lactate concentrations in the culture with pitched-blade impeller. Viable cell concentration begins to decrease when all the glucose is consumed in the vessel due to it being a batch-style run.

Culture with spin filter
The bioprocess with the spin filter was run at an agitation speed of 100 rpm with a 10 µm filter screen. With the spin filter, the culture was run using continuous perfusion. One of the CelliGen 310 cabinet pumps was calibrated and run at varying rates of input as needed to maintain a glucose level above 1 g/L and to keep waste metabolites low. Another pump was cascaded to a level sensor so media was automatically removed from the vessel anytime it reached a volume over 1.6 L. Since all of the cells were trapped in the Fibra-Cel disks and could not be counted using standard methods, the cell number was determined using the amount of glucose consumption. Due to glucose levels being too high during the run, the cells transitioned from a log phase to stationary phase resulting in a plateau in cell growth, as seen in Figure 6. Higher cell numbers were expected.

![Figure 5](image)

**Figure 5:** Viable cell concentration and glucose and lactate concentrations in the culture with spin filter. Perfusion prevented glucose from being totally consumed from the vessel and lactate levels from getting too high.

![Figure 6](image)

**Figure 6:** Calculated viable cell concentration as well as glucose and lactate concentrations in the culture with the packed-bed basket impeller. Perfusion prevented glucose from being totally consumed from the vessel and lactate levels from getting too high.

Culture with packed-bed basket impeller
The packed-bed basket impeller was run at an agitation speed of 100 rpm and the basket was filled with 70 g of Fibra-Cel disks. This culture, like the spin filter, was run using continuous perfusion using the same methods as described above, except that media was removed from a normal harvest tube, not from inside of the basket. Since all of the cells were trapped in the Fibra-Cel disks and could not be counted using standard methods, the cell number was determined using the amount of glucose consumption. Due to glucose levels being too high during the run, the cells transitioned from a log phase to stationary phase resulting in a plateau in cell growth, as seen in Figure 6. Higher cell numbers were expected.
Discussion

Each impeller and cell culture method results in a different growth pattern and it is necessary to determine what is best for the desired process. When comparing the viable cell growth curves for each of the impellers (Figure 7), it can be seen that each results in a different cell concentration and rate of growth. More importantly, as discussed earlier, some of the impellers/methods allow for perfusion (packed-bed basket and spin filter) resulting in higher and possibly continually sustainable cultures. The pitched-blade impeller provided a simple way to grow a low-density culture, but it is not possible to grow the culture to a higher density without extra cell separation equipment to allow for perfusion. The spin filter resulted in almost 4-fold the number of cells as the pitched-blade impeller due to its ability to run in perfusion mode. The perfusion process usually does not last as long as the Fibra-Cel basket due to the tendency of clogging at very high cell densities. However, the cost of the spin filter is much less than that of the Fibra-Cel basket. It is reusable and it does not rely on consumable Fibra-Cel disks. The packed-bed basket impeller resulted in 8-fold the number of cells as the pitched-blade impeller and over 2-fold the spin filter. The packed-bed impeller culture also grew faster than the spin filter culture which was most likely due to the lack of direct physical agitation and bubble shear on the cells while they are trapped in the Fibra-Cel disks. Table 5 shows a general list of the advantages for each impeller type. Every cell line is different and what will work best for each culture and purpose can vary.

Table 5: Advantages for each impeller type

<table>
<thead>
<tr>
<th>Impeller</th>
<th>Advantages</th>
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<tr>
<td>Pitched-blade impeller</td>
<td>&gt; Axial and radial flow&lt;br&gt; &gt; Simple design&lt;br&gt; &gt; Suspension or microcarrier attached cultures</td>
</tr>
<tr>
<td>Spin filter impeller</td>
<td>&gt; Easy to use with perfusion&lt;br&gt; &gt; Capable of higher cell densities</td>
</tr>
<tr>
<td>Basket impeller</td>
<td>&gt; Higher cell densities without the risk of clogging&lt;br&gt; &gt; Gentler environment for cells</td>
</tr>
</tbody>
</table>

Figure 7: A comparison of viable CHO cell concentration for all three impeller experiments. The packed-bed basket impeller provided long term, high-density cell growth. The spin filter also provided high density cell growth compared to the pitched-blade impeller. Since the pitched-blade impeller was run as a batch-style reactor, a lower viable cell density was reached which eventually drops due to all the glucose being consumed in the vessel.

Literature

# Ordering Information

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<td>Pitched-blade impeller kit, for CelliGen® 310</td>
<td>Contact us</td>
</tr>
<tr>
<td>Spin-filter impeller kit, for CelliGen® 310</td>
<td>Contact us</td>
</tr>
<tr>
<td>Basket impeller kit, for CelliGen® 310</td>
<td>Contact us</td>
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<td><strong>BioFlo® 320</strong></td>
<td>1379963011</td>
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<td>Base control station: All configured units include the same base control station.</td>
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* The New Brunswick CelliGen 310 bioprocess control station has been discontinued. Its successor is the BioFlo 320 bioprocess control station.

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eppendorf@eppendorf.com

www.eppendorf.com
Abstract

The study presents a typical protocol for the setup and operation of the Eppendorf New Brunswick CelliGen BLU single-use, stirred-tank bioreactor, a versatile new benchtop system for the culture of a wide range of mammalian cells. This bioreactor has been designed to provide research and production facilities with a single-use vessel which combines the benefits of both traditional stirred-tank design and single-use technology, capable of seamless process scale-up. The system can be operated in batch, fed-batch or continuous modes. A procedure for culturing Chinese Hamster Ovarian (CHO) cells in a 5.0 L vessel, using CD CHO serum-free medium in a batch culture is described.

Introduction

Historically, stirred-tank fermentors and bioreactors have been the trusted design for culturing all types of submerged cultures including suspension and anchorage-dependent mammalian cells, insect, yeast, plant and microbial cultures. The tried and tested tank design offers scalability and proven reproducibility which is pivotal for cost-saving process development and productivity. In the last decade, there has been an increasing acceptance and use of single-use technologies, due to their convenient operation and low start-up cost. Single-use systems eliminate the need for cleaning and sterilization, reduce validation requirements, provide rapid turn-around between runs, and significantly reduce the risk of cross contamination and microbial contamination because the culture vessel is only used once and then discarded. Although single-use, stirred-tank systems in the 75 – 2000 L scale have been on the market for some time, as have small-scale single-use bags that are gently rocked rather than stirred, until now there has been no single-use stirred-tank system for small-scale work. The new Eppendorf New Brunswick CelliGen BLU fills that void, offering a proven stirred-tank design as well as the benefits of single-use technology in a benchtop system.

Materials and Methods

Single-Use Vessels

BioBLU® single-use vessels are offered in 5.0, 14.0 and 50.0 L total volume capacities. The vessels are delivered preassembled with pitched-blade impeller, porous microsparge, and all the necessary tubing, filters, and connectors; and come sterilized, ready for use right out of the package. All components in product contact are made of materials that meet USP Class VI standards and have been tested for leachables and extractables, making these vessels appropriate for cGMP environments. In this protocol, we describe use of a CelliGen BLU with 5.0 L vessel.
Controller
CelliGen BLU’s compact control station is designed to provide advanced process management and monitoring capability, ranging from three fixed-speed pumps for additions and harvesting, to a powerful controller with 15 in. industrial color touchscreen monitor. Multiple options, including gas flow control, a weight scale, validation packages and more, enable customization to your needs.

The control station used in this protocol was configured with one 2 – 100 cubic centimeters per minute (ccm) Thermal Mass Flow Controller (TMFC) for direct sparging of gases and an integrated gas overlay with 0.1 – 3.0 Standard Liters Per Minute (SLPM) flow rate also regulated by a TMFC. Both the gas flow and gas overlay are capable of 4-gas mixing for automatic pH and Dissolved Oxygen (DO) control. Pumps, temperature control, agitation, as well as all of the other process loops, were controlled and monitored through the powerful Reactor Process Controller (RPC) firmware installed in the controller. DO was monitored using a noninvasive reusable polarographic DO probe; and pH was monitored using a non-invasive optical pH probe and fluorescence sensor.

Inoculum Preparation
One 2.5 mL vial of CHO cells was thawed and used to inoculate a 125 mL shake flask which contained 25 mL of serum-free CD CHO medium (Life Technologies® 10743-029) which was pre-warmed to 37 °C.

On day 4, when the viable cell density reached 1.5 x 10⁶ cells/mL, the cells were transferred into a 500 mL shake flask which contained 100 mL of freshly made, pre-warmed medium and allowed to incubate for 3 additional days at the same conditions as earlier. The cells were then transferred to two 1 L shake flasks, each containing 250 mL of the freshly made medium. The inoculum was grown in the shake flasks until cell density reached 2.0 – 3.0 x 10⁵ cells/mL, with greater than 90 % cell viability, sufficient for the bioreactor inoculation.

Bioreactor Set-Up and Inoculation
One day before the cells reached inoculation density, the growth medium was warmed to 37 °C and the DO probe was polarized. For this study, 3.0 L of sterile CD CHO serum-free medium was prepared by pre-warming at 37 °C for 24 hours in a CO₂ incubator. During this time, the DO probe was connected to the controller for at least 6 hours to enable polarization, as per the manufacturer’s recommendation. Once the medium was warmed and the inoculum grown to sufficient starting density, the CelliGen BLU bioreactor vessel was removed from its sterile packaging and the heat blanket supplied with the unit was wrapped around the outside of the vessel. Next, the vessel containing the cell culture medium was connected to one of the bioreactor vessel’s inlet lines using a tube welder. (A tube welder is offered as an optional accessory to the CelliGen BLU. A pre-sterilized medium filter with an attached quick connect or Luer connection can also be used if a tube welder is not available). Since this was a batch process, all of the medium was pumped into the bioreactor vessel. All additional connections to the controller including sparge, overlay, RTD, pH, and agitation were also made.

pH and DO were calibrated through the sparge overlay controller, and all process setpoints were entered on the touchscreen using the Control Setpoint values shown on the next page. Once the parameters were at their setpoints, the inoculum flasks were connected to the addition line in a sterile manner using a tube welder and contents were pumped into the bioreactor vessel.

Operational Parameters
Cultivation of animal cells in an environment optimal for manufacture of desired end products require monitoring and control of a substantial number of physical and chemical parameters. Physical parameters include temperature, fluid flow (gas flow and liquid flow) rates and agitation rates. Chemical parameters include the dissolved oxygen (DO) concentration and pH.

Control Setpoints

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>37 °C</td>
</tr>
<tr>
<td>pH</td>
<td>7.0</td>
</tr>
<tr>
<td>DO</td>
<td>40 %</td>
</tr>
<tr>
<td>Agitation</td>
<td>80 rpm</td>
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</tbody>
</table>

pH Control Parameters
pH control was set to Auto mode, which automatically adds base solution or CO₂ gas to the system based on culture demands.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dead-band</td>
<td>0.10</td>
</tr>
<tr>
<td>PID values</td>
<td>Factory set default values</td>
</tr>
<tr>
<td>Base</td>
<td>Sodium bicarbonate, 7.5 % solution</td>
</tr>
<tr>
<td>Base Solution Transfer tubing</td>
<td>Narrow bore silicone tubing with Luer-connection (1/18 in. ID &amp; 1/4 in. OD)</td>
</tr>
<tr>
<td>Vessel inlet</td>
<td>1/8 in. inlet tubing in the vessel headplate</td>
</tr>
</tbody>
</table>
Dissolved Oxygen (DO) Control
DO control was set to Auto mode, which automatically regulates gas mixing based on culture demand. PID values: factory set default values.

Gas Control
The gas control was set to 4-gas mode, which automatically maintains DO and pH. The gas flow rate was based on the vessel size.

Up until day 3, gases were introduced into the vessel headspace only through the overlay port at a rate of 0.30 L/min using 4-gas mixing to maintain pH and DO. On day 3, and for the remainder of the run, 5 – 10 ccm of gas were directly sparged into the system using a porous sparger and automatic 4-gas mixing. The overlay gas flow in the vessel headspace was kept at the previous settings.

A built-in sampling device enabled sterile sampling. Daily offline measurements of glucose and lactate concentration were read using a YSI® 2700, and cell density and cell viability was measured using an Automated Cell Counting System (New Brunswick NucleoCounter®).

All data was logged via BioCommand® Batch Control PC-compatible Supervisory Control and Data Acquisition (SCADA) software (New Brunswick).

Results and Discussion
As shown in Figure 1, the CHO cells in this study grew steadily, reaching a maximum viable cell density of 5.55 x 10⁶ cells/mL on day 5.

Cell viability, shown in Figure 2, ranged between 97.1 and 97.9 % through Day 5, until the nutrient source, glucose, was depleted from the medium, as shown in Figure 3.
As expected, lactate production steadily increased as the available glucose in the medium was consumed. As glucose in the medium became exhausted, consumption of lactate as a secondary carbon source also declined[1].

This data presented here, and in Table 1, demonstrates that the CelliGen BLU bioreactor is an easy-to-use, efficient system for the culture of CHO cells. No effort was made to optimize either the medium or the cell culture process control parameters. This study was only intended to document a general guide to bioreactor setup and operation, and present typical results you could expect to achieve with your mammalian cell line. For protocols on other cell lines, or for additional information on the CelliGen BLU, see eppendorf.com.

<table>
<thead>
<tr>
<th>Day</th>
<th>Total [10^6 cells/mL]</th>
<th>Viable [10^6 cells/mL]</th>
<th>Viability [%]</th>
<th>Glucose [g/L]</th>
<th>Lactate [g/L]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.31</td>
<td>0.30</td>
<td>97.9</td>
<td>5.83</td>
<td>0.23</td>
</tr>
<tr>
<td>1</td>
<td>0.69</td>
<td>0.68</td>
<td>97.1</td>
<td>5.14</td>
<td>0.52</td>
</tr>
<tr>
<td>2</td>
<td>1.42</td>
<td>1.39</td>
<td>97.6</td>
<td>4.71</td>
<td>0.87</td>
</tr>
<tr>
<td>3</td>
<td>2.57</td>
<td>2.51</td>
<td>97.6</td>
<td>3.74</td>
<td>1.27</td>
</tr>
<tr>
<td>4</td>
<td>4.02</td>
<td>3.92</td>
<td>97.5</td>
<td>1.47</td>
<td>2.10</td>
</tr>
<tr>
<td>5</td>
<td>5.70</td>
<td>5.55</td>
<td>97.3</td>
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</tr>
<tr>
<td>6</td>
<td>5.98</td>
<td>4.52</td>
<td>76.6</td>
<td>0.00</td>
<td>0.32</td>
</tr>
<tr>
<td>7</td>
<td>6.71</td>
<td>3.21</td>
<td>47.8</td>
<td>0.00</td>
<td>0.01</td>
</tr>
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</table>

Table 1.

References
Abstract

This study illustrates a protocol for the scale up of CHO cells using New Brunswick™ CelliGen BLU stirred-tank bioreactors equipped with 5-Liter (L) and 50-Liter (L) single-use vessels. CelliGen BLU is a versatile benchtop system for the culture of a variety of cell lines. This bioreactor has been designed to provide research and production facilities with single-use vessels that combine the benefits of both traditional stirred-tank design and single-use technology, capable of seamless process scale-up. Eppendorf has recently launched the CelliGen BLU 50 L system to address larger volume batch demands.

Introduction

Historically, stirred-tank bioreactors have been the standard for culturing all types of submerged cultures including suspension and anchorage-dependent mammalian, insect, yeast, plant and microbial cultures. This well-tested vessel design offers scalability and reproducibility, which enhance productivity and provide cost savings in process development. In the last decade, there has been an increasing acceptance of single-use technologies, due to their convenient operation and low start-up cost. Single-use systems eliminate the need for cleaning and sterilization, reduce validation requirements, provide rapid turn-around between runs, and significantly reduce the risk of cross contamination and microbial contamination. Until recently, the CelliGen BLU single-use bioreactor system has been limited to 5 L and 14 L sizes. The new 50 L CelliGen BLU vessel is a direct response to customer feedback, accommodating much larger process volumes while maintaining the benefits of single-use technology in the same proven, rigid-walled, stirred-tank design, all in a benchtop platform. All three vessel sizes have the capability to be operated in batch, fed-batch or perfusion style. This protocol describes a cell culture process using Freestyle™ Chinese Hamster Ovarian (CHO-S) cells (Invitrogen® Corp.) starting from the smaller 5 L vessel and finishing up in the larger 50 L vessel.

Materials and Methods

Single-Use Vessels

CelliGen BLU single-use vessels are now offered in 5.0 L, 14.0 L, and 50.0 L volumes. The vessels are delivered pre-assembled with a pitched-blade impeller. The vessels have either a porous micro-sparge or a macro-sparge element configuration (selected at time of purchase), and also include all the necessary tubing, filters, and connectors. The vessels come sterilized and ready for use right out of the package. All components in contact with cell culture are made from materials that meet USP Class VI standards and have been tested for leachables and extractables, making these vessels appropriate for cGMP environments. In this protocol, we describe the use of 5 L and 50 L CelliGen BLU vessels with pitched blade impellers and the macrosparge element configurations. The 5 L culture was conducted in a batch style while the 50 L culture was completed as a fed-batch.
Inoculum Preparation

For the 5 L bioreactor inoculation, Freestyle Chinese Hamster Ovarian (CHO-S) cells were used to inoculate a 125 mL shake flask which contained 30 mL of serum-free CD CHO medium (Invitrogen) supplemented with 8mM L-glutamine (JRH Biosciences) and 1 % Penicillin-Streptomycin (Invitrogen).

This initial shaker culture was expanded to a one liter shake flask containing 240 mL; the inoculum was then grown until day 4, when the viable cell density reached 3.16 x 10^6 cells/mL with a viability of 99.3 %, a density sufficient for transfer into the 5 L bioreactor.

Bioreactor Set-Up and Inoculation

**Inoculation of 5 L Bioreactor**

The DO probe was connected to the controller for at least 6 hours for polarization.

On inoculation day, the CelliGen BLU bioreactor vessel was removed from its sterile packaging and the heat blanket was wrapped around the outside of the vessel. Next, the vessel containing the cell culture medium was connected to one of the bioreactor vessel’s inlet lines using a quick connect. A Luer connection or tube welder can also be used with the CelliGen BLU. All additional connections to the controller including sparge, overlay, RTD, pH, and agitation were also made.

Approximately 2 L of sterile CD CHO serum-free medium was pumped into the vessel and warmed to 37° C. After the growth medium was stabilized at 37° C, the traditional polarographic probe (DO probe) was calibrated to an electronic zero, and then spanned after the agitation was set at 50 rpm and the airflow was set to 100 % at 1 SLPM for ~20 minutes (may vary depending on how long it takes the raw value to stabilize). The optical pH calibration was performed using the pH probe raw data (located on the vessel and packaging; preconfigured for optical pH using fluorescence sensor technology), and an offline sample was taken to re-zero the medium within the bioreactor (after the fluorescence spot was hydrated ~20 mins). DO and pH were calibrated through the touchscreen controller, and all process setpoints were entered on the touchscreen using the Control Setpoint values represented within the following pages below. Once the parameters were at their setpoints, the inoculum flasks were connected to the addition line in a sterile manner using a quick connect and the cells were pumped into the bioreactor vessel for a total volume of ~2 L with an inoculation density of 0.3 x 10^6 cells/mL.

**Inoculation of 50 L Bioreactor**

Medium warming and DO polarization were conducted in a similar fashion to the preparation prior to the inoculation of the 5 L vessel.

Once culture growth within the 5 L bioreactor had achieved sufficient density (4.84 x 10^6 cell/mL; viability 99.4 %), the 50 L CelliGen BLU vessel was removed from its sterile packaging and the heat blanket was wrapped around the outside of the vessel. Next, the bag containing the cell culture medium was connected to one of the 50 L bioreactor vessel’s inlet lines using a quick connect. Since this portion of the experiment was a fed batch process with starting volume less than 20 L, only the initial 17.8 L of the medium was pumped into the bioreactor vessel. All additional connections to the controller including sparge, overlay, RTD, pH, and agitation were also made. The base pump was also calibrated and primed for use with 20 % Sodium Bicarbonate for pH control.

The polarographic probe on the 50 L vessel was calibrated to an electronic zero once the growth medium was stabilized at 37° C, and then spanned when the agitation was set at 50 rpm and the airflow was set to 100 % at 7.5 SLPM.
(~20 minutes to stabilize the raw value). The optical pH calibration was performed using the pH probe raw data, and an offline sample was taken to re-zero the medium within the bioreactor. DO and pH were calibrated through the touchscreen controller, and all process setpoints were entered on the touchscreen using the Control Setpoint values shown on the next page. Once the parameters were at their setpoints, the 5 L harvest line was connected to the inoculation/addition line on the 50 L in a sterile manner using a quick connect, and then the calculated 1.2 L of high density CHO cells were pumped in for a total volume of 19 L with final starting cell density of 0.3 x 10^6 cells/mL. The 50 L vessel was then fed with an additional 21 liters of pre-warmed Gibco CD CHO serum-free medium on day 5 to support high cell growth and viability.

**Operational Parameters**

Cultivation of animal cells for manufacturing of desired end products requires monitoring and controlling of a substantial number of physical and chemical parameters. Physical parameters include temperature, gas flow rates, fluid flow rate, and agitation speed. Chemical parameters include the dissolved oxygen (DO) concentration and pH.

### 5 L and 50 L Control Setpoints

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>37°C</td>
</tr>
<tr>
<td>pH</td>
<td>7.1</td>
</tr>
<tr>
<td>DO</td>
<td>50%</td>
</tr>
<tr>
<td>Agitation</td>
<td>50 rpm (clockwise)</td>
</tr>
</tbody>
</table>

### 5 L and 50 L pH Control Parameters

Both vessels’ pH control was set to Auto mode, which automatically adds base solution or CO₂ gas to the system based on culture demands. Base addition was utilized for pH control on the 50 L culture due to the higher density expected at end of the 50 L run but was not needed for the 5 L run.

<table>
<thead>
<tr>
<th>Parameter</th>
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<tbody>
<tr>
<td>pH Dead-band</td>
<td>0.05</td>
</tr>
<tr>
<td>PID values</td>
<td>factory set default values</td>
</tr>
<tr>
<td>Base (50 L only)</td>
<td>Sodium bicarbonate, 20 % solution</td>
</tr>
</tbody>
</table>

### 5 L and 50 L Dissolved Oxygen (DO) Control

DO control was set to 3-gas Auto mode, which automatically regulates gas mixing based on culture demand. Factory-set default PID values were used.

### 5 L and 50 L Gas Control

The gas control was set to 3-gas Auto mode for both bioreactors, automatically maintaining DO and pH. For the 5 L bioreactor, the low flow limit set at 0.002 SLPM with a high flow limit set at 1.00 SLPM, and for the 50 L Bioreactor, the low flow limit was set at 0.04 SLPM with a high flow limit set at 7.5 SLPM. In addition, overlay air flow was supplied to both bioreactors at 0.10 SLPM.

Gases were introduced via the macrosparge element for aeration supplementation to maintain DO and pH and into the vessel headspace through the overlay port during the entire run for both the 5 L and the 50 L bioreactors.

A built-in sampling device enabled sterile sampling. Daily off-line measurements of glucose and lactate concentration were read using an YSI® 2700; cell density and cell viability were measured using an Automated Cell Counting System (Vi-CELL®).

### Results and Discussion

All vessel data was logged via BioCommand® Batch Control Supervisory Control and Data Acquisition (SCADA) software (Eppendorf). The bioreactors’ total cell density and viability are shown in Figure 1. CHO cells exhibited steady and consistent growth on both the 5 L and 50 L bioreactors. Cell growth reached a viable cell density of 4.82 x 10^6 cells/mL on day 4 in the 5 L, and 8.58 x 10^6 cells/mL on day 8 in the 50 L vessel. Cell viability was maintained around 99 % for the entire culture duration. As expected, lactate production steadily increased as the available glucose in the medium was consumed (Figure 2).

---

**Figure 1.** CHO Cell growth and viability plots for the scale-up from 5 L to 50 L CelliGen BLU bioreactors in a combined cell culture process of 8 days. The dip of cell count seen on the 50 L graph represents the single feeding event and cell density dilution resulted from 21 L media addition introduced on day 5.
The data presented here demonstrates that the CelliGen BLU bioreactor is an easy-to-use, efficient system for the scale-up of CHO cell culture up to the 50 L vessel size. No efforts were made to optimize either the medium or the cell culture process control parameters.

This study was only intended to document a general procedure for CelliGen BLU bioreactor setup and operation, and present typical results one could expect to achieve with mammalian cell line.

Although it is possible to perform such scale-up using a single CelliGen BLU controller, two separate CelliGen BLU systems were used for this application note. For protocols on other cell lines, or for additional information on the CelliGen BLU, see eppendorf.com.

References


## Ordering Information

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<tr>
<th>Description</th>
<th>International order no.</th>
<th>North America order no.</th>
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<td>CelliGen® BLU Control Station</td>
<td>M1374-120-HSA (120V)</td>
<td>M1374-120-HSA</td>
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<tr>
<td>100 - 120 V, 50/60 Hz, high flow sparge, overlay w/TMFC, and scale</td>
<td>M1374-230-HSA (230V)</td>
<td>M1374-120-LSA</td>
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<tr>
<td>CelliGen® BLU Control Station</td>
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<td>M1363-0121</td>
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<tr>
<td>100 - 120 V, 50/60 Hz, low flow sparge, overlay w/TMFC, and scale</td>
<td>M1374-230-LSA (230V)</td>
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<td>CelliGen® BLU 5L vessel</td>
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<tr>
<td>Single-use 50.0L vessel with macrosparge (pack of 1)</td>
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<td>CelliGen® BLU vessel kit</td>
<td>M1363-0105 (5L kit)</td>
<td>M1363-0105 (5L kit)</td>
</tr>
<tr>
<td>Includes heat blanket, RTD temperature sensor, pH and DO probes, with cables and needle-free syringes</td>
<td>M1363-0114 (14L kit)</td>
<td>M1363-0114 (14L kit)</td>
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<td>M1374-0151 (50L kit 120V)</td>
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<td>M1374-0115 (50L kit 230V)</td>
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<tr>
<td>M1374-0150 (50L kit 230V)</td>
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<tr>
<td>Eppendorf Research® plus, adjustable pipette</td>
<td>3120 000.089</td>
<td>3120000089</td>
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<td>Single channel pipette – 1 -10 mL</td>
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<tr>
<td>Easyet®</td>
<td>4421 000.013</td>
<td>022230204</td>
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<tr>
<td>Pipette dispenser – suitable for pipettes from 0.1 to 100 mL</td>
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</table>
Abstract
The objective of this study was to compare Eppendorf BioBLU single-use packed-bed bioreactor vessel and the traditional glass vessel counterpart used in New Brunswick™ CelliGen® 310. Alkaline phosphatase (ALKP)-secreting Chinese Hamster ovary (CHO) cells were used to measure ALKP production in each bioreactor. Overall, the results from these comparisons suggest that there is no significant difference between the reusable and single-use FibraCel basket systems for bench-scale production of recombinant proteins. Productivity of cells and collection of secreted proteins will not be hindered by the implementation of single-use bioreactor systems.

Introduction
The packed-bed basket technology, developed by Eppendorf, provides a shear free environment for production of animal cells. At present, little information is available on the utility of the Eppendorf BioBLU single-use bioreactor system for the production of secreted proteins, especially in perfusion mode of operation. Thus, this study was conducted to measure the growth and productivity of alkaline phosphatase (ALKP)-secreting Chinese Hamster ovary (CHO) cells were used to measure ALKP production in each bioreactor. Overall, the results from these comparisons suggest that there is no significant difference between the reusable and single-use FibraCel basket systems for bench-scale production of recombinant proteins. Productivity of cells and collection of secreted proteins will not be hindered by the implementation of single-use bioreactor systems.

Culture procedures
In order to evaluate the impact of these bioreactor systems on protein production, we utilized a recombinant alkaline phosphatase-secreting CHO cell line (rCHO), a proprietary cell line provided by CDI Bioscience, Inc. (Madison, WI). The rCHO cells were engineered with the IPTG-regulated RP Shift vector so that the rCHO cells stop replicating and shift to protein production when induced with IPTG. Serum free CD-CHO medium (Gibco®, Life Technologies®, Grand Island, NY) was used throughout these experiments. The media contains 6.3g/L glucose and was supplemented with 8 mM L-glutamine and 100µg/ml of an antibiotic/antimycotic solution (Invitrogen®, Life Technologies). Frozen rCHO cells were thawed and transferred to T-75 flasks with CD-CHO medium and allowed to expand. Once a sufficient number of cells were achieved, sterile disposable spinner flasks were utilized to further expand the cells. Subculture of the cells continued until a sufficient number of viable cells was achieved for use as a seed culture at the density of 5 x 10^5 cells/ml. Two New Brunswick CelliGen 310 advanced bench-top stirred-tank bioreactors were utilized to grow the rCHO cells. One of the New Brunswick CelliGen consoles was connected to an adaptor kit (available from Eppendorf) for use of the Eppendorf BioBLU single-use vessel.
Results and discussion

Glucose utilization and lactate production
Glucose is the main energy source for cell proliferation and ALKP production. Thus, glucose levels were expected to directly correlate with ALKP production in each experiment. Because lactate is a secondary energy source, lactate levels were expected to decline following this initial increase and the utilization of glucose in the media. Lactate metabolism is beneficial to the system by reducing a major metabolic by-product from the system. Glucose levels measured at the time of induction (day 3) were nearly 0 g/L in both experiments (Fig. 1). Media lactate concentrations increased in response to decreasing glucose availability. The use of lactate as a secondary energy source can also be observed as lactate levels decrease at each 2 L perfusion.

Figure 1. Glucose consumption and lactate production by rCHO cells cultured in two packed-bed bioreactor systems. Values shown are the amounts of glucose and lactate measured in the culture media at each media exchange. The time and volume of the media exchange is indicated at each dashed line. Induction of ALKP activity by IPTG began on culture day 5 and continued every two days throughout the remainder of the experiment. Results of two experimental trials are shown (A, reusable; B, single-use).

Table 1: Comparison of perfusion volumes

<table>
<thead>
<tr>
<th>Perfusion</th>
<th>Glass</th>
<th>BioBLU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>0.5 L</td>
<td>1 L</td>
</tr>
<tr>
<td>Day 2</td>
<td>1 L</td>
<td>2 L</td>
</tr>
<tr>
<td>Days 3 - 15*</td>
<td>2 L</td>
<td>4 L</td>
</tr>
</tbody>
</table>

* Perfusion occurred every other day.

Packed-bed basket impeller operated in perfusion mode
Two experimental trials were performed using the packed-bed vessels in perfusion mode: 3 L autoclavable vessel (1.25 - 3.75 L working volume) and a BioBLU 5p single-use vessel (3.75 L working volume, pre-loaded with 150g of Fibra-Cel disks). The perfusion process was initiated once the cells reached the exponential growth phase as shown in table 1. Both experimental trials had the following parameters shown in table 2.

Table 2: Bioreactor parameters (setpoints)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Glass</th>
<th>BioBLU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>37°C (± 0.1°C)</td>
<td>37°C (± 0.1°C)</td>
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<td>DO</td>
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<tr>
<td>pH</td>
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Biomarkers of cell growth and productivity
Cell productivity was assessed by measuring activity of the secreted ALKP protein using an enzyme assay (AnaSpec, Freemont, CA) according to the manufacturer’s protocol. For simplicity unit measurements were used in this study. A unit (U) of ALKP activity was defined as the amount of enzyme that hydrolyzes 1µmol of p-nitrophenylphosphate to p-nitrophenol in a total reaction volume of 1 ml in 1 minute at 37°C. The YSI® 2700 Select Biochemistry Analyzer (YSI, Inc., Yellow Springs, OH) was utilized to monitor the glucose and lactate levels in the culture media every 24 hr for the duration of each trial.
Comparison of bioreactor systems for ALKP production

Figure 2. ALKP production by rCHO cells cultured in two packed-bed bioreactor system. (A) ALKP concentration in culture media measured each day of each experimental trial. IPTG induction of ALKP began on culture day five and continued every two days for the remainder of the experiment. (B) Stacked bar charts show the cumulative production of ALKP throughout the experiment, with each bar representing a perfusion.

The average total ALKP production per experiment trial is shown in Figure 2; overall, there is not a significant difference in ALKP production between the two bioreactor systems. The total amount of ALKP measured after five media exchanges in the reusable vessel was 17.44 U/mL and 16.22 U/mL in the single-use vessel.

In summary, these results demonstrated comparable yields in ALKP production (within the usual biological fluctuations) between the two packed-bed bioreactor systems when operated in perfusion. Given the greater productivity of cells cultured in the packed-bed bioreactor and the multitude of advantages of this system operated in perfusion mode, researchers desiring to scale up mammalian cell culture for protein production should strongly consider utilization of the Eppendorf BioBLU packed-bed, single-use bioreactor system.

Note: This application note study was partially funded by Eppendorf Inc.

References

### Ordering information

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Cultivation of Human CAP® Cells: Evaluation of Scale-Down Capabilities using Single-Use Bioreactors

Claudia M. Hüther-Franken1, Christiane Schlottbom1*, Helmut Kewes2 and Dr. Michael Schomberg2

1 Eppendorf AG Bioprocess Center, Juelich, Germany; 2 Cevec Pharmaceuticals GmbH, Cologne, Germany; * Corresponding author: schlottbom.c@eppendorf.com

Abstract

Increasing process complexity coupled with rising cost pressures and rapidly evolving regulatory requirements makes today’s process development efforts a special challenge. The pressure of achieving faster time-to-market for new and innovative biotechnological products has led to the need to optimize every element of the total development workflow.

The following application note illustrates how the DASbox® Mini Bioreactor System combined with the BioBLU® 0.3c single-use vessels supports bioprocess development in human cell culture. Scale-down capabilities were investigated by comparison of 500 mL cultures in a DASGIP® Parallel Bioreactor System with 170 mL cultures in the DASbox using the BioBLU 0.3c single-use vessel.

Introduction

Initial bioprocess development involves cell line optimization, clone selection, and screening for media, feed components and strategies, and other process conditions. Shake flasks, the most common vessels used in early cell and microbial work, have served the biotechnology industry well over the decades but their limitations for optimizing cell culture or fermentation conditions are well known. Equipment used during screening should mimic the physical and mechanical characteristics of production-scale bioreactors to the highest degree possible in order to assure consistency throughout development phases. Ideally, these best practices will support the aims of QbD: that quality measures initiated during development are carried forward and manifested in product quality. DASGIP Parallel Bioreactor Systems have the potential to address process consistency and harmonization of unit operations between development and production. Today’s state-of-the-art benchtop systems use sensors and information technology to control, monitor, and record critical process parameters such as temperature.

Figure 1: BioBLU® 0.3c Single-Use Bioreactor for cell culture
pH, dissolved oxygen, and agitation. As in production-scale bioreactors, gassing and feeding proceed according to defined settings.

CEVEC® Pharmaceuticals GmbH (Cologne, Germany), a global solution provider focussing on the development of top notch human expression systems with highest ethical standards, has established a master cell bank (MCB) of CAP® cells growing in suspension, tested and certified according to ICH guidelines and European Pharmacopeia. The platform expression technologies CAP and CAP-T are based on specific, amniocyte-derived human cell lines. CAP and CAP-T were designed for stable and transient protein production and achieve highest protein yields with authentic human glycosylation patterns. Simple and reliable protocols allow for the fast generation of customized producer cell lines for pharmaceutically relevant proteins based on the parental permanent CAP cells under controlled and optimized conditions. For the required human cell line screening as well as for media optimization, the small working volumes of 100 – 250 mL make the extendable 4-fold DASbox and the BioBLU 0.3c single-use vessel a perfect fit. Bioprocesses are controlled as precise and effectively as they are in larger scale bioreactors while cell material, media and supplements as well as lab space are saved.

Several experiments were carried out aiming at verifying the scale-down capabilities from the DASGIP Parallel Bioreactor System, which CEVEC generally uses in process development, to the Mini Bioreactor System DASbox. To overcome the risk of cross-contamination and to reduce time for cleaning, sterilization and assembly they evaluated the novel developed BioBLU 0.3c single-use vessel. Which comes with a magnetic coupled stirrer and pitched blade impeller and holds several short and long dip-tubes as well as two standard PG13.5 ports facilitating full industry standard instrumentation. A specifically designed port including a gas permeable membrane allows for DO measurement using a reusable probe which can be plugged easily in directly on the bench. Recuperation of liquid from exhaust gas is carried out via a novel liquid-free operated condenser.

Materials and Methods

To evaluate the scale-down capability of the new DASbox Mini Bioreactor System and the usability of the BioBLU 0.3c single-use vessel experimental series with two different systems were carried out and compared. A 4-fold Parallel Bioreactor System for cell culture was used in 500 mL scale experiments (PBS). The corresponding small-scale approaches were carried out in a (parallel) DASbox system using single-use vessels with 170 mL (DASbox SU).

The recombinant human CAP cells producing a pharmaceutically relevant protein were batch cultivated for 7 d (170 h) in CEVEC’s serum-free, chemically defined CAP medium supplemented with 40 mM glucose and 6 mM glutamine at 37 °C. Initial viable cell density was 3*10^5 cells/mL. The DO set-point of 40 % was maintained by a constant stirrer speed and the oxygen concentration in the inlet gas. Stirrer speed was adjusted to 160 rpm (PBS) and 150 rpm (DASbox SU). The pH value was regulated to 7.1 by addition of 1 M Na2CO3 (feeding, speed rate regulated) and CO2 (submerged gassing). Inlet gas (air, O2, CO2 and N2) was mixed continuously mass flow-controlled. The bioreactors were equipped with pitched blade impellers and liquid-free operated exhaust gas condensers. The pre-cultures were cultivated in 125 mL Erlenmeyer flasks (Corning) with 25 mL working volume using a shaker incubator (37 °C, 5 % CO2) agitating at 185 rpm (Multitron 2, Infors AG). The cells were expanded up to a viable cell density of 3*10^6 cells/mL in the same medium used for bioreactor runs.

Figure 2: Viable Cell numbers of all experiments with DASGIP Parallel Bioreactor Systems (PBS) and BioBLU 0.3c vessels with average growth rate of 0.02 h⁻¹.

Figure 3: Comparism of metabolic activity by glucose consumption.
The critical process parameters were monitored, controlled and visualized online while additionally offline parameters were added manually for collective analysis and storage in a joint database. Daily samples were taken in place. Viable cell numbers, the concentrations of glucose as well as the target protein were determined via semi-automated trypan blue cell counting (Cedex XS, Roche Innovatis), an automated glucose biosensor (YSI 7100 MBS, YSI Life Sciences) and ELISA, respectively.

Results and Discussion

The highly comparable results shown in figures 2 and 3 prove the reliability of the process control in both independent experimental series.

The viable cell density increases exponentially within all cultivation studies in a reproducible manner with an average growth rate of 0.02 h⁻¹. The corresponding anti-cyclic glucose consumption thereby illustrates the similar metabolism of the different cultures. Cell viabilities ranged in between 90 – 95 % for each sample. As shown in figure 4 the final product yield reached 80 – 121 % in respect to the average protein concentration gained with the Parallel Bioreactor System (PBS) commonly used at CEVEC. No differences in cell growth, metabolic activity and protein expression could be observed using the BioBLU 0.3 c single-use vessels. The results show the successful scale-down from a 500 mL (PBS) to 170 mL (DASbox SU) bioreactor working volume.

Conclusion

Summarized, the presented results give direct evidence to the scale-down capability of the DASbox Mini Bioreactor System used with single-use vessels. This proves the DASbox to be a superior tool for process development with human cell cultures. The small working volumes save material and consumable costs while utilizing single-use vessels drastically reduce turnover-times and thereby labour costs and development times.
## Ordering information

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Abstract
Heterologous expression of membrane proteins remains a bottleneck for structural characterization by x-ray crystallography. Such proteins represent approximately 30% of the proteome and are not sufficiently represented in the Protein Data Bank (PDB)[1]. G-protein-coupled receptors (GPCRs) are an area of particular interest as it is estimated that one third of current FDA-approved drugs act through this class of receptors.

Introduction
We have been studying rhodopsin with an interest in determining the conformational change that leads to signal transduction in this class of receptors. Although there has been some success in expressing select members of the large GPCR family in bacterial systems, the best characterized expression systems have generally been in mammalian tissue culture. In our case, we isolated stable cell lines in which the desired receptor is expressed upon exposure to tetracycline. The cell line was derived from HEK293 cells, which can be grown in suspension. Attempts to scale up production of recombinantly-expressed protein by the use of spinner flasks were unsuccessful.

Based on our initial experiments using tissue culture plates, we had expected approximately 1 mg of recombinant protein for 1 L of cells grown in suspension, but found that expression levels in spinner flasks were closer to 0.1 mg per L. Use of a stirred-tank bioreactor allowed for optimization of cell growth, as described below, and resulted in higher cell densities with concomitant higher levels of expression of our recombinant protein.

Materials and methods
Cell line
The cell line, HEK293 Gnt (N-acetylglucosaminyltransferase I), was a generous gift from Phillip Reeves and H. Gobind Khorana[2]. It is a derivative of the standard HEK293 cell line that was selected by mutagenesis and ricin treatment to be deficient in N-acetylglucosaminyltransferase I activity. GPCRs expressed in this cell line have a more uniform pattern of glycosylation which should result in a higher likelihood of crystallization. We also utilized the vector that Reeves, Callewaert et al. have described[2] which places receptor expression under the control of tetracycline exposure to the cells.

Bioreactor
We used the New Brunswick™ BioFlo®/CelliGen® 115 bioreactor equipped with a thermal mass flow controller and four-gas mix module. A pitched-blade impeller was driven by a magnetic motor, and the cultures were grown in a 5 L (2.0 - 5.6 L working volume) water-jacketed vessel.

Culture media
DMEM/F12 supplied as a powder from Atlanta Biologicals (Lawrenceville, Georgia, USA) was used as the base media. This was supplemented with sodium bicarbonate (3.7 g/L), Primatone® RL-UF (0.3 g/L), 10 % heat-treated FBS, penicillin G (100 units/mL), streptomycin (100 μg/mL), glutamine (292 μg/mL), dextran sulfate (300 μg/mL), and pluronic F-68 (0.1 % w/v). The media was sterilized by filtration through a 0.2 μm membrane and pumped into the vessel.
Control software
All equipment was monitored using New Brunswick BioCommand® software with data logging set at one-minute intervals.

Method
On the day before inoculation, the 5 L bioreactor vessel was filled with 4 L of phosphate buffered saline. The various ports were connected to appropriate tubing for removal of the saline, introduction of media, introduction of cells, and the pumping of the four-gas mix through the sparger. The pH electrode was calibrated and then disconnected and the protective cover was installed. The oxygen probe was examined and also covered by a protective cover. The jacket of the vessel was filled with water, and the assembly was set in autoclave for a 30-minute sterilization cycle. Afterward the vessel was returned to the tissue culture room and allowed to cool overnight. The following day the calibration of the pH electrode and oxygen sensor was checked after allowing the oxygen sensor to charge by the control unit.

A stable cell line which contains the expression cassette for the GPCR, under the control of a cytomegalovirus promoter/tetracycline-responsive promoter was selected using the neo gene. These cells were maintained in tissue culture plates with DMEM/F12 medium supplemented with 10 % fetal bovine serum, G418, and blasticin. For inoculation of a 5 L bioreactor vessel, thirty 15 cm plates were grown to approximately 80 % confluence. On the day of inoculation, 4 L of media were prepared and transferred to the vessel with a peristaltic pump after removal of PBS from the vessel.

All setpoints were programmed from BioFlo/CelliGen 115 control station as follows: temperature at 37 °C and a pH of 7.2. Oxygenation was maintained at 50 % using the four-gas mixture of air, nitrogen, oxygen, and carbon dioxide, and the thermal mass flow controller was set to deliver 0.5 L per minute. The pH was maintained by a combination of carbon dioxide and a solution of 7.5 % sodium bicarbonate that was controlled by pump 2. Agitation with the pitched blade impeller was set to 30 rpm.

The cells were recovered from the tissue culture plates by brief trypsinization and resuspension in the culture medium. The cells were pumped into the vessel with an auxiliary peristaltic pump. A small sample was removed and the starting cell density was determined with a hemocytometer.

Over the next five to seven days, the cell density was checked on a daily basis. Once the density reached 0.8 – 1.0 x 10⁶, the culture was supplemented with 40 mL of 20 % (w/v) glucose and 120 mL of 10 % (w/v) Primatone RL-UF. The following day, expression was induced by the addition of tetracycline (2 µg/mL) and sodium butyrate (5 mM) to the culture (Figure 1). One day later, the cells were recovered from the bioreactor and pelleted by centrifugation. A 1 mL aliquot was reserved for analysis by Western Blot to determine the level of expression (Figure 2).
Results and discussion

The expressed GPCR was solubilized by lysing the cell pellet from the small aliquot with a buffer containing 1 % (w/v) dodecyl-maltoside. The expressed GPCR was detected using a Western Blot with a monoclonal antibody, and the signal detected was compared to rhodopsin purified from cow retinae. We detected approximately 1 mg of recombinant GPCR per L of cell culture. The migration of the recombinant protein was probably due to differences in glycosylation. This was a dramatically improved result when compared to cell growth in suspension with spinner flasks where a cell density above $0.5 \times 10^6$ was hard to achieve. In experiments using the same cell line performed in spinner flasks, the expression level of recombinant GPCR ranged from 0.1 – 0.2 mg/L of culture (Table 1).

A large-scale prep (two 4.5 L runs) was subsequently performed, and 10 mg of purified rGPCR were obtained in a detergent solubilized form. A G-protein activation assay in which uptake of a radio-labeled non-hydrolyzable analog of GTP by transducin was measured as a function of time in the presence of the recombinant receptor, confirmed the bioactivity of the recombinant protein. The reaction was started by the addition of GTPγS, and aliquots of the reaction were applied to nitrocellulose filters at various times. In the absence of a receptor, very little spontaneous uptake of the radio-labeled nucleotide was detected. The form of the receptor expressed in the experiments contained mutations in which residues were altered to cause constitutive activation. The receptor expressed in the bioreactor caused an increase in the rate of nucleotide uptake by transducin, as expected (Figure 3).

### Table 1. Recombinant protein (rGPCR) expression comparisons.

<table>
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<tr>
<th>Yields</th>
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<td>Maximum Cell Density</td>
<td>$0.5 \times 10^6$/mL</td>
<td>$1.4 \times 10^6$/mL</td>
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<tr>
<td>Protein Culture Volume</td>
<td>0.1–0.2 mg/L</td>
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<td>10 mg/L</td>
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Figure 3. The bioactivity of the expressed rGPCR protein in the bioreactor was measured using G-protein activation assay using [$^{35}$S]-GTP binding assay. Uptake of a radio-labeled nonhydrolyzable analog of GTP by transducin was measured as a function of time in the presence of the recombinant receptor, confirming the bioactivity of the recombinant protein.

**Conclusion**

This study demonstrates that by being able to control the cell culture process parameters using a cell culture bioreactor, both the HEK293 cell density and expression levels of the rGPCR dramatically increased in comparison to using a spinner flask or tissue culture plates. The bioactivity of the rGPCR was good, however a change in the level of glycosylation of the recombinant protein was indicated by the positions of the rGPCR bands relative to the standard protein band in the Western Blots.

**References**


### Ordering information

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Abstract

The Fibra-Cel packed-bed basket technology has been established as an excellent method for the growth of suspension and anchorage-dependent cell lines. The three dimensional structure of the Fibra-Cel disk provides an excellent solid-support matrix for the entrapment or attachment of animal cells, allowing constant perfusion of nutrients in a low-shear environment. In this application note, we show that Hybridoma cells can be successfully cultivated in high densities in the 2.5 L packed-bed Fibra-Cel basket controlled by a CelliGen 310 bioreactor.

Introduction

Packed-bed bioreactor cell culture is generally accepted as one of the best methods to simulate the conditions of animal cell growth in vivo since cells are maintained in a low-shear environment with constant refreshment of nutrients and removal of waste. The growth of attachment-dependent cells on Fibra-Cel has been shown to increase both cell and product yields. In particular, Hybridoma cells are inherently sensitive to waste buildup and the implementation of packed-bed Fibra-Cel growth conditions in addition to perfusion production methods has greatly increased yields. To demonstrate that the CelliGen 310 2.5 L basket impeller bioreactor is capable of robust, reproducible high density Hybridoma culture under perfusion conditions, two independent trials were conducted using the suspension-adapted DA4.4 hybridoma cell line.

Materials and Methods

Inoculum preparation

DA4.4 Hybridoma cells (ATCC® #HB-57; Manassas, VA) were grown in 1 L shake flasks at 37 °C with 5 % CO₂ and agitation set at 95 rpm. Culture medium was prepared using Gibco® Hybridoma-SFM complete DPM powder supplemented with 5 % Hyclone® Fetal Bovine Serum and 1 % Gibco liquid Pen/Strep before sterile filtration using a 0.2 µm Millipore® Millipak® gamma gold filter into sterile Hyclone bags (5 L and 10 L, as necessary). Medium was stored at 2 – 8 °C until use. The 1.75 L vessel working volume was inoculated with a target total of 4.1 x 10⁸ cells. Actual viable cell numbers were 3.5 x 10⁸ cells (2.2 x 10⁵ cells/mL) for the first run and 4.8 x 10⁸ cells (3 x 10⁵ cells/mL) for the second run. The table below shows the origin of the materials used in this study.

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Figure 1. Left: The packed-bed basket impeller including Fibra-Cel disks. Right: The CelliGen 310 bioreactor with 2.5 L vessel.
Bioreactor conditions
For both runs, hybridoma cells were cultured in the same vessel, using the same CelliGen 310 cabinet for 9 consecutive days, using the basket impeller system packed with 75 g of Fibra-Cel disks.

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Perfusion was initiated for each bioreactor on day 3 and continued through day 9. Initially, the main objective was to increase the perfusion rate to maintain a glucose concentration above and near 1 g/L. For the second bioreactor experiment, the perfusion rate was adjusted to match the first bioreactor rate in order to make the two runs as identical as possible. The tables illustrate the experimental parameters and perfusion volumes for both trials.

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<td>8</td>
<td>4.75</td>
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<td>9</td>
<td>5</td>
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Biochemistry analysis
Daily off-line measurements of glucose concentration were performed using a YSI® 2700 analyzer (YSI, Inc., Yellow Springs, OH). The glucose consumption was calculated for each time point and plotted as an average of the two independent trials. Error bars indicate standard error of the mean.

Results and Discussion
As presented in the graph below (Figure 2), the rate of glucose consumption across both trials is indicative of reproducible growth of hybridoma cells in this environment. We conclude that the use of Fibra-Cel in the basket impeller system on the CelliGen 310 is an excellent method for high density hybridoma culture. In a batch run with the CelliGen pitch blade bioreactor, hybridoma cells usually peak at approximately 5 g/day of glucose consumption. The packed-bed basket impeller system presents significantly higher productivity with glucose consumption peaking at, on average, 25 g/day. In addition, if growth conditions are maintained by continued fresh media perfusion and glucose concentration is never allowed to fall below 1 g/L, hybridoma can be continuously cultured in the basket many days after the 9 day window provided in this study; this further increases productivity and decreases overall antibody production costs. No optimization of growth conditions were attempted for either bioreactor run.

![Average glucose consumption](image)

**Figure 2.** The glucose consumption was calculated daily for each bioreactor and the mean is presented. Error bars indicate standard error of the mean. Comparable consumption was observed across the two bioreactors.
## Ordering information

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<td>2.5 L Basket Impeller Kit</td>
<td>M1287-1140</td>
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</table>

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Eppendorf AG • 22331 Hamburg • Germany
eppendorf@eppendorf.com
This study describes a simple procedure for improving insect cell yields in a benchtop cell culture bioreactor. Here, yields of Spodoptera frugiperda (Sf9) cells were increased by nearly 29% through monitoring dissolved carbon dioxide (dCO₂) levels in the culture and adding air to the vessel headspace to reduce dCO₂. The method can also be used to maximize yields in a wide range of mammalian cell types.

Abstract

This study describes a simple procedure for improving insect cell yields in a benchtop cell culture bioreactor. Here, yields of Spodoptera frugiperda (Sf9) cells were increased by nearly 29% through monitoring dissolved carbon dioxide (dCO₂) levels in the culture and adding air to the vessel headspace to reduce dCO₂. The method can also be used to maximize yields in a wide range of mammalian cell types.

Introduction

Producing high yields of protein from insect cells usually requires maintaining high levels of dissolved oxygen (DO) in the culture. However, as cell concentrations grow, they generate an ever-increasing level of dCO₂, which can inhibit cell growth. We compared growth of Sf9 insect cell yields in two runs, first without control of dCO₂ levels and then by continuously flowing air into the vessel headspace above the liquid media level to reduce dCO₂ concentration. This insect cell culture protocol has not been fully optimized to obtain the highest yields possible, but it is meant to serve as a guide for basic procedures and materials. The method can also be used to maximize yields in a wide range of mammalian cell types.

Materials and Methods

Bioreactor

We used a 2.5 L total volume New Brunswick CelliGen 310 benchtop autoclavable bioreactor* with a pitched-blade impeller. The CelliGen 310 is a cGMP-compliant system designed for high-density growth of mammalian, plant, and insect cell lines. It comes standard with a large 15-inch industrial color touchscreen interface with an advanced controller to simplify setup, calibration, and control. The bioreactor includes three built-in pumps, pH/DO and level/

* The New Brunswick CelliGen 310 bioprocess control station has been discontinued. Its successor is the BioFlo 320 bioprocess control station.
foam probes, and one thermal mass flow controller (TMFC) for regulating gas flow. The CelliGen 310 used in this study had a TMFC range of 0.1 – 5 L/min (other flow ranges or choice of multiple TMFCs are also available).

We added three optional accessories. A Mettler-Toledo® dCO₂ sensor and transmitter were connected to the CelliGen 310 to measure dCO₂ concentration throughout the process.

A New Brunswick accessory gas overlay controller with a flow range of 0.1 – 5 L/min (capable of regulating four gases) was used to regulate addition of air to the vessel headspace. And an optional New Brunswick BioCommand® supervisory software package was also used to automatically log data. Additionally, a gas overlay vessel kit that includes necessary tubes, filters, and fittings is highly recommended.

**Overview**

> Autoclave the vessel with phosphate buffer solution (PBS) for 60 minutes.
> Remove PBS from the vessel.
> Add 1.5 L of insect cell media to the vessel.
> Inoculate 500 mL of insect cell suspension with a starting cell count of 4.0 × 10⁵ cells/mL.

**Medium**

We used Sf-900 II serum-free media.

**Inoculum**

A proprietary Sf9 cell line was supplied by a leading biotechnology company. The inoculum was cultivated in an Eppendorf New Brunswick open-air Innova® 2000 shaker placed inside an incubator for temperature regulation.

**Control setpoints**

The following setpoints were keyed into the touchscreen controller prior to inoculation:

> Temperature 28 °C
> DO 40 %
> Agitation 70 rpm, gradually increased to 100 rpm over the course of the run

**DO control**

The DO probe provided as part of the CelliGen 310 kit was calibrated at 0 % (obtained by briefly disconnecting the cable), then calibrated at 100 % (obtained using 100 rpm agitation and 5 L/min air flow rate). The control was set to 4-gas mode to automatically maintain the DO setpoint by sparging three gases (air, O₂ and N₂).

**pH control**

Although pH control capability is built into the CelliGen 310 bioreactor, it is not generally needed for controlling insect cell growth and was therefore not regulated in this study.

**Fed-batch control**

Pumps were calibrated using standard supplied tubing to keep track of liquid quantities entering and exiting the vessel. Samples were taken several times a day (as described below) to measure glucose and cell density.

**Pump Calibration**

To assure the most accurate flow rate, calibrate the pump each time you change tubing. (1) Use about two feet of tubing for each line attached to a pump head. (2) Insert tubing into the appropriate pump head. (3) Set the pump assignment to “None.” (4) Select “Calibrate” and either “15”, “30,” or “60” seconds as the time interval. Record the quantity of water pumped into a graduated cylinder for the defined time period, and enter that into the “Flow Rate” field, then hit “OK.” Your flow rate will be automatically calculated.

**Control Program**

For these studies, we used one of three built-in pumps. When cells reached a density of 6 × 10⁶ cells/mL, 20 mL/L of Gibco® Yeastolate (Life Technologies® Catalog No. 18200-048) was automatically added by Pump 1. In both runs, O₂ was sparged into the liquid media and controlled by the CelliGen 310’s built-in 4-gas controller. In run 1, we added no gas overlay into the vessel headspace. In the second run, all conditions remained the same, but 300 mL/min of air was continuously added to the vessel headspace to reduce dCO₂ concentration (control screen shown in Figure 2).
Results and Discussion

As shown in Figure 3, maximum viable cell density in run 1 reached $9.18 \times 10^6$ cells/mL on day 9. In run 2, maximum cell density increased by 28.5 %, reaching $11.8 \times 10^6$ cells/mL on day 6. This study shows that by reducing dCO₂ concentration, maximum cell density not only was significantly improved, but was also achieved at a faster growth rate.

It should be noted that the CelliGen 310 bioreactor can simultaneously control more than 120 process loops (32 loops per vessel, four vessels simultaneously), making it an extremely powerful research tool. It can be operated in batch, fed-batch, or perfusion modes and comes with a choice of four interchangeable vessels (2.5 – 14.0 L) as well as a wide range of specialized impellers to maximize yields. For secreted products, a packed-bed basket option is available to maximize cell productivity regardless of cell type. The system includes multiple analog inputs/outputs for easily integrating data from up to 10 ancillary devices, such as additional TMFCs, sensors, scales, or on-line gas and glucose analyzers for optimized process control. However, we did not take advantage of the full potential of the 310, intending only to provide an easy technique for increasing yields in insect cell culture. This technique is also very useful in maximizing yields in a wide range of mammalian cultures. For system specifications or to request additional information see www.eppendorf.com.
## Ordering information

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<td><strong>M400 transmitter, Mettler Toledo®</strong></td>
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* The New Brunswick CelliGen 310 bioprocess control station has been discontinued. Its successor is the BioFlo 320 bioprocess control station.
Insect Cell Culture Using the New Brunswick™ BioFlo®/CelliGen® 115 Benchtop Fermentor/Bioreactor with Spin Filter Assembly

James Jarvis
Eppendorf Inc., Enfield, CT, U.S.A.
Corresponding author: sha.m@eppendorf.com

Abstract

This application report presents a simple protocol for achieving high-density culture of Spodoptera frugiperda (Sf9) cells using a New Brunswick bench-top, autoclavable stirred-tank reactor with a spin-filter assembly. Factors such as substrate concentration and metabolite buildup can be limiting for culture growth and viability at high densities. Using the spin filter in a 2 L vessel (0.8 – 2.2 L working volume) attached to the BioFlo/CelliGen 115 cabinet, a cell density of 18.24 x 10^6 cells with viability over 90 % was achieved, outperforming the batch or fed-batch process.

Introduction

Stirred-tank bioreactors are widely used for research and industrial applications for cultivating a wide variety of cell types, including insect cells, hybridoma, CHO, BHK21, HEK293, and others; these cultures manufacture viral vaccines and monoclonal antibodies, blood clotting factors, etc. Spodoptera frugiperda, also known as the Fall Armyworm or Sf9, are insect cells commonly used for the production of proteins of interest in pharmaceutical research due to their unique ability to replicate mammalian post-translational modifications such as glycosylation. Insect cells produce a variety of proteins utilizing the Baculovirus Expression Vector System (BEVS). Cell lines such as Sf9, Hi-5, SF21, etc., are proven to express high levels of end products.

Insect cell culture can be achieved by using batch, fed batch or perfusion methods. For this study, the perfusion method was used in conjunction with New Brunswick spin filter assembly. The spin filter allows for the removal of exhausted media without removing the cells in suspension, making room for fresh media addition, thus achieving and maintaining the highest culture densities possible.

Figure 1: BioFlo/CelliGen 115 systems feature a compact control station capable of either fermentation or cell culture operating modes to accommodate growth of a wide variety of cell types. A built in color touch-screen interface facilitates setpoint control and monitoring. The BioFlo/CelliGen 115 system (left) is equipped with a 2 L water-jacketed vessel with pitched-blade impeller and four rotameters.
Materials and Methods

Bioreactor
For this application, a standard 2 L BioFlo/CelliGen 115 advanced cell culture kit with a magnetic drive and water-jacketed vessel was used. A Suspension-Cell Spin Filter with 10µm screen was used to grow the insect cells in a continuous, high flow rate perfusion mode. In addition, BioCommand® Batch Control software was used to monitor the system and control the feeding schedule.

Medium
This application used an animal component-free chemically-defined ESF-921 medium from Expression Systems (Woodland, CA).

Inoculum
The cell stock used was Sf9 cells derived from ATCC® CRL-1711 adapted to a serum free environment, obtained from Expression Systems (Woodland, CA). The inoculum was cultivated in an Eppendorf shaker (New Brunswick Innova® 40, order no.).

Controller setpoints
Calibrate pH probe prior to autoclaving. Enter controller set points prior to inoculation and allow the media equilibrate to prior to proceeding. The DO may remain high after calibration and before inoculation due to the absence of cells consuming it. An initial DO value of > 95 % is acceptable; it will decrease as the cells start to metabolize it. Normal setpoints for Spodoptera cells are controlled by the Primary Control Unit (PCU) and are as follows:

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<tr>
<td>pH</td>
<td>6.3</td>
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<tr>
<td>Agitation</td>
<td>100 rpm</td>
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<tr>
<td>Gas control</td>
<td>4-gas mode</td>
</tr>
<tr>
<td>Inoculum</td>
<td>4.1 x 10⁵ cells/mL</td>
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DO calibration
The DO probe is calibrated using a standard two-point calibration method: 0 % (often referred to as the zero point) and 100 % (often referred to as span). The zero can be achieved by either disconnecting the DO cable (the electronic zero; used in this process) or by sparging N₂ into the media to achieve a level stable near zero. The 100 % calibration point is achieved by bringing the vessel filled with medium to all of the operational setpoints, i.e. agitation, temperature, etc. DO should be calibrated post-autoclave and pre-inoculation after a six hour polarization period. After calibration, the DO may remain around 100 % until after inoculation.

pH calibration
The pH probe was calibrated prior to the autoclave cycle outside the vessel using a two-point calibration method with standard pH buffers. The pH 7.0 buffer is used to zero the probe and the pH 4.0 or 10.0 can be used as the span (Refer to the BioFlo 115 operating manual).

pH control
The pH for insect cells normally does not drift much from setpoint, but at higher culture densities the pH may drop. The pH parameters are maintained by the addition of CO₂ to lower the pH or an 8 % sodium bicarbonate solution to raise the pH. The dead band was set to 0.1 for this run.

Gas control
The BioFlo/CelliGen 115 was set to the 4-gas mode to maintain the DO and pH setpoints automatically. The cascade in 4-gas mode was set to gas flow and the O₂ control was set to 4-gas mode.

Continuous feed (perfusion)
All pumps were calibrated using the standard, supplied tubing to track liquid quantities entering and exiting the vessel. Samples were taken several times a day to measure the density of the culture as well as nutrient consumption.

<table>
<thead>
<tr>
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<td>Pump 2</td>
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<td>Pump 3</td>
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Control program
For this study, a BioFlo/CelliGen 115 for the control of the pH, DO, level sensor, and pumps was used; BioCommand Plus software was also used to monitor the culture parameters.

Results and Discussion
Insect cells generally have a high demand for oxygen during protein production. Maximum growth rate and high cell densities are achieved by keeping the DO at a constant set point.

Factors such as substrate concentration and metabolite buildup can be limiting factors; these were made more controllable through the abilities of the BioFlo/CelliGen 115 bioreactor with the Advanced Cell Culture Kit coupled with a spin filter kit.

The BioFlo/CelliGen 115 system allowed for the growth of insect cells to a final density of 18.24 x 10⁶ cells/mL. The inclusion of the TMFCs (thermal mass flow controller) provided the ability to mix the four gases according to culture needs and further enhanced the final culture density.

Conclusion
Considering the above results, we can view the viable cell density of 18.24 x 10⁶ as proof of the fundamental capabilities of the BioFlo/CelliGen 115 system.

The temperature of the system remained steady and was controlled by using un-chilled tap water as the coolant.

DO and pH control remained stable and consistent throughout the experiment.

Overall, the BioFlo/CelliGen 115 system performed extremely well. The BioFlo/CelliGen 115 advanced cell culture system with spin filter assembly is recommended for insect cell culture to achieve high cell densities.

Figure 3: Insect cell Sf9 perfusion culture in a 2 L BioFlo/CelliGen 115 bioreactor with spin filter impeller. The cells were inoculated from 1000 mL shaker flask culture. The inoculum cell density was 4.9 x 10⁵ cells/mL. After two days of the batch process, medium perfusion was started at the rate of 0.5 – 2 L working volumes per day.
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eppendorf@eppendorf.com

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An Update on the Advantages of Fibra-Cel® Disks for Cell Culture

Rich Mirro
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Corresponding author: sha.m@eppendorf.com

Abstract
With a renewed acceptance of perfusion processes in cell culture, both in the laboratory and for production applications [1], New Brunswick™ packed-bed bioreactors using Fibra-Cel disks are seeing an upsurge in interest. This application note examines Fibra-Cel technology and its many advantages, from increased secreted protein yields to labor savings in applications including rabies vaccine production.

What Is Fibra-Cel?
Eppendorf Fibra-Cel is a solid-support growth matrix (Figure 1) for anchorage-dependent and suspension cell cultures. It is used predominantly in perfusion processes for the production of secreted products—such as recombinant proteins and viruses—and it is currently being evaluated for stem cell research [2].

Since the 1980s, scientists around the globe have been using Fibra-Cel to grow a wide range of cell types (see inset, right), including hybridomas and insect cultures. Originally used in New Brunswick™ CelliGen® autoclavable cell culture bioreactors, Fibra-Cel technology has now been successfully scaled up for commercial production in sterilizable-in-place systems as large as 150 liters. BioBLU® packed-bed, single-use vessels containing Fibra-Cel are also available for those who prefer the advantages of a disposable system.

Manufactured according to cGMP guidelines, Fibra-Cel is composed of two layers of nonwoven material—polyester and polypropylene—which are sonicated together, cut into disks, and electrostatically treated to attract cells and facilitate their attachment to the disks. Normally it takes about six hours for cells to attach to microcarriers (with a normal inoculum of $1 \times 10^6$ cells/mL), whereas cells can attach within 15 – 60 minutes on Fibra-Cel disks.

Cells successfully used on Fibra-Cel disks
Hybridoma: 123A, 127A, GAMMA, 67-9-B, DA4-4
Anchorage-dependent: 3T3, MRC-5 and other human fibroblasts, HEK-293, BHK, COS, CHO, stroma cells, hepatocytes
Insect cells: Tn-368, Sf9, rSf9, Hi-5

Figure 1. Scanning electron micrograph of Fibra-Cel disks (left); mouse–mouse hybridoma DA4.4 immobilized on Fibra-Cel disks during production at $1 \times 10^8$ cells/cm$^3$ of packed-bed volume (right)

Moreover, the growth process for microcarrier cultures can require extended delays for periodic stoppage of stirring to allow time for cells to become attached. By comparison, the Fibra-Cel bed is inoculated ($3 \times 10^5$ cells/mL of bed volume) in a single step.
Fibra-Cel in a New Brunswick bioreactor is also advantageous over microcarriers because it enables sustained long-term periods of high-density growth in perfusion mode, without danger of clogging because there are no filters. Perfusion is a mode of cell culture in which a fresh nutrient medium is continuously added to the culture while simultaneously removing the spent medium that contains the product of interest. In a New Brunswick bioreactor, cells growing on or in the Fibra-Cel disk bed are retained within the vessel, inside the packed bed, where they continue the production of the desired product.

The packed bed comprises two horizontally positioned screens that extend to the bioreactor vessel walls. Enclosed between the screens, a bed of Fibra-Cel disks serves as solid support for the growing cells (Figure 2). Cells growing in the disk bed become immobilized on or between the disks, where they remain throughout the culture run, protected from external shear forces. The process is favored for manufacturing because product yields can be increased by as much as tenfold over comparable processes [3]. Once the bioreactor is set up and inoculated, the culture can be maintained to produce proteins for long periods of time thus saving labor, time, and money.

Like the proprietary Eppendorf Cell Lift impeller, rotation of the discharge ports in the proprietary packed-bed impeller creates a low differential pressure at the base of the impeller tube, which circulates the medium throughout the system. The medium receives gases through a sparger located at the bottom of the inner tube, protecting the cells from being exposed to the gas liquid interface. This results in low turbulence and low shear stress on the culture. Exceptionally high cell densities are achievable due to the high surface-to-volume ratio provided by the disk bed, coupled with the ability to use perfusion. In comparison with other cell support systems, it was found that higher titers and cell densities were achieved in trials using Fibra-Cel disks [4].

**Other benefits**
Because higher yields are possible, smaller bioreactors can be used to substantially reduce the initial capital expenditure as well as reduce the utilities required for operation (such as electricity, water, and steam if required). In addition, because the cells remain entrapped, the packed bed eliminates the need for cell filtration to separate cells from the end product, thus simplifying harvesting. Last, product recovery and downstream processing can be more easily controlled because users can determine the volume of harvest material that is to be processed at any given time.
Commercial production

Bioreactors using Fibra-Cel have been used in the production of a variety of commercial products. One example of commercial production includes end products such as EPO, which can now be commercially produced on the bench using Fibra-Cel technology, eliminating the need for labor-intensive and space-consuming roller bottles. A substantial portion of the world’s human rabies vaccine is also produced using Eppendorf Fibra-Cel technology. Additionally, many of our customers are currently using Fibra-Cel in their proprietary processes to produce interferons, monoclonal antibodies, and hormones.

In summary, Fibra-Cel provides benefits in research laboratories as well as in commercial production. Our customers have found that because yields are high, bioreactors containing Fibra-Cel packed beds can outperform much larger-sized bioreactors, thereby achieving commercial-scale production in a bioreactor with a far smaller footprint. Production space requirements are reduced, as are costs associated with labor, start-up, and operations.

For protocols on other cell lines, or for additional information on the Fibra-Cel, see eppendorf.com.

References


Ordering information

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Today, the rapidly expanding demand for vaccine products for viral diseases such as rabies has necessitated the development of more sophisticated production techniques based around cell culture systems. This article reviews vaccine production strategies, with a focus on rabies, looking specifically at the use of the Vero cell line – used worldwide and approved by the US Food and Drug Administration – as well as media technology and the bioreactor options available.

Growing Demand

Viral diseases, including rabies, are worldwide challenges for the international biomedical community. The World Health Organization (WHO) notes that over 32,000 rabies-related deaths were reported in 1998, while annual deaths worldwide from the virus grew to 55,000 by 2006 (1,2). Rabies is often transmitted to humans from infected domestic animals. Dogs infected with rabies can become extremely aggressive and attacks on humans are widespread, especially in certain Asian countries where using unleashed dogs for home security is common. It is spread through the saliva of infected animals and bites can be fatal.

Since the 18th century, vaccination has proven to be the most successful route to the total elimination of viral diseases such as rabies. From the early work of pioneers such as Jenner and Pasteur, vaccination was put on a sound scientific footing. Demand for vaccine products has continued to increase ever since, prompting the advances in production technology that we see today.

Viral Cultivation

The expanding vaccine requirements have led to techniques for growing large quantities of antigenic proteins. Traditionally, viruses have been grown in embryonated hens' eggs, but numerous shortcomings compromise their utility. These include a bottleneck in the availability of high-quality, pathogen-free eggs, as well as low titers of emerging viruses (3).

A major concern is that, when viruses are cultivated through extended passages in hens' eggs, there is an evolutionary process in the amnion or allantoic cavity of the egg, resulting in the selection of a virus subpopulation, antigenically and biochemically distinct from the original inoculum (4). Because of these and other factors, permanent cell lines are coming to dominate the field as an alternative method.

Mammalian cell culture systems provide much shorter lead times; a more controlled production process that takes advantage of closed-system bioreactors; a reduced risk of microbial contamination; and the opportunity to cultivate viral stocks without significant egg passage-dependent antigenic changes (5). These systems provide...
a flexible and scalable platform that can make use of existing biopharma infrastructure for vaccine production, and could replace egg-based vaccines in the foreseeable future (6).

Cell Line Options

In the past few years, several continuous cell lines have been approved by regulatory authorities for virus production. These include the *Spodoptera frugiperda* insect cell line from Protein Sciences (7), Madin-Darby canine kidney (MDCK), the PER.C6 cell line, designed for growth to high densities (8), and the widely used Vero line.

It is important to note that certain cell lines may provide an environment favouring selection of viral subpopulations, and these types may be inappropriate for vaccine production. In light of this, various lines of investigation support the Vero cell line as the candidate of choice for viral vaccine production, including:

- Efficiency of primary virus isolation and replication to high-infectivity titers
- Genetic stability of the haemagglutinin molecule, while maintaining the antigenic properties of human-derived viruses
- Similarities in the pattern of protein synthesis and morphological changes between virus-infected Vero and MDCK cells (9)

Media Alternatives

There is a variety of different Vero isolates available from commercial suppliers, but all are quite similar, and their nutritional needs are comparable (10). The search for the ideal mammalian cell culture medium began in the 1950s, with the holy grail being an economical, protein-free, serum-free medium that would provide strong growth support and have the property of scalability to large volumes, up to thousands of litres, while coming in at an affordable price.

Serum provides a protective function to cultured cells, and binds toxins and other contaminating materials. As such, serum-free media must be extremely carefully formulated (11). Albumin can be substituted for serum, but it may impede the downstream steps of purification (12).

Chen *et al* tested five different serum-free media, combined with Cytodex 1 microcarriers (12). The following were evaluated: OptiPro SFM (Invitrogen), VP-SFM (Invitrogen), EX-CELL Vero SFM (SAFC Biosciences), Provero-1 (Lonza) and HyQ SFM4MegaVir (HyClone). EX-CELL Vero SFM gave one of the highest cell densities, demonstrating that the use of serum-free media has become routine for Vero cell cultivation. Comparable results were observed with a commercial serum-free medium MDSS2N (AXCEVIR-VeroTM by Axcell Biotechnologies).

Rabies Strategies

The Brazilian group led by Frazatti-Gallina has been active in the field of rabies vaccine production (13). Using Vero cells adhered to microcarriers, and cultivated in a bioreactor with

It is important to note that certain cell lines may provide an environment favouring selection of viral subpopulations, and these types may be inappropriate for vaccine production.
serum-free medium, they generated an effective rabies vaccine. With the aid of tangential filtration, they purified the rabies virus by chromatography and inactivated it using beta-propiolactone.

Their protocol states that 350cm² T-flasks were harvested and inoculated into a 3.7-litre CelliGen bioreactor, at a proportion of 16 cells per microcarrier (Cytodex 3-GE), yielding an initial seeding proportion of 2.5×10⁷ cell/ml. The cells were grown in serum-free MD552 medium.

The serum-free VP-SFM medium, according to the manufacturer, was developed for Vero, BHK-21 and Chinese hamster ovary cell growth. This medium drives the adherence of the Vero cells to the microcarriers. After four days of cultivation in VP-SFM medium, the cells were infected with Pasteur vaccines (PV) strain rabies virus (multiplicity of infection = 0.08). The harvest of the culture supernatant were carried out three days after the virus inoculation and four times thereafter at 24-hour intervals. During this period, culture conditions were maintained at 60rpm at a pH of 7.15 and 5 per cent dissolved oxygen. Only the temperature varied, from 36.5°C in the cellular growth phase of the culture to 34°C after virus inoculation. In the course of the programme, seven batches of virus suspensions were produced in the bioreactor (16 litre per cycle) at a mean viral titer of 10⁶. FFDS0/0.05ml.

The effectiveness of the preparation was demonstrated by immunising mice with three doses of the new vaccine, and comparing it with the commercial Verorab and human diploid cell rabies vaccine. Mean titers of neutralising antibodies of 10.3-34.6, 6.54 and 9.36 IU/ml were found, respectively.

The choice of the serum-free medium was fortunate. In this case, the amount of contaminating DNA was very low and tolerable – less than 22.8pg per dose of vaccine. The authors argue that this protocol is especially applicable in the developing world, where rabies is a constant hazard and a major public health problem.

**Increasing Yield**

Yu et al sought methods to increase yield in Vero cell culture systems over that obtained using roller bottles (14). In a recent review, they summarised the production technology developed over the course of the last seven years. They have adopted the 30-litre BioFlo 4500 fermentor/bioreactor. The cells were cultivated in media containing 10 per cent serum, first grown as a monolayer, and when the cell density reached 1.0-1.2×10⁶ cells/ml, they were transferred to the bioreactor containing 25g/litre of Cytodex-1 for perfusion culture. The virus preparations, also cultured in roller bottles, were infected with the PV2061 virus strain, harvested and transferred to the bioreactors.

**Purified Vaccine**

Wang et al have described a purified Vero cell rabies vaccine that has been widely produced in China, referred to as ChengDa (Liaoning ChengDa Biological) (15,16). It is grown on a Vero cell line utilising the PV2061 strain, inactivated with beta-propiolactone, lyophilised, and reconstituted in 0.5ml of physiological saline. It fulfils WHO recommendations for potency.

The process used for ChengDa was developed by Aycardi (17). A single bioreactor was capable of producing one million doses of rabies vaccine per year. The method uses ultra-high-density microcarrier cell cultures adapted to a 30-litre CelliGen bioreactor equipped with a patented cell lift impeller, specifically configured for a perfusion system to feed the growth media into the bioreactor. A specially designed decanting column was used to prevent perfusion loss of microcarrier and keep the cells in high concentration. The system delivers high oxygen transfer, high nutrient level and low shear stress, allowing cell growth of up to 1.2×10⁷ million cells/ml under continuous perfusion for up to 20 days.

**Important Step**

The combination of advances reviewed here provides strong support for the use of cell culture systems for virus production of vaccines. The fact that Vero cells have been approved for clinical products represents an important step on the road to technologies that do not rely upon hens’ eggs for generation of adequate quantities of viruses.

Advances in culture media enable the elimination of serum, thus driving the rapid and efficient purification of proteins. The use of carrier beads adds to the efficiency of culture technology, allowing greatly increased cell densities to be reached. Improvements in bioreactor design, combined with these various technological advances, results in a greatly improved and more functional production train.

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Stem Cells
Expansion of Human Bone Marrow-Derived Mesenchymal Stem Cells in BioBLU® 0.3c Single-Use Bioreactors

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Abstract

The use of adult stem cells holds great promise for new cell-based therapies and drug discovery. For their routine application, large numbers of cells have to be produced with consistently high quality. Two-dimensional cultivation systems, such as T-flasks, are widely used, but they are limited in terms of control and scalability. Stem cell expansion in rigid-wall, stirred-tank bioreactors, however, facilitates the precise control of critical process parameters like pH and dissolved oxygen, and allows a more straightforward scale-up to larger process dimensions. We tested the suitability of Eppendorf BioBLU 0.3c Single-Use Vessels controlled by a DASbox® Mini Bioreactor System for the expansion of human bone marrow-derived mesenchymal stem cells on microcarriers, and obtained 1 x 10⁸ cells in a working volume of 250 mL. The cells were able to differentiate into osteocytes and chondrocytes, respectively, demonstrating that their expansion in stirred-tank bioreactors did not affect multipotency. BioBLU Single-Use Vessels with maximum working volumes of 250 mL and 3.75 L were previously used for the expansion of human induced pluripotent stem cells as cell-only aggregates [1] and of adipose-derived mesenchymal stem cells on microcarriers [2]. These results, and our current data, suggest that Eppendorf BioBLU Single-Use Vessels are widely applicable for the expansion of different stem cell types at various scales.

Introduction

Although the term stem cells had already appeared in the scientific literature by the mid-1800s, the number of research studies on these cells only increased at the beginning of the twenty-first century. Stem cells are unspecialized cells which have the ability to self-renew, and the capacity to differentiate into specialized cells. According to their origin, they are classified into embryonic stem cell (ESCs), derived from an embryo, and adult stem cells (ASCs), found in adult tissues. Mesenchymal stem cells (MSCs) are adult stem cells, which can originate from a large variety of tissues such as bone marrow, adipose tissue, placenta, muscle or umbilical cord. MSCs are defined as multipotent, meaning they are capable of differentiating into more than one, but not all, cell types. In contrast, ESCs are pluripotent, meaning they can generate all cell types. MSCs are attractive candidates for therapeutic applications, especially in the field of regenerative medicine [3]. Despite their limited differentiation potential, MSCs offer great advantages
compared to ESCs, as they do not pose ethical issues, they can be isolated from various sources, and they reduce the risk of rejection reactions. The doses of human MSCs (hMSCs) needed for clinical trials are estimated at between one and 200 million cells per patient, depending on the disease being tackled [4]. One of the most important challenges in providing hMSCs for curative use is the production of large quantities of cells in a robust manner. Indeed, whatever the tissue source, the number of hMSCs extracted is very low, and not sufficient for clinical use; hence the hMSCs have to be expanded following isolation. Besides providing the needed cell quantities, hMSC production must also comply with the manufacturing process regulations required of a fully controlled production system. hMSC expansion in stirred-tank bioreactors can be monitored and is scalable, and hence can fulfill these requirements from experimental quantities to production. An Eppendorf DASbox Mini Bioreactor System equipped with BioBLU 0.3 Single-Used Vessels has already been successfully used for the expansion of pluripotent stem cells in suspension [1]. In the study presented here, we used the same bioreactor system to culture adherent multipotent stem cells on microcarriers, and reached a clinically relevant number of cells.

Material and Methods

Initial cell culture in T-flasks
We obtained human bone marrow-derived mesenchymal stem cells (hMSCs-BM), cryopreserved after the second passage, from Lonza®, Switzerland. To reach the cell quantity needed for this study, we first expanded hMSCs-BM in Eppendorf Cell Culture Flasks T-175 for four passages. hMSCs-BM were cultured in MSCGM™ Mesenchymal Stem Cell Growth BulletKit™ Medium (Lonza), including both the basal medium and the necessary supplements. At passage six, we harvested the cells to be used for three-dimensional microcarrier culture in a stirred-tank bioreactor.

Preparation of microcarriers
We prepared Cytodex® type 1 (GE Healthcare® Bio-Sciences, Sweden) and Cytodex type 3 microcarriers (Sigma-Aldrich®, USA) according to the manufacturer’s instructions. This included four steps: the microcarrier hydration, a washing with PBS, the microcarrier sterilization by autoclaving, and a final rinsing in the culture medium.

Culture of hMSCs-BM on microcarriers in BioBLU 0.3c Single-Use Vessels
We processed the experiments in parallel in a 4-fold Eppendorf DASbox Mini Bioreactor System for cell culture (Figure 1), equipped with Eppendorf BioBLU 0.3c Single-Use Vessels. We inoculated the cultures with hMSCs-BM cultured in T-flasks, combined with Cytodex (type 1 or 3) microcarriers. To ensure a similar cell-to-bead ratio (10 cells/bead), we used 140 mg of Cytodex type 1 and 200 mg of Cytodex type 3 microcarriers. The initial number of cells per bioreactor was $6 \times 10^6$ cells, which corresponds to a cell density of 9,700 cells/cm² for Cytodex type 1 and 11,000 cells/cm² for Cytodex type 3. We cultured hMSCs-BM in MSCG Mesenchymal Stem Cell Growth BulletKit Medium containing both the basal medium and the necessary supplements, in an initial working volume of 100 mL. To promote the initial cell adhesion, we did not agitate the culture for 24 hours. After this attachment period, we manually adjusted the culture volume to 200 mL, and set the agitation speed to 60 rpm for the entire proliferation phase. The cells were cultured at 37°C. The pH of the growth medium was controlled at 7.6 by automatic addition of CO₂ in the vessel headspace. We set the dissolved oxygen (DO) level to 40 %. The DO setpoint was maintained by delivering gas (N₂, air, and O₂) into the medium with the gas flow set at 0.1 sL/h. To maintain the culture until its maximum yield, we exchanged 50 % of the culture medium after 6 and 8 days of culture. After 9 days, up to 70 % of the medium was replaced almost daily. During those refreshment steps, agitation was stopped to let the microcarriers sediment. The cells were cultivated for 20 days on Cytodex type 1 and for
27 days on Cytodex type 3 microcarriers.

**Analysis of cell viability**
During the proliferation phase, we regularly analyzed the cell viability on the microcarriers by 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) coloration. To do so, we supplemented 400 μL of cell/microcarrier suspension with 40 μL of 5 % MTT solution (Sigma-Aldrich), and incubated the suspension at 37°C for 45 minutes. MTT produces a yellowish solution that is converted to dark blue, water-insoluble MTT formazan by the mitochondrial dehydrogenases of living cells. The dark blue crystals precipitate inside the cells, and can be seen by light microscopy.

**Cell counting and metabolite measurement**
To evaluate cell growth, we counted the cells at days 6, 9, 13, 15, 16, and 20. At each of these points in time, we collected independent samples of 3 mL of the cell/microcarrier suspensions. Cells were detached from microcarriers by addition of 0.25 % Trypsin-EDTA, and the cell number was determined using the CASY® Cell Counter and Analyzer, model TT (Omni Life Science®, USA).

We used the supernatants collected during cell counting to quantify glucose and lactate concentrations, using the Glucose RTU™ Kit (Biomérieux®, France) and L-Lactate Assay Kit (Abcam®, United Kingdom), respectively.

**hMSC-BM differentiation—osteogenic lineage**
For osteogenic lineage differentiation, we detached hMSCs-BM from microcarriers by trypsin treatment, and seeded them into BD BioCoat™ Fibronectin Cellware, 24-well plates at a density of 7 x 10³ cells/well. One day after seeding, we replaced the expansion medium with hMSC Osteogenic Differentiation BulletKit Medium (Lonza). This serum-containing induction medium is designed to induce osteogenic differentiation of hMSCs into mature, functionally active osteoblasts. We exchanged the medium 3, 7, 10, 14, and 18 days after the first induction. At days 14 and 21 we assessed the bone cell mineralization using the OsteoImage™ Mineralization Assay (Lonza). Moreover, at day 21 we stained calcium deposits with an anthraquinone dye (Alizarin Red S Staining Kit, ScienCell, USA).

**Results**

**hMSC-BM expansion on microcarriers in BioBLU 0.3c Single-Use Vessels**
In order to investigate the suitability of BioBLU 0.3c Single-Use Vessels for the scalable production of multipotent stem cells, we cultured hMSCs-BM in parallel on two microcarriers types, Cytodex type 1 and Cytodex type 3. Cytodex 1 microcarriers are based on a dextran matrix covered with positively charged groups, while a layer of denatured collagen is covalently bound on the Cytodex 3 dextran surface.

The quantity of MSCs in the bone marrow is very low, and hMSCs-BM have to be strongly expanded to obtain clinically relevant cell numbers. One possibility to expand cells in a microcarrier culture is the use of a technique called bead-to-bead transfer or colonization. By adding fresh microcarriers into the existing culture, cells can switch from one carrier to another, and start to grow on the empty beads [4]. This allowed us to avoid subculturing techniques traditionally used with adherent cells, and was successfully employed for the culture of cell lines such as Vero cells [5], as well as for hMSC expansion [6]. We used MTT coloration to visually monitor cell proliferation, and to detect the appropriate
moment to add fresh microcarriers. When stained cells were visible on all beads (Figure 2A and 2C) we added fresh microcarriers to offer additional growth surface. Figure 2B and Figure 2D demonstrate that the colonization already started the day after carrier addition.

Because the cell proliferation rate is different depending on the microcarrier type used, we followed each culture independently. In the culture performed on Cytodex type 1 we added fresh microcarriers at days 6 and 10, while additional beads were added at days 6 and 15 into the culture using Cytodex type 3 carriers.

We cultured hMSCs-BM for 20 days on Cytodex type 1 microcarriers and for 27 days on Cytodex type 3 microcarriers. During the expansion we counted the cells at different time points. As shown in Figure 3, we obtained the best proliferation rate on Cytodex type 1 microcarriers. The cell number increased 17.5 fold to a maximum cell density of $1 \times 10^8$ cells/bioreactor at day 14, corresponding to $4 \times 10^5$ cells/mL. On Cytodex type 3 microcarriers, 20 days of culture were needed to reach a maximum cell number of $7 \times 10^7$ cells per bioreactor, which is 11.5-fold higher than the initial seeded quantity and corresponds to $2.5 \times 10^6$ cells/mL.

Close glucose monitoring revealed a rapid nutrient consumption of the hMSC-BM culture on Cytodex type 1 microcarriers (Figure 4A) and on Cytodex type 3 microcarriers (not shown). Two days after cell seeding, the glucose concentration had decreased by 40 %. Despite the addition of 100 mL of fresh medium at day 2 the glucose concentration continued to drop. To maintain the culture until its maximum yield, we regularly exchanged the medium as described in the Material and Methods section. As a result of glucose consumption and feeding, the glucose concentration varied in a characteristic pattern, but never dropped below 0.1 g/L. As by-products of their glucose metabolism, the cells produce metabolites, such as lactate and ammonia, which accumulate in the medium. High lactate concentrations can affect hMSCs growth as well as cell morphology. We monitored the lactate concentration throughout the entire cultivation period and maintained it below 25 nM to ensure that a concentration of 35.4 nM, which was found to inhibit growth [7], was never reached (Figure 4B).

Multipotency analysis of hMSC-BM cultured in BioBLU 0.3c Single-Use Vessels

To confirm that hMSCs-BM cultured on microcarriers in BioBLU 0.3c Single-Use Vessels retained their differentiation capacity, we harvested cells from the microcarriers and used them for in vitro osteogenic and chondrogenic differentiation assays. Since the highest cell density was obtained on Cytodex type 1 microcarriers, only data generated from cells expanded on this support are shown. However, we obtained
equivalent results with cells cultured on Cytodex type 3 microcarriers.

Osteogenic differentiation is divided into three stages: cell proliferation (from day 1 to 4), extracellular matrix maturation (from day 5 to 14), and finally, matrix mineralization (from day 14 to 28). This last step is characterized by high expression of osteocalcin and osteopontin, followed by calcium and phosphate deposition [8]. Bone is composed of the organic protein collagen and the inorganic mineral hydroxyapatite. By specific staining of the hydroxyapatite portion, bone mineralization can be quantified during the differentiation process. As shown in figure 5A, bone mineralization was detectable already after 14 days of induction and was clearly increased at day 21. Hydroxyapatite was only detected on induced cells.

After 21 days of osteogenic lineage induction, calcium deposits, indicating the matrix mineralization phase, were furthermore detected by Alizarin Red S staining on induced hMSCs-BM, while we observed no deposition in the not-induced negative control (Figure 5B).

Next we assessed chondrogenic differentiation, which is a complex, multi-stage process characterized by the production of cartilage-specific molecules such as type II collagen and proteoglycans. Detectable by Alcian Blue staining, proteoglycans are a good indicator of cartilage formation and are considered to be a marker of cell chondrogenesis. After 14 days of chondrogenic lineage induction, we detected strong cartilage proteoglycan synthesis, while the level stayed low in the negative control (Figure 6).

These results clearly demonstrate that the differentiation potential of hMSCs-BM is maintained after intensive expansion on microcarriers in BioBLU 0.3c Single-Use Vessels. We successfully differentiated cells into osteocytes and chondrocytes, indicating that multipotency is maintained when hMSCs are cultured in a stirred-tank bioreactor.

Figure 4: Metabolic profiles of the hMSC-BM culture on Cytodex type 1 microcarriers.
A: Glucose consumption profile of the hMSC-BM culture on Cytodex type 1 microcarriers. A representative result is shown.
B: Lactate production in the hMSC-BM culture on Cytodex type 1 microcarriers. A representative result is shown.

Fig. 5: Osteogenic lineage differentiation
B: Alizarin red S staining of hMSCs-BM cultured on Cytodex type 1 microcarriers. Negative control (left) and induced cells (right) on type 1 microcarriers. A representative result is shown.
Conclusion

In this application note we demonstrate the successful expansion of hMSCs-BM in an Eppendorf DASbox Mini Bioreactor System equipped with BioBLU 0.3c Single-Use Vessels. We evaluated Cytodex type 1 and Cytodex type 3 microcarriers as growth surfaces for these adherent stem cells. We obtained the highest cell number on Cytodex type 1 microcarriers, with a 17.5-fold expansion, corresponding to a maximum cell density of $1 \times 10^8$ cells/batch at day 14. On Cytodex type 3 microcarriers we reached a maximum cell number of $7 \times 10^7$ cells/bioreactor, which is 11.5-fold higher than the initial seeded quantity. In this study, we furthermore demonstrated that expansion in BioBLU 0.3c Single-Use Vessels did not affect the hMSC multipotency, as they conserved their ability to differentiate into osteocytes and chondrocytes. Taken together, these results demonstrate that the DASbox Mini Bioreactor System equipped with BioBLU 0.3c Single-Use Vessels is suitable to expand multipotent stem cells in a safe and controlled manner.
Literature


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Large-scale Production of Human Mesenchymal Stem Cells in BioBLU® 5c Single-use Vessels

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Abstract

Stem cell-based regenerative medicine has great potential to revolutionize human disease treatments. Among the various stem cell platforms, mesenchymal stem cells (MSCs) represent one of the highest potentials. Although successful expansion of MSCs in vitro has been well established, the large-scale production of MSCs remains a bottleneck. In this study, we demonstrate a successful large-scale bioprocess application of adipose-derived mesenchymal stem cells (AdMSCs) in an industrial single-use vessel at 3.75 liter (L) scale (working volume).

The vessel offers a precision controlled environment for the ideal growth of stem cells under simulated physiological conditions. Stem cells and culture media were monitored, analyzed, and controlled, thus allowing us to produce AdMSCs in large-scale quantities while maintaining healthy stem cell properties as evidenced by stem cell marker assays and differentiation assays performed at the end of the culture. Furthermore, every cell culture step from T-flask to shake flask to bioreactor vessel was conducted strictly using single-use consumables.

Introduction

Stem cells are undifferentiated cells which have the capability of self-renewal and the potential to differentiate into a variety of cell types, thus performing a critical role in tissue repair and regeneration. Stem cells can be broadly classified as: embryonic, adult, and induced pluripotent stem cells (iPSCs). Adult stem cells can be further characterized by their tissue of origin, such as: hematopoietic, mammary, intestinal, mesenchymal, endothelial, neural, and hair follicle stem cells. According to recent market reports, mesenchymal stem cells (MSCs) are the most studied stem cells [1 – 3].

Like other adult stem cells, adipose-derived mesenchymal stem cells (AdMSCs) express all of the common stem cell markers and can be differentiated into various types of specialized cells under appropriate growth conditions. AdMSCs have a unique advantage over other MSCs, since they can be isolated in large quantities from fat tissue and are resistant to apoptosis [2, 4 – 8].

Although MSCs have enormous potential for regenerative medicine, drug screening, and drug discovery, their applications are limited by the quantities required for industrial applications [8]. Here in this study, we scaled-up AdMSC culture from shake flasks, a method previously developed in our lab [9], into a BioBLU 5c (Eppendorf) single-use vessel. In the vessel, cell samples, and medium can be analyzed throughout the expansion process and the growth process can be tightly controlled (e.g., oxygen, pH, temperature, glucose, glutamine, lactate, ammonia, etc.), thus allowing us to produce AdMSCs in large-scale quantities.
Materials and Methods

Initial cell culture in T-flasks
AdMSCs were obtained from American Type Culture Collection (ATCC®, PCS-500-011™) at passage 2 and cells were seeded at a density of 5,000 cells/cm² into a T-75 cm² flask (USA Scientific®, CC7682-4175) using 15 mL of mesenchymal stem cell basal medium (ATCC, PCS-500-030™). The medium was supplemented with components of the Mesenchymal Stem Cell Growth Kit (ATCC, PCS-500-040™) at the following concentrations: 2 % fetal bovine serum (FBS), 5 ng/mL rh FGF basic, 5 ng/mL rh FGF acidic, 5 ng/mL rh EGF, and 2.4 mM L-alanyl-L-glutamine.

Preparation of microcarrier
Prior to the start of the experiment, polystyrene (SoloHill® Engineering, P-221-040) and collagen coated microcarriers (SoloHill Engineering, C102-1521) were prepared according to the manufacturer’s instructions, including sterilization.

Cultivation of cells on microcarriers in shake flasks
Cultivation of AdMSCs on microcarriers in shake flask culture was performed as described previously [9].

pH mixing study
In order to determine the lowest speed of agitation required for sufficient mixing, a pH-based mixing study was performed at various speeds such as: 25, 35, and 55 rpm according to Xing, Kenty, Li, and Lee [10]. Briefly, a pH sensor was calibrated using different standard buffer solutions and placed inside a bioreactor containing PBS buffer. 4 N NaOH at 0.5 % vessel working volume (3.75 L) was added to the bioreactor which created a pH disturbance. The pH value was continuously recorded until reaching a steady state. After each run, the pH value of the bioreactor was brought back to initial pH using 4 N HCl. The homogeneity (H) of pH mixing was calculated and plotted against elapsed time using the following equation:

\[ H(t) = \frac{\text{pH}(t) - \text{pHi}}{\text{pHf} - \text{pHi}} \times 100 \]

> H(t) = homogeneity at time t
> pH(t) = pH value at time t
> pHf = final pH value under the complete homogenized condition
> pHi = initial pH value upon trace (NaOH) addition

Optimization and cultivation of AdMSCs in BioBLU 5c single-use vessels
Two independent large-scale experiments were performed in BioBLU 5c single-use vessels using two different microcarriers: the first experiment was performed with polystyrene and the second experiment with collagen coated microcarriers. The New Brunswick™ CelliGen® BLU benchtop bioreactor used for each independent experiment was configured with low-flow thermal mass flow controllers (TMFCs) providing a gas flow range of 0.002 – 1.0 Standard Liters per Minute (SLPM) and an included overlay with a control range of 0.01 – 5.0 SLPM.

For the first experiment, polystyrene microcarriers containing AdMSCs were harvested from shake flask cultures after 15 days of growth and seeded directly into the BioBLU 5c single-use vessel containing 1.5 L of AdMSC complete medium with microcarriers at a concentration of 15 g/L. Following the day of inoculation, another 1.5 L of AdMSC complete growth medium was added to the vessel with microcarriers at a concentration of 45 g/L, to reach the final concentration of microcarriers (30 g/L). The agitation speed was set at 25 rpm. The temperature was set at 37 °C. The pH of the bioreactor was maintained at 7.0 by the controller using automatic addition of CO₂ gas and 7.5 % sodium bicarbonate (NaHCO₃) solution. During the experiment, the dissolved oxygen (DO) level was set to 10 % and the controller was set to 4-gas mode to automatically maintain the DO setpoint by delivering 4 gases (air, CO₂, N₂, and O₂) through the overlay (vessel head space). The overlay gas flow was maintained at 0.1 SLPM during the first 10 days of the experiment. After 10 days, the overlay gas flow was increased to 0.3 SLPM. A 25 % medium exchange was performed at day 5 and an additional 0.75 L AdMSC complete medium was added at day 11 to reach the maximum working volume of the vessel (3.75 L). Furthermore, a 50 % medium exchange was performed at day 14.

For the second experiment, collagen coated microcarriers containing AdMSCs were harvested from shake flask cultures after 15 days of growth and seeded directly into the BioBLU 5c single-use vessel (Figure 1) containing 3.5 L AdMSC complete medium with collagen coated microcarriers at a concentration of 17 g/L. The initial agitation speed was again set to 25 rpm. After 1 h of incubation, the cell culture volume was adjusted to total 3.75 L with 0.25 L of serum-containing medium to reach a final FBS concentration of 4 % and the targeted level of final concentration of growth supplements (10 ng/mL final concentration of rh FGF basic, rh FGF acidic, and rh EGF, and 2.4 mM final concentration of L-alanyl-L-glutamine). Most of the bioreactor control parameters were the same as the first bioreactor run, except that the agitation speed was increased to 35 rpm after 6 days of cell culture. In addition, the overlay gas flow was increased to 0.3 SLPM and N₂ gas was introduced at 0.01 SLPM through the macrosparger to maintain the DO level at 15 %. A 50 % medium exchange was performed at days 4, 8, and 12 with AdMSC complete medium containing 0.1 % Pluronic®-F68 surfactant (Thermo Fisher Scientific®, 24040-032) and 0.5 g/L of glucose was added to the vessel at day 15 to sustain cell growth without additional media exchange.
Cell counting and metabolite measurement

Cells on microcarrier beads were counted by NucleoCounter® NC-100™ (ChemoMetec® A/S) according to the manufacturer’s protocol. The supernatants collected during cell counting were used for metabolite measurement using the automated Cedex® Bio Analyzer (Roche®). In addition to the NucleoCounter, a Vi-CELL® XR (Beckman Coulter®) was also used to count the cells that were collected from T-75 cm² flasks. Vi-CELL was not used for counting cells from the microcarrier culture due to the risk of jamming the Vi-CELL’s needle sipper with microcarriers.

Stem cell surface marker assays

To assess the quality of AdMSCs after expansion and to confirm that the stem cell markers were retained during cultivation in the vessel, CD44, CD90, and CD105-specific fluorescent immunoassays were performed using the following procedure: cells on the microcarrier beads were fixed with 4 % paraformaldehyde for 30 min, followed by Dulbecco’s PBS (DPBS), Ca²⁺ and Mg²⁺ free (ATCC, 30-2200™) wash 3 times, and blocked with 5 % FBS at room temperature for 1 h. Immunostaining was performed using BioLegend® FITC-conjugated anti-human CD44 antibody solution containing the nuclear stain 4’, 6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific, P36935) for 1 h at room temperature. For immunostaining of the CD90 and CD105 markers, cells were fixed and blocked using the same protocol as described above. The cells were incubated with mouse anti-human CD90 and CD105 antibodies (Abcam®, ab23894 and ab44967) for 1 h and washed 5 times with room temperature DPBS for 5 min each. The cells were further incubated with Alexa-Fluor® 546 and Alexa-Fluor 594 anti-mouse secondary antibodies (Thermo Fisher Scientific, A21123 and A21125) and DAPI solutions at room temperature for 1 h. The cells were washed 5 times with room temperature DPBS for 5 min each and visualized under an EVOS® FL LED-based fluorescence microscope (Thermo Fisher Scientific).

Isolation of cDNA and polymerase chain reaction (PCR) amplification of stem cell markers

Total RNA was isolated from the AdMSCs grown on the microcarrier beads and T-75 cm² flasks using TRIzol® reagent (Thermo Fisher Scientific, 15596-018). cDNA was synthesized using the High-capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, 4374966) in a Mastercycler® pro thermocycler (Eppendorf). The primer sequences and PCR conditions used for the CD45, CD105, and beta actin genes were described previously [11]. The Oct3/4 and Sox2 genes were amplified using primer pair kits from R&D Systems® (RDP-321 and RDP-323). The Human CD44 gene was amplified using forward 5’ AGAAGAAAGCCAGTGCGTCT 3´ and reverse 5’ GGGAGGTGTTGGATGTGAGG 3´ primers, which were designed using the BLAST program with Entrez Gene: 960 human as a template. The following program was used for amplification: Step 1: 94 °C for 4 min; 35 cycles of Step 2: 94 °C for 45 sec, 60 °C for 45 sec, 72 °C for 45 sec; Step 3: 72 °C for 10 min and Step 4: 4 °C hold. All the primers were validated by aligning with respective gene sequences using the BLAST program.

Stem cell differentiation assays

AdMSCs were harvested from the bioreactor into 50 mL conical tubes (USA Scientific, 1500-1200). Once the microcarrier beads settled to the bottom of the tube, the supernatants were removed and cells were washed with DPBS. Afterwards, the microcarrier beads were treated with 5 mL of prewarmed trypsin-EDTA solution (ATCC, PCS-999-003”) at 37 °C for 10 min. During incubation, the tubes were occasionally vortexed for 2 s and then neutralized by adding an equal volume of trypsin neutralizing solution (ATCC, PCS-999-004”). Microcarrier beads were allowed to settle to the bottom of the
tube and the supernatants were collected as soon as possible. Microcarrier beads were washed 2 – 3 times with DPBS and the supernatant was combined into a 50 mL tube. Following washing, AdMSCs were collected from the bottom of the tube by centrifugation at 120 x g for 5 min and resuspended into 5 mL of mesenchymal stem cell medium. Cells were seeded at a density of 18,000 cells/cm² into 6-well plates (USA Scientific, CC7682-7506). Differentiations were induced with Adipocyte (ATCC, PCS-500-050™) and Osteocyte (ATCC, PCS-500-052™) Differentiation Toolkits. Following manufacturer’s instructions, differentiated adipocytes were identified by Oil Red O staining (ScienCell™, 0843) and osteocytes were identified with Alizarin Red S staining (ScienCell, 0223). Both were visualized using an Olympus® CK40 inverted microscope equipped with an Infinity2 CCD camera (Lumenera®).

Results and Discussion

From the mixing study, it was found that 100 % homogeneity was achieved by 120 s with agitation at 55 rpm, whereas 90.4 % and 84 % homogeneity were achieved by 35 and 25 rpm agitation, respectively (Figure 2). MSCs are very sensitive to shear force damage; gentle agitation at lower rpm is preferred whenever possible. Since a significant amount (≥ 84 %) of homogeneity was achieved in the BioBLU 5c at 25 or 35 rpm within 2 min, the bioreactor agitation speed was maintained between 25 and 35 rpm during the entire experiment. AdMSCs were initially expanded under shake flask culture conditions using single-use polycarbonate flasks. Microcarriers containing AdMSCs were collected from these flasks and used to inoculate the BioBLU 5c single-use vessel with an initial cell density of 5,000 cells/mL. For the first experiment, 30 g/L of microcarrier was used in order to explore the maximum microcarrier concentration for AdMSCs cultured under a controlled environment. Although AdMSCs quickly expanded in the bioreactor within 24 h of inoculation, there was a 4 day lag phase in cell growth following the addition of high concentration of microcarriers. This might be due to collisions between microcarriers and shear forces resulting from the ultra-high density microcarrier use. The initial culture also showed that the DO level could not be maintained at the 10 % setpoint. Thus, the overlay gas flow was increased to 0.3 SLPM after 10 days of cell growth. However, the 0.3 SLPM overlay gas flow was still not enough to bring the DO down to the 10 % setpoint. Direct gas sparging was not used in this experiment, but was subsequently used in later experiments. The actual DO fluctuated around 20 % throughout the bioreactor run. After the 50 % medium exchange on day 14, cell growth increased and reached its maximum density of 3.9 x 10⁴ cells/mL by day 18. The final density was ~7-fold higher than the initial cell density (Figure 3).

Figure 2: Homogeneity curves during the pH-based mixing study at various rpm in a BioBLU 5c single-use vessel

Figure 3: Growth profile of AdMSCs in BioBLU 5c single-use vessel with polystyrene microcarrier beads
A: Cell density in single-use vessel
B: Glucose and Lactate concentrations over time
Since the maximum expected AdMSC density was not achieved from the first bioreactor experiment using polystyrene microcarriers, a second experiment was performed using collagen coated microcarriers. Recent studies have shown that collagen coated microcarriers may support higher MSC density in single-use vessels [12, 13]. In the second experiment, microcarriers containing AdMSCs were collected from shake flasks and inoculated into the bioreactor for a final density of 17,500 cells/mL. Medium exchanges were performed every 4 days during the experiment. The DO was set to a more controllable 15 % and maintained using N₂ addition through the overlay. Beginning on day 6, N₂ gas was also introduced through the sparger at 0.01 SLPM. Since 100 % DO was calibrated using 100 % air, 15 % DO setpoint represents only ~3 % O₂ in the medium, still within the targeted hypoxic physiological conditions (2 – 5 % O₂). Furthermore, the agitation speed of the bioreactor was increased to 35 rpm to support the complete suspension of AdMSCs containing microcarriers in the BioBLU vessel. Pluronic-F68 surfactant (0.1 %) was also introduced into the medium to reduce foaming resulting from N₂ sparging. Pluronic-F68 is also known to protect cell membranes and reduce the shear force during cell culture agitation [14]. Cell growth steadily increased in the bioreactor from day 6 which was accompanied by an increase in glucose consumption and lactic acid production. Although cells were still metabolically active at day 15 as seen from continued glucose consumption and lactic acid production, the addition of 0.5 g/L glucose at day 15 did not result in a significant increase in cell growth (Figure 4), which indicated that AdMSCs reached a stationary state. This might be due to cell growth being limited by either space for propagation or exhaustion of certain essential nutrients other than glucose. After 16 days of cell culture, AdMSCs in the vessel reached a maximum density of ~2.4 X 10⁵ cells/mL (0.24 million cells/mL), which was about 14-fold higher than initial seeding density.

Figure 4: Growth profile of AdMSCs in BioBLU 5c single-use vessel with collagen coated microcarriers
A: Cell density
B: Glucose and Lactate concentrations over time: 50 % medium exchanged was performed every 4 days and 0.5 g/L glucose was added at day 15
To confirm that AdMSCs retained their stem cell properties during expansion in the bioreactor, immunostaining of stem cell surface markers was performed. MSCs express various cell surface markers such as: CD73, CD90, CD105, and CD44 [3 – 5, 7]. Microcarrier beads that contained AdMSCs were characterized based on surface marker expression using CD44, CD90, and CD105-specific antibodies followed by fluorescence imaging. The results revealed that AdMSCs retained stem cell surface markers during the experiment (Figure 5).

**Figure 5**: Stem cell marker identification immunoassays for AdMSCs expanded on microcarriers in bioreactor  
**A**: AdMSCs on microcarrier beads are positive for CD44 stem cell marker, as indicated in green by fluorescence imaging  
**B**: AdMSCs on microcarrier beads are positive for CD90 stem cell marker, as indicated in red by fluorescence imaging  
**C**: AdMSCs on microcarrier beads are positive for CD105 stem cell marker, as indicated in red by fluorescence imaging
In addition to immunostaining, PCR was also performed to monitor gene expression of additional stem cell markers. PCR data revealed that AdMSCs collected towards the end of the bioreactor culture were positive for CD44, CD90, CD105, Oct3/4, and Sox2 gene expression, whereas they were negative for CD45 gene expression. The post-bioreactor stem cell marker gene expression was compared to cells cultured on T-75 cm² flasks. From the comparison, it was observed that AdMSCs collected from the bioreactor and T-75 cm² flasks prior to bioreactor culture had the same stem cell marker gene expression pattern (Figure 6).

Figure 6: PCR analysis of multipotency markers in AdMSCs cultured in T-flasks and in BioBLU single-use vessels

For gel A & B: M: DNA ladder; Lane 1: Positive control synthetic DNA sequence for Oct3/4 or Sox2; Lane 2: PCR negative control; Lane 3: Sample at 0.2 million cells/mL; Lane 4: Sample at 0.24 million cells/mL; Lane 5: Sample from T-75 cm² flask at passage 4; Lane 6: Sample from T-75 cm² flask at passage 5

For gel C, D, E, & F: M: DNA ladder; Lane 1: PCR negative control; Lane 2: Sample at 0.2 million cells/mL; Lane 3: Sample at 0.24 million cells/mL; Lane 4: Sample from T-75 cm² flask at passage 4; Lane 5: Sample from T-75 cm² flask at passage 5
To further confirm that the AdMSCs cultured in the bioreactor retained their differentiation capacity, adipocyte and osteocyte differentiation assays were performed. AdMSCs were collected from the microcarrier beads and seeded into 6-well plates containing either adipocyte or osteocyte differentiation media. In the osteocyte differentiation medium, cells transformed into long polygonal shaped osteocytes and produced calcium deposits in the extracellular matrix.

On the other hand, when cells were treated with adipocyte differentiation medium, cells became oval shaped and accumulated lipid droplets. After 21 days of culture, the plates were stained with Oil Red O or Alizarin Red S staining solutions. Microscopic observation revealed that the AdMSCs from the bioreactor were successfully differentiated either into adipocytes or osteocytes (Figure 7).

Since the Vi-CELL could not be used for cell counting in the presence of microcarriers, the NucleoCounter NC-100 was used to conduct daily cell counts throughout the bioreactor run. However, the NC-100 appears to have a smaller dynamic range as compared to Vi-CELL, thus giving inaccurate readings at higher cell densities. In order to provide more accurate cell counts, a comparative study was performed between the NC-100 and the Vi-CELL. For this purpose, AdMSCs were collected from T-75 cm² flasks and counted with both the Vi-CELL and the NC-100 counter after a 3-fold dilution. The cell count results indicated that at high cell concentrations, the NC-100 undercounts the cells significantly as compared to the industry standard Vi-CELL. In the high cell concentration range, Vi-CELL reported on average 1.8-fold higher than the NC-100 from the same sample (Figure 8).

**Figure 7:** Differentiation assays for AdMSCs expanded on microcarriers in bioreactor
A: Adipogenic differentiation formed lipid droplets as indicated by Oil Red O positive staining
B: Osteogenic differentiation caused calcium mineralization of extracellular matrix as indicated by Alizarin Red S positive staining

**Figure 8:** Comparison between Vi-CELL and NC-100 for measuring cell counts from the same AdMSC samples
A corrected bioreactor cell growth profile was provided based on NC-100 to Vi-CELL correlation using the averaged correction factor of 1.8 (Figure 9). The peak cell density reached ~0.43 million cells/mL in the BioBLU single-use vessel’s 3.75 L maximum working volume, resulting in a total cell number yield of ~1.6 billion cells (1.62 x 10⁹) on day 16. Such a large quantity is necessary for stem cell therapy using MSCs. It was estimated that the average human would require approximately 1 billion cells per treatment dose [15].

**Figure 9:** Vi-CELL corrected growth profile of AdMSCs in BioBLU 5c single-use vessel using collagen coated microcarriers

Conclusion

Our study clearly demonstrated the feasibility of using BioBLU 5c single-use vessels for the production of large-scale MSCs. The BioBLU 5c single-use vessel has a maximum working volume of 3.75 L, capable of producing large-scale MSCs in a single run. In addition, BioBLU 5c is equipped with a pitched-blade impeller which allows stem cells to be cultured under low rpm conditions to avoid shear force damages.

In this study, we have also shown that AdMSCs cultured in BioBLU 5c single-use vessels retained their differentiation and multipotency properties as evident by immunostaining, PCR, and differentiation assays. The above studies validated the general applicability of the CelliGen BLU benchtop bioreactor and BioBLU single-use vessels for large-scale process optimization and production of stem cells.

Besides the BioBLU 5c, Eppendorf also manufactures BioBLU 0.3c, BioBLU 1c, BioBLU 14c, and BioBLU 50c single-use vessels which are equipped with a range of reaction volumes (up to 40 L working volume). The larger single-use vessels will allow for the production of larger numbers of MSCs from a single bioreactor run.
Literature


## Ordering information

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Scalable Expansion of Human Pluripotent Stem Cells in Eppendorf BioBLU® 0.3 Single-Use Bioreactors

Ruth Olmer1, Christina Kropp1, Claudia Huether-Franken2, Christiane Schlottbom2*, and Robert Zweigerdt1

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Abstract

The routine application of human pluripotent stem cells and their derivatives in regenerative medicine and innovative drug discovery will require the constant supply of high cell numbers in consistent, high quality. Well monitored and controlled stirred-tank bioreactors represent suitable systems to establish up-scalable bioprocesses enabling the required cell production. The following application note describes the successful cultivation of human pluripotent stem cells in suspension culture using Eppendorf BioBLU 0.3 Single-use Vessels in a DASbox® Parallel Mini Bioreactor System.

Introduction

Human pluripotent stem cells (hPSCs), comprising human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs), and their derivatives are considered promising cell sources for novel regenerative therapies [1]. Cell therapies aim at the replacement of cell or tissue loss induced by degenerative disorders such as cardiovascular and neurodegenerative diseases, diabetes and many others, which cannot be healed by currently established, conventional treatments. Moreover, specific human cell types derived from hPSCs by differentiation can be utilized for the development of yet unavailable in vitro disease models, novel drug discovery strategies and more predictive drug safety assays.

Most of the envisioned clinical and industrial applications will require billions of lineage-specific cells which cannot be produced by conventional surface-adherent 2-dimensional (2D) cultures. Stirred-tank bioreactors, which are widely used in the biopharmaceutical industry for the generation of recombinant proteins expressed in mammalian (tumor) cell lines, provide numerous advantages for process development, as they allow for online monitoring and control of key process parameters such as pH, oxygen tension and biomass formation. Advanced bioreactor systems which have been developed in a wide range of culture vessels also facilitate the straightforward scale-up...
to larger process dimensions. However, cultivation and differentiation of hPSCs in stirred bioreactors apparently require the adaptation of cell cultivation from the established 2D surface-adherent culture to 3-dimensional (3D) suspension culture. It was recently demonstrated that hPSCs can be successfully grown as free floating, “cell only aggregates” in small-scale suspension [2-4]. Based on this knowhow the transfer to a DASGIP® Parallel Bioreactor System with four individually controlled glass vessels having a working volume of 100 - 250 mL each, was established [5]. Optimization of stirring-controlled aggregate formation from single cell inoculated hiPSCs led to an approximately four-fold cell expansion resulting in 2 x 10^8 cells per vessel (100 mL) using a fed-batch process. However, with regard to the envisioned clinical application of hPSCs, the possibility to utilize single-use culture vessels, which will support the development of GMP-conform processes, is of great interest. Subsequently, aim of this work was to establish a suspension culture of hiPSCs in a parallel DASbox Mini Bioreactor System equipped with fully instrumented BioBLU 0.3 Single-Use Vessels.

Materials and Methods

Experiments were performed utilizing the cord blood derived hPSC line hCBiPSC2 [6]. Suspension cultures were initiated by detachment and dissociation of hiPSC monolayer cultures with accutase (Life Technologies). Single cells were suspended in mTeSRTM1 (STEMCELL Technologies, Vancouver, Canada) supplemented with the ROCK inhibitor Y-27623 (10 µM). Each BioBLU 0.3 single-use vessel was equipped with probes for pO_2 and pH. The pH probes were calibrated by two-point calibration. pO_2 probe calibration was conducted under process conditions: headspace gassing with 3 sL/h air plus 5% CO_2, stirring at 70 rpm utilizing an pitched-blade impeller [5, 7], 37°C in 100 mL mTeSRTM1; after stable pO_2 values were reached a slope calibration was performed. For culture inoculation 25 mL of a single-cell suspension were added to achieve a density of 5 x 10^5 cells/mL in the final 125 mL culture volume. After 48 h the entire medium was replaced daily (batch feeding) excluding cell loss. For cell counting and other analysis a sampling volume of 3.5 mL was harvested daily without medium replacement to prevent culture dilution. This strategy resulted in subsequent culture volume reduction from 125 to approximately 100 mL during the 7 day process duration. Beside pO_2 and pH, glucose and lactate concentrations, viable cell counts and the expression of pluripotency markers were monitored. Daily viable cell counts were performed via a trypan blue exclusion assay after cell-aggregate dissociation by collagenase B (Roche) treatment. Pluripotency assessment was performed by flow cytometry analysis specific to SSEA4 and TRA1-60.

Results and Discussion

24 h after inoculation of respective single cell suspensions to BioBLU 0.3 Single-Use Vessels small cell aggregates with an average diameter of 58.1 ± 23.1 µm emerged in the stirred cultures. These aggregates, which showed a highly homogeneous size distribution throughout the process, increased in size over the cultivation period resulting in an average diameter of 139.25 ± 25.37 µm (figure 2) on day 7.

![Figure 2](image2.png)
A robust ~4-fold increase in viable cell count was achieved in this fed-batch process resulting in an average cell concentration of \(2.1 \times 10^6\) cells/mL on day 7 and thus a total cell yield of \(~2.1 \times 10^8\) cells per vessel (figure 3).

Monitoring the metabolic activity revealed ~47% of glucose consumption and accumulation of 7.4 mM lactate at 48 hours. The metabolic activity was also followed by online measurements of pH and pO₂. Increasing cell numbers over time resulted in a maximum pH drop to 6.8 (figure 4; as compared to pH 7.4 in fresh medium) and dissolved oxygen levels decreased to 57% (data not shown). The expression of pluripotency-associated surface markers TRA 1-60 and SSEA4 were determined at the process endpoint to evaluate the quality of the expanded hPSCs. Flow cytometry revealed that the majority of the yielded cell population retained expression of these markers i.e. 84% positivity for TRA 1-60 and 90% for SSEA4 (figure 5) was observed suggesting maintenance of pluripotency in this cultivation process.

**Conclusion**

This set of experiments demonstrates the successful expansion of human pluripotent stem cells applying the DASbox system in combination with BioBLU 0.3 Single-Use Vessels. In a 7 day-lasting expansion process in stirred suspension culture cell yields of up to \(2.3 \times 10^8\) cells /100 mL were obtained, which is in good agreement with our previous data in the DASGIP Parallel Bioreactor System, stirred glass vessel system (DS0200TPSS; 100-250 mL working volume) [5]. Notably cells generated by the described process retained expression of established, pluripotency associated cell surface markers. The work confirms the general applicability of the culture system for hPSC expansion in stirred suspension and reveals the DASbox system in combination with BioBLU 0.3 Single-Use Vessels to be an excellent platform for further process optimization and future adaptation to lineage-specific hPSC differentiation processes.

**References**

### Ordering information

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Massively Expanding Stem Cell Suspensions

Achieving Optimal Cultivation and Maintaining Pluripotency and Differentiation Potential Counts

**Ruth Olmer, Ph.D., Sebastian Selzer, and Robert Zweigerdt, Ph.D.**

Human pluripotent stem cells (hPSCs) and their derivatives have gained increased importance for industrial applications in recent years. They have a great potential for therapeutic applications as well.

In vitro assays and novel regenerative therapies will require large cell quantities produced under defined conditions.

In conventional mammalian cell culture, the utilization of bioreactors is well established, e.g., for the production of recombinant therapeutic proteins, vaccines, and antibodies. Established protocols are used for process development and manufacturing of mammalian cells in 100–1,000-L scale and beyond.

Cell cultivation in stirred tank bioreactors allows for tight control and online monitoring of all relevant process parameters such as temperature, agitation, pH or dissolved oxygen, and scaleup.

This know-how can serve as a basis for creating processes to cover the demand on hPSCs, including human induced pluripotent stem cells (hiPSCs) and human embryonic stem cells (hESCs).

There already have been investigations on cultivating adherence-dependent stem cells in suspension culture. These studies mainly focused on the modification of matrix-attached hESC cultivation on microcarriers, a method that is widely used in conventional mammalian cell culture.

However, culture heterogeneity due to the preference of undifferentiated hPSCs to stick to each other rather than to prescreened types of microcarriers might be challenging. Recent studies in our labs and elsewhere have now demonstrated the potential of cultivating undifferentiated human ESCs and iPSCs as cell-only-aggregates in suspension.

More recently, we have demonstrated the feasibility of translating this approach into stirred tank reactors, paving the way for the envisioned mass production of pluripotent stem cells and their derivatives. Key features of this technology include utilization of a fully defined serum-free culture medium, single cell-based inoculation, and significant long-term expansion of hPSCs in easy-to-scaleup suspension independent of extracellular matrices or scaffolds.

The method was successfully applied to several human hPSC lines and cynomolgus monkey ES cells as well. In a previous step, transfer from static suspension in culture dishes to stirred spinner and shaken Erlenmeyer flasks was also enabled.

Such dynamic cultivation of cell-only-aggregates turned out to be robust regarding the reproducibility of cell expansion, karyotype stability, and overall preservation of the stem cells’ pluripotency. Somewhat lower expansion rates in dynamic conditions further suggested a high potential for culture optimization by applying a more controlled environment.

To improve culture monitoring and control capabilities and to pave the way for larger-scale cultivation, the method was transferred to a stirred tank bioreactor system.

Studies were carried out in a Dasgip parallel bioreactor system consisting of four 250 mL cultivation vessels.

**Ruth Olmer, Ph.D., is senior scientist and Robert Zweigerdt, Ph.D., is principal investigator, both at Hannover Medical School, Leibniz Research Laboratories for Biotechnology and Artificial Organs supported by the REBIRTH cluster of Excellence. Sebastian Selzer (s.selzer@dasgip.de) is engineer for hardware development at Dasgip. Web: www.dasgip.com.**
(100–250 mL working volume) including an integrated Dasgip control unit and software. Allowance was made for independent monitoring and control of temperature, pH, oxygen tension, and stirring conditions.

To test and ensure reproducibility, all approaches were performed in four independent experimental repeats in a culture volume of 125 mL each. Utilizing cord blood endothelial cell-derived hiPSCs, preliminary experiments showed that the inoculation density and the agitation mode were highly critical for successful process initiation in impeller-stirred bioreactors.

Experiments revealed that 5x10^5 hPSCs/mL was an efficient inoculation density in the stirred bioreactor setting. Subsequently, experiments were carried out to compare impeller designs and stirring speed modulation aiming at optimal control of cell aggregation and homogeneity of aggregate size distribution.

### Impeller Design

Axial 8-blade pitched impellers and modified stirring bars were designed...
and tested for comparative agitation studies (Figure 1). The basic design of the pitched blade impeller was previously developed and successfully applied to control single cell-inoculated mouse ESC aggregate formation in a 2-L bioreactor scale.

In the context of the 250 mL vessels inoculated with 125 mL culture volume, impeller dimensions were down-scaled to ensure similar geometries of the reactor-impeller design. Impeller variants also differed in blade size and ankle as outlined in Figure 1.

To initially evaluate suitability of the agitation system, cell-free assessment of microcarrier distribution in the bioreactor was tested providing a meaningful and cost-efficient substitute of cell aggregates. All impeller designs and stirring bars were analyzed at agitation speeds varying between 30–60 revolutions per minute (rpm; Figure 1). These experiments revealed that all three 8-blade 40 mm impeller variants as well as a 60 mm stirring bar resulted in homogeneous carrier distribution at 40 or 60 rpm, respectively. Having the blade impellers’ applicability for the common use with bioreactors in mind, only these devices were chosen for the subsequent cell culture experiments.

Undifferentiated hiPSC cultures (expanded in conventional 2D culture) were dissociated and inoculated as single cell suspensions at 4–5 x 10⁵ cells/mL in a total volume of 125 mL and stirred at 60 rpm. To visualize the impact of the three different 8-blade impeller design variants, aggregate diameter analysis and size distribution was carried out by light microscopy. Notably, all approaches resulted in successful aggregate formation from single cells whereby minor variability between the three impeller designs was detected (Figure 2). Growth kinetics and metabolic activity revealed robust and reproducible hiPSC expansion in the stirred bioreactor system.

Evaluation of viable cell numbers in eight independent bioreactor runs over the time revealed, on average, a robust, about four-fold expansion of the inoculated hiPSCs after seven days of cultivation (Figure 3). Monitoring the metabolic activity by determining glucose, lactate, and amino acid concentrations as well as pH and dissolved oxygen levels confirmed efficiency and high reproducibility of the culture system.

Importantly, further analysis proved maintenance of pluripotency-associated marker expression of suspension culture expanded cells, and functional assessment confirmed their multilineage differentiation in vitro.

**Conclusions**

In summary, advanced parallel bioreactor systems are highly suitable for the transfer of single cell inoculated human pluripotent stem cell suspension to fully controlled cultivation. The precise process control, detailed online monitoring as well as evaluation and optimization of complex, multifactorial culture parameters will further allow significant process optimization in ongoing studies.

Capable of operating with even small working volumes of 125 mL (while the functionality and geometry is similar to larger-scale stirred bioreactors), these reactor systems are matching demands of process development and optimization for human stem cell cultivation. The yield of 2 x 10⁸ pluripotent cells in a single process run in the 125 mL scale shown in our previous study has proven the technology to be suitable to enable the mass expansion of human pluripotent stem cells.

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**Figure 3. Growth kinetics and metabolic activity of hiPSCs suspension cultures: Up to 5.5 fold increase in cell numbers was achieved in individual runs over seven days. The dashed lines on glucose and lactate concentration, pH, and pO₂ graphs illustrate medium exchange.**
Stem cell culture in stirred-tank bioreactors makes scale-up easier and allows comprehensive monitoring and control of parameters like temperature, pH, and dissolved oxygen. Here are some tips to help you transfer your stem cell culture from dishes and flasks to bioreactors.

1. Culture surfaces
   In bioreactors, adherent stem cells can be expanded in suspension as cell-only aggregates or on microcarriers. The size of cell-only aggregates can be influenced by seeding density, stirring speed, and the bioreactor impeller design. Culture on microcarriers under restrictive cell culture conditions (e.g., a serum-free medium) requires coating them with peptides or proteins like fibronectin or collagen.

2. Inoculation
   Some guiding values for culture on microcarriers:

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<td>Microcarrier loading density</td>
<td>1–4 g dry beads/L</td>
</tr>
<tr>
<td>Cell-to-bead ratio</td>
<td>min. 3–5 cells/bead</td>
</tr>
</tbody>
</table>

   To improve cell attachment:
   > Reduce initial culture volume
   > Do not agitate during the first few hours

3. Cell expansion
   Bead-to-bead transfer: The progressive addition of fresh microcarriers increases the surface area for growth while avoiding dissociating cells from the beads (passage step).
   > Visually monitor the carrier occupation percentage closely to determine the optimal timing for addition of fresh carriers.

4. Case-by-case optimization needed
   Due to cell heterogeneity (tissue sources, storage conditions, preexpansion conditions, culture medium, and others) and the large number of interactive process parameters (dissolved oxygen, pH, stirring speed, cell substrate, bioreactor type, and the like), each process will require individual optimization.
   > Software-aided monitoring and control of critical process parameters helps to improve process stability and reproducibility.
Fermentation
Setup of a Microbial Hyaluronic Acid Production Process Using the BioFlo® 120 Bioprocess Control Station

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Contact: becken.u@eppendorf.com

Abstract

Achieving optimal process conditions for microbial growth and production of a desired bioproduct needs hardware and software to monitor and control the bioprocess parameters, including the pH, temperature, and agitation of the fermentation broth. Intuitive bioprocess software and an easy-to-use bioprocess controller can accelerate process development, because they reduce the time needed to train staff, prepare the system, and start the process run. Researchers at Praj Industries, India used the BioFlo 120 bioprocess control station to produce hyaluronic acid (HA) in \textit{Streptococcus zooepidemicus} at bench scale. In this application note we describe how they set up, monitored, and controlled critical process parameters, and thus present an impression of the controller’s integrated, touchscreen-accessible software.

Introduction

HA is a polymer composed of repeating disaccharide units of \(\beta-1,3-N\)-acetyl glucosamine and \(\beta-1,4\)-glucuronic acid. High concentrations of HA are present in the skin, the vitreous humor, and the umbilical cord. HA is also a component of the capsules of certain microbial strains. It is industrially produced for a variety of biomedical applications and cosmetics, mainly by microbial fermentation of natural producers like \textit{Streptococcus spec.} or recombinant bacterial strains.

Praj Industries Limited based in Pune, India, offers innovative solutions for beverage alcohol and bioethanol plants, brewery, water and wastewater treatment plants, critical process equipment and systems, and bioproducts. They tested the BioFlo 120 bioprocess control station (Fig. 1) for HA production in \textit{Streptococcus zooepidemicus}, and in this application note describe the setup and operation of the fermentation process.

Fig. 1: The BioFlo 120 bioprocess control station equipped with fermentors for microbial applications.
Material and Methods

Bacterial strain, preculture, and inoculation
The researchers at Praj Industries used *Streptococcus equi subsp. zooepidemicus* (ATCC® 39920) to produce HA. To prepare the preculture for the fermentation process, they inoculated 50 mL of medium with a single colony grown on brain heart infusion agar and incubated the culture at 37°C for 12 to 16 hours. The medium contained 2 g/L glucose, 10 g/L beef extract, 20 g/L polypeptone, 5 g/L yeast extract, 2 g/L NaCl, 1 g/L Na₂HPO₄, and 0.12 g/L K₂HPO₄.

The main culture was inoculated with 5 % (v/v) of the preculture. The main culture medium contained 40 g/L glucose, 20 g/L polypeptone, 10 g/L yeast extract, 2 g/L NaCl, 1 g/L MgSO₄, and 2.5 g/L K₂HPO₄.

Cultivation in the BioFlo 120 fermentor
The researchers carried out the fermentation in a 2 L glass vessel in a working volume of 1.3 L. The culture was agitated using a direct-drive motor. The vessel was equipped with analog sensors to measure pH and dissolved oxygen, and the temperature was controlled using a heat blanket. The bioprocess system’s integrated software gives an overview of these vessel parameters in the system setup screen (Fig. 2).

Process parameters were controlled with the Eppendorf bioprocess software built into the controller. The fermentation was performed at 37°C with agitation at 300 to 400 rpm. The pH was controlled at pH 7.0 by automatic addition of 5 M NaOH. Figure 3 shows the user interface to set up the pH control. The pH interface screen can be used to alter the pH setpoint for process control, the Proportional and Integral Settings for the PID controller, and the deadband used. The setpoints of other process parameters can be adjusted in a similar way.

The summary screen (Fig. 4) offers an overview of the settings for the various process parameters, showing the process value, setpoint, control mode and controller output for each of them.

With the Eppendorf bioprocess software all data measured online can be seen in a single trend screen (Fig. 5). It is simple and easy to export the data from the controller to Excel®, using a USB flash drive.

Culture feeding
Ten hours after the start of the fermentation the researchers began to feed the culture with a solution containing 60 % glucose. Using the system’s integrated pump 0.416 mL of feed solution was added per minute.

Offline analytics
For offline measurements the researchers took samples every three to six hours.

They monitored bacterial growth by measuring the optical density of the culture at 600 nm (OD₆₀₀). The glucose

* The screenshots shown do not originate from the described experiments and values may differ.
concentration in the medium was determined using the Accu-Chek® Active glucose monitoring system (Roche®, Switzerland). The researchers measured the viscosity of the culture to monitor HA production qualitatively. After 24 hours, they determined the concentration of HA in the culture supernatant quantitatively using a carbazole assay with D-glucuronic acid as the standard [1].

Results and Discussion

Figure 6 summarizes the results of the fermentation run of Streptococcus zooepidemicus. Within 24 hours the culture reached an OD_{600} of 18. During the first 13 hours of the fermentation the glucose concentration in the medium decreased constantly and then increased again due to the addition of feed solution. HA production caused an increase of the viscosity of the culture up to 2142 cps at 24 hours. This corresponds to a final titer of 3.5 g/L HA.

Although the process conditions were not optimized it is clear that the BioFlo 120 bioprocess control station provides favorable conditions for microbial production of HA in a standard glass stirred-tank bioreactor.

Conclusion

Using the example of a microbial HA production process we show several software features for the setup, monitoring, and control of critical process parameters. The combination of a local touchscreen and the user-friendly Eppendorf bioprocess software has proven to make process control on the BioFlo 120 simple and straightforward.

Literature

Ordering information

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<thead>
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<td>B120AVB007</td>
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</tbody>
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For more information on these and other configurations visit www.eppendorf.com/BioFlo120

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eppendorf@eppendorf.com · www.eppendorf.com

www.eppendorf.com

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Push-Button Simplicity: Automatic Fermentation with the BioFlo® 120 Auto Culture Mode

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¹Eppendorf Inc., Enfield, CT, USA; ²Eppendorf Manufacturing Corp., Enfield, CT, USA.
Contact: becken.u@eppendorf.com

Abstract

The Auto Culture modes, standard on the BioFlo 120 bioprocess control station, offer push-button automatic process control for some of the most common microbial (E. coli) and mammalian (Chinese Hamster Ovary, CHO) cultures. When activated, process control loop modes and setpoints are automatically turned on and populated with values recommended by our experienced applications development team. The Auto Culture modes allow users, who are less familiar with bioreactors and fermentors, to achieve quick and easy initial culture success while undergoing a minimal learning curve. All setpoints and modes of operation can be adjusted, optimized, and saved as user-defined recipes, which are collected into the Auto Culture library for future use. For this application note, an E. coli batch fermentation was conducted using an Auto Culture mode to demonstrate this novel feature of the BioFlo120 control station.

Introduction

Learning to use a bioprocess controller is a complex and often intimidating endeavor for the beginner. Indeed, even with previous bioprocess experience, moving to a new software platform can entail much learning and reduce efficiency. Although many textbooks and manuals exist on the subject, they are no substitute for hands-on experience. With the Auto Culture modes of the BioFlo 120 bioprocess control station, the user can select either a pre-defined E. coli batch fermentation protocol or a CHO batch cell culture process, which begins at the push of a button (Figure 1). The Auto Culture modes are populated with setpoints and cascades tested by our applications development team and backed by the expertise developed over hundreds of experiments in our applications lab. The user needs only select the vessel size and type from the list of available choices, and make standard preparations (sensor and pump...
calibration, vessel preparation) for the run. Once ready, the user simply presses the “play” button and the system does the rest.

Here we give an overview on the settings and parameters of the Auto Culture mode for the cultivation of *E. coli* and test the feature in a batch fermentation process.

Material and Methods

**Inoculum preparation**

We used an *E. coli* strain (ATCC® 25922GFP™) which produces green fluorescent protein (GFP). The inoculum and fermentation medium was Terrific Broth (TB), prepared as described previously [1]. We prepared the inoculum by inoculating two 1 L baffled shake flasks (VWR®, USA), each containing 200 mL of TB medium, using a frozen vial from a mini cell bank [2]. The flasks were then incubated overnight at 37 °C and 200 rpm in an Eppendorf Innova® 44 shaker.

**Fermentation**

To demonstrate the ease of use of the Auto Culture mode, we performed a standard *E. coli* batch fermentation. This process involved three steps:

1. **Preparation of the control station**

   In preparation for push-button fermentation, we selected the correct vessel configuration on the Setup screen. For the experiment, a 2 L autoclavable, heat-blanketed, direct-drive vessel was selected. We calibrated the gel-filled pH sensor according to standard protocol using pH 7 and pH 4 buffers. We calibrated the pumps per the protocol outlined in the BioFlo 120 Operating Manual. The BioFlo 120 used in this experiment had the hardware configuration shown in Table 1.

2. **Vessel preparation**

   We added 2 L of TB medium to the fermentor before sterilizing the vessel.

   We calibrated the DO sensor according to the protocol outlined in the BioFlo 120 Operating Manual.

   A sterile bottle containing 25 % (v/v) ammonium hydroxide was connected to a liquid addition port for pH control. The tubing was connected to pump 1, which served as the base pump. Acid was not connected for this experiment. If the user desires, an acid bottle can be connected through pump 2. Auto Culture pH control would call for base from pump 1 and acid from pump 2, as needed.

   Finally, the vessel was inoculated with 100 mL of the inoculum (5 % of the initial working volume).

3. **Culture start**

   As shown in Figure 1, the Auto Culture mode offers push-button control. To begin the culture, the “play” button for *E. coli* was pressed. After confirming that the sensors were calibrated, the process began when all the relevant control loop modes were automatically changed to the appropriate state. The setpoints for each control loop were auto-populated as outlined in Table 2.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Configuration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gas mix</td>
<td>Automatic gas mix</td>
</tr>
<tr>
<td>Gas flow control</td>
<td>One thermal mass flow controller (TMFC) with 0-20 standard liters per minute (SLPM) flow range</td>
</tr>
<tr>
<td>Vessel</td>
<td>Heat-blanketed glass vessel with baffle assembly (maximum working volume of 2.2 L)</td>
</tr>
<tr>
<td>Motor</td>
<td>Direct drive</td>
</tr>
<tr>
<td>Impeller</td>
<td>Two Rushton-type impellers</td>
</tr>
<tr>
<td>Sparger</td>
<td>Ring sparger (macrosparger)</td>
</tr>
</tbody>
</table>
Sampling and monitoring the fermentation

The fermentor was monitored offline by taking a 5 mL sample hourly using the swabable Luer Lock port. Cell growth was monitored by offline measurement of the OD_{600} value with an Eppendorf BioSpectrometer® kinetic photometer. To measure GFP production, a Bacterial Cell Lysis Kit (GoldBio®, USA) was employed to release the GFP from the cells into the supernatant. The GFP yield was then quantified using an Eppendorf BioSpectrometer fluorescence photometer.

DO control during fermentation

Since oxygen supply is often the critical limiting factor during fermentation, care was taken to ensure that the Auto Culture mode responds to culture demand appropriately.

Usually, user-defined cascades for DO control that adjust the agitation speed, gas flow, and oxygen concentration are established over time, after optimization of a process by the scientist. In the Auto Culture mode, a tested cascade is provided for every vessel configuration and automatically populated and activated when Auto Culture is initiated. This DO control cascade is shown in Figure 3 for the 2 L autoclavable vessel used at maximum working volume. For each control loop on the summary screen, CSC (Cascade) indicates that the control loop is involved in a user-defined automatic control algorithm. The maximum gas flow rate is set to 1 Vessel Volume per Minute (VVM) as had been determined sufficient in previous experiments [3].

The control loops that are enabled in the DO cascade operate in series, resulting in the first loop (in this case, agitation) reaching maximum setpoint before the next control loop (in this case, gas flow) responds. Therefore, in this experiment, agitation will increase to a maximum of 1,200 rpm to attempt to maintain DO at setpoint before gas flow will begin to increase from a minimum of 0 SLPM to a maximum of 2.2 SLPM. By the time the cascade is completely executed, agitation reaches 1,200 rpm, gas flow reaches 2.2 SLPM, and O_{2} as a percentage of total flow reaches 100 %. All of this occurs automatically, without user intervention.

Table 2. E. coli Auto Culture mode setpoints and loop modes which are populated upon start. Loop setpoints listed as “Auto” are determined by the DO control cascade. *Maximum flow rate is determined upon pump calibration.

<table>
<thead>
<tr>
<th>Loop name</th>
<th>Mode</th>
<th>Setpoint</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agitation</td>
<td>Cascade</td>
<td>Auto</td>
</tr>
<tr>
<td>Temperature</td>
<td>On</td>
<td>37°C</td>
</tr>
<tr>
<td>pH</td>
<td>On</td>
<td>7.0; deadband = 0.1</td>
</tr>
<tr>
<td>DO</td>
<td>On</td>
<td>30 %</td>
</tr>
<tr>
<td>Gas flow</td>
<td>Cascade</td>
<td>Auto</td>
</tr>
<tr>
<td>Air</td>
<td>On</td>
<td>Auto</td>
</tr>
<tr>
<td>O_{2}</td>
<td>Cascade</td>
<td>Auto</td>
</tr>
<tr>
<td>Pump 1</td>
<td>On – base assignment</td>
<td>25 % of maximum flow rate*</td>
</tr>
<tr>
<td>Pump 2</td>
<td>On – acid assignment</td>
<td>25 % of maximum flow rate*</td>
</tr>
</tbody>
</table>

Sampling and monitoring the fermentation

The fermentor was monitored offline by taking a 5 mL sample hourly using the swabable Luer Lock port. Cell growth was monitored by offline measurement of the OD_{600} value with an Eppendorf BioSpectrometer® kinetic photometer. To measure GFP production, a Bacterial Cell Lysis Kit (GoldBio®, USA) was employed to release the GFP from the cells into the supernatant. The GFP yield was then quantified using an Eppendorf BioSpectrometer fluorescence photometer.

DO control during fermentation

Since oxygen supply is often the critical limiting factor during fermentation, care was taken to ensure that the Auto Culture mode responds to culture demand appropriately.

Usually, user-defined cascades for DO control that adjust the agitation speed, gas flow, and oxygen concentration are established over time, after optimization of a process by the scientist. In the Auto Culture mode, a tested cascade is provided for every vessel configuration and automatically populated and activated when Auto Culture is initiated. This DO control cascade is shown in Figure 3 for the 2 L autoclavable vessel used at maximum working volume. For each control loop on the summary screen, CSC (Cascade) indicates that the control loop is involved in a user-defined automatic control algorithm. The maximum gas flow rate is set to 1 Vessel Volume per Minute (VVM) as had been determined sufficient in previous experiments [3].

The control loops that are enabled in the DO cascade
Results

The batch *E. coli* fermentation using a GFP-expressing strain in Auto Culture mode was completed successfully. As shown in Figure 4, within 6 h, the OD<sub>600</sub> value reached 14 and the GFP production was 650 relative fluorescence units/mL. Since a batch culture protocol does not include nutrient or carbon source addition, nutrients were depleted and the culture entered stationary phase around 7 hours, and we ended the experiment. The growth curve is typical for a batch fermentation and provides necessary strain characterization information to begin designing a fed-batch or continuous bioprocess.

Auto Culture mode allows for the optimization of parameters based on experimental need, with the option to save a new custom recipe which is then available in the Auto Culture menu for future use. Each time a new production strain is developed, the batch culture allows the scientist to determine the appropriate growth parameters. In this case, our GFP-expressing strain grew satisfactorily at 37 °C and at pH 7.0. If, on the other hand, the experiment had required a custom temperature or other setpoint, those changes could be made at any time. When the experiment is finished and the ideal setpoints determined, the recipe can be saved as a custom Auto Culture mode available to be automatically employed just like the pre-loaded *E. coli* template. In this way, the number of available Auto Culture modes grow with experience, allowing for the creation of a library of custom recipes.

![Fig. 4: E. coli growth curve and GFP production yield.](image)

RFU: Relative fluorescence units

Fig. 4: *E. coli* growth curve and GFP production yield.

Conclusion

The BioFlo 120 (Figure 2) is a benchtop bioprocess system that uses proprietary software to monitor and control a wide array of fermentation and cell culture applications, and can be employed for batch, fed-batch or continuous cultures. The BioFlo 120 is equipped for use with BioBLU® Single-Use Vessels up to 40 L working volume as well as industry-standard glass autoclavable vessels up to 10.5 L working volume. With the option of mass-flow-controlled gassing and automatic mixing of up to four gasses, the control station is well equipped for dissolved oxygen (DO) control in a variety of applications. The push-button bioprocess concept reduces the complexity of the design of a new bioprocess. The setpoints and cascades recommended by our experienced application team help to achieve satisfactory bioprocess results in a short time and offer a starting point for further optimization.

In this application note we used the Auto Culture mode for *E. coli* fermentation in a batch process. The BioFlo 120 also offers an Auto Culture mode for the cultivation of CHO cells.
Literature

### Ordering information

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For more information on these and other configurations visit www.eppendorf.com/BioFlo120
Transfer of an Itaconate Production Process in *Ustilago maydis* to the BioFlo® 120 Bioprocess Control Station

Hamed Hosseinpour Tehrani¹, Ulrike Becken², Lars M Blank¹, Nick Wierckx¹

¹Institute of Applied Microbiology, RWTH Aachen University, Aachen, Germany. ²Eppendorf Bioprocess Center, Juelich, Germany

Contact: becken.u@eppendorf.com

**Abstract**

The unsaturated dicarboxylic acid itaconate is used as a building block for the production of pharmaceuticals and adhesives, as a copolymer for synthetic resins, and is a promising starting material for biofuel production. Researchers at RWTH Aachen University have previously optimized a production process for itaconate using a genetically engineered strain of the fungus *Ustilago maydis*. The following application note highlights the simplicity and ease of use of the new BioFlo 120 bioprocess control station, through a successful process transfer from it's predecessor, the New Brunswick™ BioFlo/CelliGen® 115.

**Introduction**

For the bio-based production of itaconate (Fig. 1) natural producer as well as heterologous hosts have been used. Itaconate is naturally synthesized by the fungi *Aspergillus itaconicus* and *Aspergillus terreus*, and *Ustilago maydis*, among others. *Aspergillus* produces itaconate via enzymatic decarboxylation of the tricarboxylic acid cycle intermediate cis-aconitate. This biosynthetic route has been identified many years back. More recently, an alternative biosynthesis pathway starting from trans-aconitate has been described in *U. maydis* [1]. For large-scale itaconate production *U. maydis* can be better, because unlike *Aspergillus spec.* it has a single-cell, yeast-like morphology that avoids problems typical of filamentous fungi, like high viscosity and clogging, hindered oxygen transfer, and sensitivity to mechanical stress.

Researchers at the RWTH Aachen and Marburg University had previously optimized fermentation of a metabolically engineered *U. maydis* strain and produced itaconate in a high-density fed-batch process controlled by a BioFlo/CelliGen 115 bioprocess control station [1]. Now they transferred process control to the BioFlo 120 bioprocess controller (Fig. 2). To compare the results obtained with the two different bioprocess systems, they analyzed cell growth and the concentrations of glucose and itaconate in the culture medium over time.

![Fig. 1: Chemical structure of itaconate](image1)

![Fig. 2: BioFlo 120 bioprocess control station](image2)
Material and Methods

**Itaconate producer strain**

The researchers had previously identified and characterized the gene cluster responsible for itaconate biosynthesis in *U. maydis*. This laid the foundation for the design of the itaconate hyper-producer strain MB215 Δcyp3 petefia1. Overexpression of the gene cluster regulator ria1 and deletion of the cyp3 gene, encoding for a P450 monooxygenase, increased itaconate production by a factor of 4.5 compared to the wildtype and abolished the formation of the byproduct 2-hydroxyparaconate [1].

**Process parameters and control**

Using a BioFlo/CelliGen 115 bioprocess control station, the researchers had previously optimized the process conditions for itaconate production in *U. maydis*. For cultivations with the BioFlo 120 bioprocess controller they used the same process parameters and medium composition. *U. maydis* was cultivated in a working volume of 0.5 L in medium containing 200 g/L glucose, 4 g/L NH₄Cl, 0.2 g/L MgSO₄·7H₂O, 0.01 g/L FeSO₄·7H₂O, 0.5 g/L KH₂PO₄, 1 g/L yeast extract, 1 mL/L vitamin solution, and 1 mL/L trace element solution. The temperature was set to 30°C. The pH was maintained at 6.0 to 6.5 by automatic addition of 10 M NaOH. Dissolved oxygen was controlled at 80 % by aeration with a rate of 1 L/min (2 vvm) and automatic adjustment of the agitation speed between 700 and 1,200 rpm. Process parameters were controlled with the Eppendorf bioprocess control software (Fig. 3) built into the controller. The researchers inoculated the cultures to an optical density (OD₆₀₀) of 0.75 and cultivated them for 168 hours. After 96 hours they fed the cultures with a single feed pulse of glucose.

**Analytics**

DO, temperature, and pH were measured online using analog sensors. The cell density was quantified offline by measuring the OD₆₀₀ of the culture. The researchers quantified the concentrations of glucose and itaconate in the culture supernatant by HPLC.

Fig. 3: Eppendorf bioprocess control software. The setup screen allows to select the vessel and sensors intended to use. All fields in the gas flow indicator and the analog input/output indicator section automatically populate based on the controller configuration.

**Results**

To compare the performance of the processes controlled by the BioFlo/CelliGen 115 and BioFlo 120 bioprocess control stations, the researchers analyzed the cell density of the culture and the concentrations of glucose and itaconate in the culture supernatant over the duration of the processes. Figures 4A and B show previously published results obtained with the BioFlo/CelliGen 115 controller [1]. Results obtained with the BioFlo 120 bioprocess controller were very similar (Figure 4C and D). Within 96 hours the glucose in the culture medium was completely consumed, but temporarily raised again by a single feed pulse at that timepoint. Glucose consumption was accompanied by an increase in cell density to an OD₆₀₀ of around 120 and then stayed almost constant until the end of the process. The itaconate concentration in the culture supernatant increased almost linearly to approximately 60 g/L after 168 hours.
**Conclusion**

By metabolic strain engineering and optimization of bioprocess parameters and medium composition, researchers at the RWTH Aachen increased the itaconate production by *U. maydis* to an industrially relevant level. In just a short period of time working with the new BioFlo 120, the researchers were able to reproduce the results from an already optimized production process on the new system. Impressed with its ease of use, Hamed Tehrani, lead researcher on the project concluded “The results were the same when compared to the BioFlo/CelliGen 115, but the BioFlo 120 proved much easier to use.”

**Literature**

### Ordering information

<table>
<thead>
<tr>
<th>Description</th>
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</table>

For more information on these and other configurations visit [www.eppendorf.com/BioFlo120](http://www.eppendorf.com/BioFlo120)
High-Density *Escherichia coli* Fermentation and Protein Production using the Eppendorf BioFlo® 120 Bioprocess Control Station

Bin Li and Ma Sha
Eppendorf Inc., Enfield, USA.
Contact: beken.u@eppendorf.com

**Abstract**

We used the BioFlo 120 bioprocess control station for the fed-batch fermentation of a GFP-expressing *Escherichia coli* strain. To quantify cell density and protein production we measured the optical density (OD$_{600}$) of the culture and the fluorescence of GFP, respectively. After nine hours of culture, the optical density had increased to 191 and the relative fluorescence units (RFU) to 12,532. These results indicate that the BioFlo 120 supports the growth of *E. coli* to high densities and the production of large amounts of protein.

**Introduction**

The BioFlo 120 (Figure 1) is a benchtop bioprocess system with the flexibility to control both autoclavable and single-use vessels. The system has proprietary software to monitor and control a wide array of fermentation and cell culture applications, and can be employed for batch, fed-batch, perfusion, and continuous cultures. The BioFlo 120 supports the use of BioBLU® Single-Use Vessels as well as industry-standard glass autoclavable vessels. With the option of mass flow-controlled gassing and automatic mixing of up to four gasses, the control station is well equipped for dissolved oxygen (DO) control in a variety of applications, including high-density mammalian cell culture and bacteria and yeast fermentations.

In the project described in this application note, we tested the suitability of the BioFlo 120 bioprocess control station for high-density *E. coli* fermentation.
Material and Methods

E. coli strain, medium, and preculture
We used a GFP-expressing E. coli strain (ATCC® 25922GFP™). A mini cell bank was prepared as described previously [1].

To prepare a preculture, we inoculated two 1 L baffled shake flasks (VWR®, USA), each containing 200 mL of Terrific Broth (TB) medium, from a frozen vial of E. coli inoculum stock. We incubated the culture at 37°C and 200 rpm overnight in an Eppendorf Innova® 44 shaker (Eppendorf, Germany).

For E. coli fermentation, we used chemically defined medium at pH 7.0. We prepared the initial fermentation medium in a 2 L glass vessel as follows: 150 mL 10 x phosphate/citric acid buffer (133 g/L KH₂PO₄, 40 g/L (NH₄)₂HPO₄, 17 g/L citric acid) and 1.3 L deionized water were added to the vessel for sterilization at 121°C for 20 min. After the medium had cooled to room temperature, the following sterile components were added aseptically to make the complete fermentation medium: 15 mL of 240 g/L MgSO₄, 0.34 mL of 20 g/L thiamine solution, 15 mL of 100 x trace element solution, and 25 mL of 70 % glucose solution. The 100 x trace element solution contained: 10 g/L iron (III) citrate, 0.25 g/L CoCl₂ · 6H₂O, 1.5 g/L MnCl₂ · 4H₂O, 0.15 g/L CuCl₂ · 6H₂O, 0.3 g/L H₃BO₃, 0.25 g/L Na₂MoO₄ · 2H₂O, 1.3 g/L zinc acetate · 2H₂O, and 0.84 g/L EDTA [2, 3].

We prepared a concentrated feeding medium in a 500 mL glass bottle. 45 mL of 240 g/L MgSO₄, 1.66 mL of 20 g/L thiamine solution, 15 mL of 100 x trace element solution, and 70 % glucose solution were added to a final volume of 500 mL.

Fermentation
We ran the fermentation in a heat-blanketed glass vessel connected to a BioFlo 120 bioprocess control station. The configurations of the controller and the vessel are outlined in Table 1. We inoculated the culture with 150 mL of the preculture (10 % of the initial working volume).

The fermentation was carried out at 37°C. The pH was controlled at 7.0 (± 0.1) and the DO was set to 30 %. Antifoam 204 (Sigma-Aldrich®, USA) was added only when needed.

pH calibration and control
We calibrated the pH sensor outside the vessel prior to autoclaving, using a two-point calibration method and standard buffers. We used the buffer of pH 7.0 to set “ZERO” and the buffer of pH 4.0 for the “SPAN” (please refer to the BioFlo 120 user manual). For pH control, we connected a sterile bottle containing 25 % (v/v) NH₄OH to a liquid addition port for pH control. The pH was automatically maintained at 7.0 by adding 25 % (v/v) NH₄OH via pump 1 (assigned as base pump). Acid was not connected for this experiment, but if the user desires, acid can be added using pump 3.

Dissolved oxygen (DO) sensor calibration and gassing control
We calibrated the analog polarographic DO sensor using a standard two-point calibration method: 0 % (set “ZERO”) was obtained by disconnecting the sensor from the cabinet and allowing the raw value to stabilize; 100 % (set “SPAN”) was obtained by agitating at 1,000 rpm and 2.0 SLPM air flow until the DO value stabilized at the maximum (please refer to the BioFlo 120 user manual).
DO was controlled using the cascade shown in Fig. 2. It is important to note that the control loops that were enabled in the DO cascade operate in series, resulting in the first loop (in this case, agitation) reaching maximum setpoint before the next control loop (in this case, gas flow) responds. Therefore, in this experiment, agitation will increase to a maximum of 1,000 rpm to attempt to maintain the DO at the setpoint before the gas flow will begin to increase from a minimum of 0 SLPM to a maximum of 2.0 SLPM. When the cascade was fully executed, the agitation maintained 1000 rpm, and the gas flow reached 2.0 SLPM (100 %). The O2 level automatically adjusts as a percentage of total flow. All the controls occurred automatically without user-intervention.

Culture feeding
Starting 3.5 hours after inoculation, the culture was fed with a concentrated feed solution following the protocol shown in Table 2. Pump 2 was assigned as the feeding pump and controlled by the BioCommand® SCADA Software. We set the period of pump control time to 10 seconds.

Analysis
To monitor the fermentation offline, we took a 3-5 mL sample hourly using the swabable valve connected to the sample tube. We monitored cell growth by measuring the optical density of the culture (OD600) with an Eppendorf BioSpectrometer® kinetic photometer. To measure GFP production we released GFP from the cells to the supernatant using a Bacterial Cell Lysis Kit (GoldBio®, USA) [4] and quantified GFP fluorescence with an Eppendorf BioSpectrometer fluorescence photometer. We determined the glucose concentration in the medium using a Stat Profile® Prime Analyzer (Nova Biomedical, USA).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>3.5</th>
<th>4.5</th>
<th>5.5</th>
<th>6.5</th>
<th>7</th>
<th>7.5</th>
<th>8</th>
<th>8.5</th>
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<tr>
<td>Pump speed (mL/min)</td>
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<td>0.3</td>
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<td>0.8</td>
<td>1.2</td>
<td>1.4</td>
<td>1.7</td>
<td>4.1</td>
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</table>

Table 2: Pump speed at different elapsed fermentation times during the fed-batch fermentation. The pump was automatically controlled by BioCommand software.
Results

Within nine hours the culture grew to an optical density of almost 200 (Figure 3). Parallel to the increase of biomass the amount of GFP rose and reached almost 12,532 relative fluorescence units after nine hours (Figure 4). The glucose concentration dropped slowly during the first four hours, increased with the addition of the glucose-containing feed medium, and then declined rapidly (Figure 3).

Conclusion

We used the BioFlo 120 bioprocess control station for an E. coli fed-batch fermentation. Within nine hours the culture grew to an OD$_{600}$ of 191 and produced almost 13,000 RFU of GFP. These results indicate that with the BioFlo 120 control station and vessels high cell densities and high protein titers can be reached.
Literature


### Ordering information

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Scale-Up of *Escherichia coli* Fermentation from Small Scale to Pilot Scale Using Eppendorf Fermentation Systems

Bin Li and Ma Sha  
Eppendorf Inc., Enfield, CT, USA  
Contact: becken.u@eppendorf.com

Abstract

The scale-up of fermentation processes is critical to the success of industrial fermentation for the production of biologics in the biopharmaceutical market. Eppendorf bioprocess systems are available with autoclavable, single-use and sterilize-in-place vessels and together cover a wide range of working volumes from less than 1 L to as large as 2,400 L. In this application note, we used *E. coli* fermentation to demonstrate the scale-up capabilities of Eppendorf fermentation systems from small scale to bench scale and pilot scale.

To determine suitable parameters and setpoints for the operation of each fermentor, we considered critical scalability-related engineering parameters. The parameters described include proportional vessel/impeller geometry, oxygen transfer rate (OTR), impeller power numbers (Np) and impeller power consumption per volume (P/V).

We carried out *E. coli* fermentation runs at three different scales (1 L, 10 L, and 100 L) following a constant P/V strategy, and represented the *E. coli* biomass growth trends by plotting optical density (at 600 nm, OD<sub>600</sub>) curves over time. The fermentation runs at each of the three scales produced very similar biomass yields over time, indicating excellent scalability within the Eppendorf fermentor product family.

Introduction

The ultimate goal in bioprocess development is large-scale commercial production. Production-scale process optimization is usually cost prohibitive, so optimization is often performed in smaller scale bioreactors and fermentors. An optimized small-scale process can then be transferred to pilot scale following established scale-up strategies.

The basics of successful fermentation scale-up start with proportional fermentor and impeller design. Eppendorf fermentation systems were designed following bioprocess industry stirred-tank design standards and provide excellent scalability from geometrical perspective. The intention of this study was to evaluate the scale-up of *E. coli* fermentation. To do so, we first investigated engineering parameters critical for scaling up fermentation processes, such as vessel and impeller geometry, oxygen transfer rate, power number, and impeller power consumption per volume. Then we utilized these data to scale-up an *E. coli* process from small scale (1 L) to pilot scale (100 L) following the constant P/V scale-up strategy.
Materials and Methods

Equipment
The Eppendorf fermentation systems, from small scale to pilot scale, used in this project are shown in Figure 1. Vessel parameters critical for scale-up are listed in Table 1.

Investigation of engineering parameters

Oxygen transfer rate (OTR)
OTR is the rate at which oxygen is transferred from air to the liquid in a vessel, and OTR values provided by equipment manufacturers are often the maximum OTR. Since oxygen is often the limiting factor during aerobic fermentation, it is important to select equipment of different sizes with similar OTR capabilities so that the small-scale success can be replicated at the large scale. We conducted the OTR measurements using a previously published protocol based on sulfite depletion [1], with the exception of air flow, which was set to 1.5 vessel volumes per minute (VVM) to match the specification of the BioFlo® 610.

Power number (Np)
The (impeller) power number (also known as Newton number) is a dimensionless number associated with different type of impellers. Np is commonly used to calculate impeller power consumption during bioprocess scale-up [2]. We determined the power number and P/V values at various scales.

Table 1: Proportionally designed vessels and impellers at different scales

<table>
<thead>
<tr>
<th></th>
<th>Bioblock 1 L</th>
<th>BioFlo 320 10 L</th>
<th>BioFlo 610 100 L</th>
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<tr>
<td>Maximum gas flow (SLPM)</td>
<td>4.2</td>
<td>20.0</td>
<td>150.0</td>
</tr>
<tr>
<td>Vessel type</td>
<td>Glass</td>
<td>Glass</td>
<td>Stainless steel</td>
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<tr>
<td>Working volume (L)</td>
<td>0.2 – 1.0</td>
<td>3.5 – 10.5</td>
<td>32.0 – 100.0</td>
</tr>
<tr>
<td>V&lt;sub&gt;max&lt;/sub&gt; height (mm)*</td>
<td>136.0</td>
<td>323.0</td>
<td>904.0</td>
</tr>
<tr>
<td>Vessel inner diameter (ID) (mm)</td>
<td>100.0</td>
<td>211.0</td>
<td>381.0</td>
</tr>
<tr>
<td>Ratio V&lt;sub&gt;max&lt;/sub&gt; height : vessel ID per impeller</td>
<td>0.7</td>
<td>0.8</td>
<td>0.8</td>
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<tr>
<td>Impeller style; impeller material</td>
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<tr>
<td>Impeller quantity</td>
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<td>2</td>
<td>3</td>
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<tr>
<td>Impeller diameter (D) (mm)</td>
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<td>165.1</td>
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<tr>
<td>Ratio impeller diameter : vessel ID</td>
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<td>0.4</td>
<td>0.4</td>
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<tr>
<td>Max. agitation (rpm)</td>
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<td>1,200</td>
<td>500</td>
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<tr>
<td>Max. tip speed (m/s)</td>
<td>3.85</td>
<td>5.30</td>
<td>4.32</td>
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* V<sub>max</sub> height = Height from bottom of the vessel to the top surface of the liquid at maximum vessel working volume

Table 2: Correlation between tip speeds (m/s) and agitation speeds (rpm) for the three systems

<table>
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<tr>
<th>Tip speed (m/s)</th>
<th>Bioblock 1 L</th>
<th>BioFlo 320 10 L</th>
<th>BioFlo 610 100 L</th>
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<tbody>
<tr>
<td>0.6</td>
<td>249</td>
<td>136</td>
<td>69</td>
</tr>
<tr>
<td>1.2</td>
<td>498</td>
<td>272</td>
<td>139</td>
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<td>1.8</td>
<td>747</td>
<td>407</td>
<td>208</td>
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<td>2.8</td>
<td>1,163</td>
<td>634</td>
<td>324</td>
</tr>
<tr>
<td>3.8</td>
<td>1,578</td>
<td>860</td>
<td>440</td>
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tip speeds toward the higher agitation rates that are usually used for fermentation. Since the controllers display rpm rather than tip speed, a translation between tip speed and agitation rpm for each system is shown (Table 2). One way to experimentally determine the Np is to measure impeller torque using a rotational torque sensor (Figure 2), and to calculate the power number using the following equation [2]:

\[
Np = \frac{2\pi (M - M_d)}{\rho N^2 d^5}
\]

where:
- \(M\): Torque (with full working volume of DI water), (N·m)
- \(M_d\): Torque (empty vessel), (N·m)
- \(\rho\): DI water density = 1,000 kg/m³
- \(N\): Agitation speed (rps)
- \(d\): Impeller outer diameter (m)

It is important to measure net impeller torque without bearing resistance. The impeller torque is equal to the torque value measured in deionized (DI) water at a given agitation rate and then subtracting the torque value measured in the empty vessel at the same agitation rate.

It is a common practice in scale-up studies to determine power numbers without gassing. However, gassing greatly reduces impeller torque, and thus has a significant impact on the apparent impeller power numbers as well as the results of aerobic fermentation. Since typical fermentation experiments are conducted under high gassing conditions, we have decided to obtain Np under a high gas flow of 1.5 VVM in addition to under “no gassing” conditions.

The purpose of determining Np is to calculate the impeller power consumption per liquid volume \((P/V, W/m^3)\). \(P/V\) can be calculated from Np using the following equation [6]:

\[
P/V = \frac{Np \times \rho \times N^3 d^5}{V}
\]

where:
- \(\rho\): DI water density = 1,000 kg/m³
- \(N\): Agitation speed (rps)
- \(d\): Impeller outer diameter (m)
- \(V\): Full working volume (m³)

Bioreactor setup and \textit{E. coli} fermentation

Mini cell bank
We used \textit{E. coli} (ATCC® 25922GFP™) in the fermentation runs of all three different scales. We aseptically thawed the stock vial into a 15 mL conical tube along with 5–6 mL of BD Bacto™ Tryptic Soy Broth (TSB) medium (Becton, Dickinson, USA). We spread 100–500 µL of TSB medium containing \textit{E. coli} onto an agar plate and incubated it at 37°C overnight. We inoculated several 500 mL baffled shake flasks each containing 100 mL TSB medium from a single colony of the agar plate. The culture was grown in a New Brunswick™ Innova® 44 Shaker at 37°C, 200 rpm overnight and then centrifuged in an Eppendorf Centrifuge 5810 R at 4°C, 3,700 rpm for 10 min to collect the \textit{E. coli} pellet. We used an equal volume of TSB medium containing 15 % glycerol to suspend the cells, and the resulting \textit{E. coli} suspensions were mixed and aliquoted into 1 mL tubes for storage at -80°C as mini cell bank.

Inoculum
We retrieved a frozen vial of \textit{E. coli} from the mini cell bank and added 20 µL into 500 mL of TSB medium in each 2 L shake flask (VWR®, UK). We incubated the culture at 37°C, 200 rpm overnight in an Innova 44 shaker to form the \textit{E. coli} inoculum stock. The number of shake flasks needed depended on the vessel sizes in the fermentor step. The OD\textsubscript{600} values of the inocula were typically about 12.

Fermentation and feeding protocol
We chose to use 90 % of the vessel maximum working volume for all three fermentors. \textit{E. coli} was cultured in a chemically defined medium of pH 7.0. The initial fermentation medium we prepared as follows: 10 %
working volume of 10 x phosphate/citric acid buffer (133 g/L KH₂PO₄, 40 g/L (NH₄)₂HPO₄, 17 g/L citric acid) and 76.5 % working volume of deionized water were added to the vessel for sterilization. After the medium was cooled to growth temperature or room temperature, we added the following sterile components aseptically to make the complete fermentation medium: 1 % working volume of 240 g/L MgSO₄, 22.7 ppm of the working volume of 20 g/L thiamine, 1 % working volume of 100 x trace element solution, 1.48 % working volume of 70 % glucose solution and 0.2 % working volume of 500 x carbenicillin stock solution.

The 100 x trace element solution contained: 10 g/L iron (III) citrate, 0.25 g/L CoCl₂ · 6H₂O, 1.5 g/L MnCl₂ · 4H₂O, 0.15 g/L CuCl₂ · 6H₂O, 0.3 g/L H₂BO₃, 0.25 g/L Na₂MoO₄ · 2H₂O, 1.3 g/L zinc acetate · 2H₂O, and 0.84 g/L EDTA [3-4].

We separately prepared a concentrated feeding medium: 5 % feeding medium volume of 240 g/L MgSO₄, 0.83 % feeding medium volume of 20 g/L thiamine solution, and 15 % feeding medium volume of 100 x trace element solution were mixed with 70 % glucose solution to the final volumes of 0.2 L, 2 L, and 20 L for the three different scales. To maintain a constant working volume throughout the fermentation process, a continuous fermentation method was used, and volumes of E. coli culture identical to the volume of feeding medium added were removed upon feeding.

The feeding-in and pumping-out protocol shown in Table 3 illustrates the adjustments made to the pump speed over the course of the fermentation.

In all three fermentors, we inoculated the growth medium with an inoculum volume of 10 % of the initial fermentation medium volume. Antifoam 204 (Sigma-Aldrich®, USA) was added only when foaming was observed. We monitored cell growth offline using samples taken every hour.

**pH calibration and control**

We calibrated the pH sensors outside the vessels prior to autoclaving them, using a two-point calibration method and standard buffers. We used the buffer of pH 7.0 to set “ZERO” and the buffer of pH 4.0 for the “SPAN”/“slope” (please refer to the DASware® control and BioFlo user manuals). The pH was automatically maintained at 7.0 by adding 25 % (v/v) NH₄OH via a pump (assigned as “base”). The deadband for pH control was set to 0.05.

**Dissolved oxygen (DO) sensor calibration and gassing control**

We performed an analog polarographic DO sensor calibration using a standard two-point calibration method: 0 % (set “ZERO”) was obtained by disconnecting the sensor from the cabinet and allowing the raw value to stabilize; 100 % (set “SPAN”) was obtained by running different agitation speeds for DASGIP 1 L, BioFlo 320 10 L, and BioFlo 610 100 L runs (822, 600, and 433 rpm, respectively) and 1.5 VVM (1.35, 13.5, and 135 SLPM, respectively) air flow until the DO value stabilized at the maximum.

### Table 3: Proportional feeding and broth removal strategy for continuous fermentation runs at different scales

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>3.5</th>
<th>4.5</th>
<th>5.5</th>
<th>6.5</th>
<th>7</th>
<th>7.5</th>
<th>8</th>
<th>8.5</th>
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<tbody>
<tr>
<td>Pump speed (mL/min)</td>
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<tr>
<td>Bioblock 1 L</td>
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<td>0.39</td>
<td>0.53</td>
<td>0.67</td>
<td>0.88</td>
<td>1.03</td>
<td>1.7</td>
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<tr>
<td>BioFlo 320 10 L</td>
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<td>1.3</td>
<td>2.0</td>
<td>3.9</td>
<td>5.3</td>
<td>6.7</td>
<td>8.8</td>
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<td>17</td>
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<tr>
<td>BioFlo 610 100 L</td>
<td>7</td>
<td>13</td>
<td>20</td>
<td>39</td>
<td>53</td>
<td>67</td>
<td>88</td>
<td>103</td>
<td>170</td>
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</table>

**Results and Discussion**

As shown in Table 1, all three Eppendorf fermentation systems from small scale to pilot scale were designed following the same vessel and impeller geometrical principles, which laid a good foundation for successful fermentation scale-up. However, it is also important to select equipment of different sizes with high OTR capabilities so that the different fermentation scales can match each other in top line performance, and the small-scale success can be replicated in large scale. As shown in Table 4, all three fermentation systems achieved high levels of...
OTR, ~350 mmol/L/h or higher. This allowed scale-up fermentation runs to be carried out at high capacities, delivering matching biomass growth curves at very high bacterial densities.

Scalable geometry and matching high OTR provide the foundation and the framework for high-density fermentation scale-up experiments, but they did not constitute the scalability strategy in itself. Various strategies have been used for fermentation scale-up including constant tip speed, but the most reliable method to date has been constant power (P/V). It requires the determination of impeller power numbers (Np). We calculated Np numbers at different tip speeds up to 3.8 m/s (limited by the maximum tip speed of the small-scale system) following direct torque measurements described in the Materials and Methods section (Figure 3). Np is a constant under turbulent conditions [5]. Under less turbulent conditions such as a lower tip speed of 0.6 m/s, the Np numbers may be slightly different than at higher tip speeds. Although the actual Np numbers varied slightly at different tip speeds (as shown in Figure 3), they were very similar to each other and the average could be considered a constant in guiding fermentation scale-up. The impeller Np for Eppendorf fermentation vessels are ~10 without gassing and ~5 with 1.5 VVM of air sparging (Table 5).

From the measured Np numbers, we calculated the impeller power consumption per liquid volume (P/V, W/m³) as described in the Material and Methods section (Figure 4). Maintaining constant P/V between vessels is one of the most accepted strategies for scale-up.

To design high-density E. coli fermentation scale-up, the Np values obtained under 1.5 VVM air flow (Figure 4B) were used. We curve fit the data points and added trend lines (Figure 5). The maximum P/V achievable by all three scales was ~2.42 kW/m³, which we selected to be the constant P/V.

### Table 4: OTR measurements from DASGIP Bioblock to BioFlo

<table>
<thead>
<tr>
<th></th>
<th>Bioblock 1 L</th>
<th>BioFlo 320 10 L</th>
<th>BioFlo 610 100 L</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTR (mmol O₂/L/h)</td>
<td>392.6</td>
<td>486.7</td>
<td>349.3</td>
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<tr>
<td>Experimental conditions</td>
<td></td>
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<td></td>
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<tr>
<td>Air flow (SLPM/ VVM)</td>
<td>1.5/1.5</td>
<td>15.75/1.5</td>
<td>150.0/1.5</td>
</tr>
<tr>
<td>Agitation (rpm)</td>
<td>1,530</td>
<td>1,200</td>
<td>500</td>
</tr>
<tr>
<td>Tip speed (m/s)</td>
<td>3.68</td>
<td>5.30</td>
<td>4.32</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Liquid</td>
<td>DI water</td>
<td>DI water</td>
<td>DI water</td>
</tr>
</tbody>
</table>

OTR, ~350 mmol/L/h or higher. This allowed scale-up fermentation runs to be carried out at high capacities, delivering matching biomass growth curves at very high bacterial densities.

### Table 5: Average impeller Np values calculated from the results shown in Figure 3. The Np values at a tip speed of 0.6 m/s were not included in the calculation of the average.

<table>
<thead>
<tr>
<th></th>
<th>Average Np without gassing</th>
<th>Average Np with 1.5 VVM of air flow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioblock 1 L</td>
<td>10.0</td>
<td>4.6</td>
</tr>
<tr>
<td>BioFlo 320 10 L</td>
<td>10.3</td>
<td>5.9</td>
</tr>
<tr>
<td>BioFlo 610 100 L</td>
<td>10.2</td>
<td>5.0</td>
</tr>
</tbody>
</table>
value governing the fermentation scale-up (Figure 5). Back-calculating the agitations from this P/V value determined that 822, 600, and 433 rpm were the agitation values to be used for Bioblock 1 L, BioFlo 320 10 L, and BioFlo 610 100 L, respectively. Furthermore, all three fermentation runs were conducted under 1.5 VVM of constant air flow.

We conducted three fermentation runs and took samples hourly to monitor the cell growth (OD₆₀₀ value) as described above. As shown in Figure 6, the growth curves from all three fermentation runs produced very similar profiles, indicating that excellent scalability has been achieved using the constant P/V scale-up strategy.

Fig. 4: P/V values calculated based on Np values from Table 5.

Fig. 5: Determining the constant P/V values for scale-up under 1.5 VVM of air flow

Fig. 6: Fermentation biomass growth curves among all three systems. Fermentations were carried out using a constant P/V-value of 2.42 kW/m³, which was determined from Fig. 5.
Conclusions

The Eppendorf fermentors from Bioblock to BioFlo are of geometrically and proportionally similar stirred-tank design. All three systems are capable of delivering high OTR values, providing excellent capability for high density aerobic fermentation in a scalable manner. Maintaining constant P/V between different vessel sizes in fermentation scale-up from Bioblock 1 L to BioFlo 320 10L to BioFlo 610 100 L produced nearly identical *E. coli* growth curves, providing solid proof for the scalability of Eppendorf fermentation systems. All three fermentation systems were able to support high-density *E. coli* growth at or near 140 OD<sub>600</sub> within 9 hours of inoculation. In addition, the Np values of the impellers can be used for further scale-up or scale-down studies between Eppendorf and the stirred-tank fermentation vessels of other manufacturers.

Literature


### Ordering information

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<td>76SR0700ODLS</td>
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<tr>
<td>2x Rushton-type impeller, L-Sparger, 400 mL – 1.5 L</td>
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<td>76SR1500ODLS</td>
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<tr>
<td>1 L Vessel bundle, Water-Jacketed, Magnetic Drive</td>
<td>M1379-0310</td>
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<td>M1379-0312</td>
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<td>M1379-0313</td>
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<tr>
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<td>M1282-0002</td>
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<tr>
<td><strong>Innova® U725</strong>, Ultra Low Temperature Freezer, 725 L, 230 V/50 Hz</td>
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<tr>
<td><strong>Eppendorf BioSpectrometer® kinetic</strong>, 230 V/50 – 60 Hz</td>
<td>6136 000.002</td>
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<tr>
<td><strong>Centrifuge 5810 R</strong>, refrigerated, with Rotor A-4-81 incl. adapters for 15/50 mL conical tubes, 230 V/50 – 60 Hz</td>
<td>5811 000.320</td>
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</table>

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High-Density Fermentation of *Corynebacterium glutamicum* for Renewable Chemicals Production

Judith Becker¹, Stefanie Kind¹, Michael Kohlstedt¹, Ulrike Becken²*, Christoph Wittmann¹

¹ Institute of Systems Biotechnology, Saarland University, Saarbruecken, Germany
² Eppendorf AG Bioprocess Center, Juelich, Germany
* corresponding author: becken.u@eppendorf.com

**Abstract**

A large variety of chemicals can be efficiently produced by microbial fermentation. They are used in a multitude of applications; for example, as biofuels, building blocks for polymers, food supplements, and ingredients for cosmetics. To be profitable, fermentation processes have to deliver the desired end product at a high yield and a high titer. In this application note we describe bioprocesses which were carried out by the team of Professor Wittmann at the University of Saarbruecken. They used engineered *Corynebacterium glutamicum* strains for the production of the compatible solute ectoine and the polyamide building block 1,5-diaminopentane. The researchers optimized culture conditions in an Eppendorf DASGIP® Parallel Bioreactor System and established high-density, fed-batch processes which delivered high titers of the desired chemicals at high yield.

**Introduction**

Professor Wittmann’s team deals with the systems biology of industrially relevant microorganisms, to develop tailored microbial cell factories for sustainable bioproduction. In this application note, they highlight the bioproduction of two different chemicals which are in great industrial demand. The first is ectoine, a compatible solute with cell protecting properties. Among other applications, it is used as an ingredient of cosmetics and healthcare products. The second is 1,5-diaminopentane, which is a promising building block for the production of polyamide. The global market demands 6.6 million tons of polyamide per year for the manufacturing of fibers for clothing, car parts, materials in medical applications, and many other products [1].

In nature, ectoine is produced by halophilic bacteria. As their cultivation in high-saline media imposes great challenges to the fermentor material, there is a growing interest in the development of bacterial strains which produce and secrete ectoine under more standard cultivation conditions.

**Fig. 1:** Ectoine and diaminopentane production.
conditions. In the work presented here, the researchers designed a *C. glutamicum* strain that produces and secretes ectoine without the need for high-saline growth conditions [2], (Fig. 1).

Polyamide is typically synthesized from petrochemical monomers, but its bio-based manufacturing is highly desirable. Producing polymer building blocks through microbial fermentation can save primary energy input and greenhouse gas emissions, and ultimately reduce dependence on fossil fuels. Using systems metabolic engineering, the Wittmann team optimized a *C. glutamicum* strain for diaminopentane production. They achieved this by the overexpression of several enzymes in its biosynthetic pathway, and the shut-down of competing metabolic pathways by gene deletion or attenuation [1], (Fig. 1).

The team used an Eppendorf DASGIP Parallel Bioreactor System to establish fed-batch processes which deliver the desired end products at high yields and high titers. For process optimization, monitoring and control of critical process parameters is key. We will exemplify basic functions of the Eppendorf bioprocess control software, DASware® control 5, in conveniently monitoring, controlling, and displaying process variables.

### Material and Methods

**Strains**

For ectoine production the research team used the *C. glutamicum* strain ECT-2 [2]. Diaminopentane was produced using the *C. glutamicum* strain DAP-16 [1].

**Fed-batch production of ectoine and diaminopentane**

Table 1 summarizes the fermentation conditions for ectoine and diaminopentane production.

<table>
<thead>
<tr>
<th>Table 1: Process parameters</th>
<th>Ectoine</th>
<th>Diaminopentane</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. glutamicum</em> strain</td>
<td>ECT-2</td>
<td>DAP-16</td>
</tr>
<tr>
<td>Initial volume</td>
<td>300 mL</td>
<td>300 mL</td>
</tr>
<tr>
<td>Temperature setpoint</td>
<td>35°C</td>
<td>30°C</td>
</tr>
<tr>
<td>DO setpoint</td>
<td>&gt; 30 %</td>
<td>&gt; 20 %</td>
</tr>
<tr>
<td>pH setpoint</td>
<td>6.9</td>
<td>7.0</td>
</tr>
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</table>

The researchers cultivated *C. glutamicum* in initial working volumes of 300 mL, in an Eppendorf DASGIP Parallel Bioreactor System (Fig. 2) equipped with 1 L DASGIP Bioblock Stirrer Vessels. They monitored and controlled process parameters using DASGIP Control software (now DASware control 5). Temperature was kept constant at 35°C (for ectoine production) and at 30°C (for diaminopentane production), respectively. They controlled the pH by automatic addition of base using an Eppendorf DASGIP MP8 multi pump module. For the control of dissolved oxygen (DO), the researchers implemented a DO cascade which varies the agitation speed and the aeration rate. Such a
DO cascade can be easily set up using DASware control 5 software. Agitation, gas flow, and oxygen enrichment can be individually defined, displayed, and edited online (Fig. 3). The researchers carried out fermentations in the media defined in [1] and [2]. To monitor bacterial growth and the production performance, they quantified optical densities, glucose concentrations, and product concentrations offline (Fig. 4). Glucose concentrations were determined enzymatically using a 2300 STAT Plus™ analyzer or a 2700 Select analyzer (YSI® Inc. / Xylem® Inc., USA). Ectoine and diaminopentane concentrations were determined by HPLC. To achieve high product yields, the Wittmann team established fed-batch processes. For ectoine production, feeding was initiated by a DO-based signal, resulting in glucose concentrations below 5 g/L. For diaminopentane production, feeding was initiated when the glucose concentration had dropped to 10 g/L, thus maintaining glucose concentration above 10 g/L.

Results and Discussion

Ectoine production
Within 8 hours C. glutamicum ECT-2 consumed the initially supplied glucose, and the optical density (OD₆₀₀) of the culture increased to 100 (Fig. 5 A). In the course of this initial batch phase, the ectoine concentration increased to 2 g/L. Growth arrest leads to reduced oxygen consumption and hence an increase in DO. Based on the DO readings feeding was initiated, resulting in glucose concentrations of around 5 g/L. In this fed-batch phase the bacteria did not produce more biomass but shifted the production towards the formation of ectoine. Ectoine concentration reached a final titer of 4.5 g/L after 16 hours (Fig. 5 A). The different cultivation phases gave markedly different product yields. In the initial batch phase, the culture produced 28 mmol ectoine per mol glucose, whereas in the following fed-batch phase the ectoine yield was 298 mmol per mol glucose.

Fig. 5: Fed-batch fermentation of the ectoine producer C. glutamicum ECT-2 in a DASGIP Parallel Bioreactor System. A: Cultivation profile. B: Ectoine yields (mmol per mol glucose) achieved in the batch- and the fed-batch phases. The data represent mean values from two independent fermentation experiments.
(Fig. 5 B). Overall the fermentation produced 6.7 g/L ectoine per day, which is among the highest yields reported in the literature so far.

**Diaminopentane production**

Within 12 hours, the *C. glutamicum* DAP-16 culture reached an optical density of 120, and consumed the initially supplied glucose. Upon glucose depletion, feeding was initiated to maintain glucose concentrations above 10 g/L. *C. glutamicum* continuously secreted diaminopentane, which reached a concentration of 88 g/L within 50 hours. (Fig. 6 A). During the initial batch phase, the culture metabolized 21 % of glucose to diaminopentane. During the subsequent feeding phase, the yield increased to 29 % (Fig. 6 B).

**Conclusion**

For profitable bio-based production, microbial fermentation processes have to deliver the desired end products at high yields and high titers. The results presented here exemplify the successful high-density fermentation of *C. glutamicum* in a DASGIP Parallel Bioreactor System. Cultivation of metabolically engineered strains in optimized fed-batch processes led to product titers which are among the highest reported so far. Bioprocess control software like DASware control 5 can help the researcher to establish efficient production processes. By allowing intuitive monitoring and control of critical process parameters, and the seamless integration of offline measured values it simplifies process optimization and facilitates fermentation under optimal culture conditions.
Literature


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Production of Polyhydroxybutyrate from Lignocellulosic Hydrolysates—Optimization of *Bacillus sacchari* Fermentation and Scale Up from 2 L to 200 L

Bruno Sommer Ferreira¹ and Christiane Schlottbom²

¹ Biotrend – Inovação e Engenharia em Biotecnologia, S.A., Cantanhede, Portugal; ² Eppendorf AG Bioprocess Center, Juelich, Germany; *Corresponding author: schlottbom.c@eppendorf.com

**Abstract**

Biopolymers such as polyhydroxybutyrate (PHB) are considered to be carbon-neutral, and thus environment-friendly, replacements for fossil fuel-derived plastics. They are more expensive, however, and production process costs must be reduced to increase market acceptance. Alternative feedstocks offer a promising way to reduce costs. This application note presents the process development and optimization of wheat straw hydrolysate fermentation to produce PHB in *B. sacchari*. Process engineers at Biotrend® (Portugal) evaluated various ratios of two sugar concentrations on a small scale, using an Eppendorf New Brunswick™ BioFlo®/CelliGen® 115. They established automated feed triggers for the New Brunswick BioFlo 415, 610 and Pro fermentation systems, allowing them to successfully scale up the process 100-fold.

**Introduction**

Research into polyhydroxyalkanoate (PHA) biopolymers (including PHB) has intensified. Since their mechanical, physical, and thermal characteristics are similar to many fossil-fuel-based plastics, such as polypropylene (PP) and polyethylene (PE), they have the potential to replace those high-volume products in certain applications. Naturally produced from sugars by various bacterial strains, they are an environmentally friendly alternative to plastics derived from petroleum and natural gas. PHAs are biodegradable, non-toxic, and can either be thermoplastic or elastomeric materials, making them suitable for applications in biomedicine, packaging, and many other fields.

In 2014, the global production of PHAs was estimated at 54 kilotonnes, with a more than 5-fold market increase expected by 2020 [1]. But although production costs have decreased substantially over the last several decades, PHA prices are still significantly higher than traditional plastics. Feedstocks account for 50% of their average production costs [2]. Using cheaper feedstocks, such as lignocellulosic sugars, would be a major breakthrough in cutting costs.

<table>
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<th>Total [g/L]</th>
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<tbody>
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<td>573</td>
<td>270</td>
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Material and Methods

Precultures

*B. sacchari* was repeatedly sub-cultured in shake flasks using a medium that contained sucrose as the sole carbon source to create a population rich in cells with improved sucrose uptake. After eight subcultures, a culture bank was prepared in cryogenic tubes and stored at -80°C.

The stored cells were used to inoculate shake flasks containing medium with sucrose as the carbon source and the growth was observed closely. The medium for the seed and flask cultures contained: 1.0 g/L (NH₄)₂SO₄, 4.5 g/L Na₂HPO₄·2H₂O, 0.2 g/L MgSO₄·7H₂O, 1.0 g/L yeast extract, and 0.1 % (v/v) trace elements solution [3], supplemented with 20 g/L sucrose. The cultures grew overnight, at 32°C and 150 rpm, in a New Brunswick Innova 44R shaker (Eppendorf AG, Germany).

Evaluation of feed composition at 2 L

A New Brunswick BioFlo/CelliGen 115 controlled the small-scale fermentation runs in a 2 L vessel (0.8 – 2.2 L working volume). The objective of these fermentations was to study the effect of feed composition on biomass and PHB formulation. The medium (1.3 L initial working volume) for the fed-batch cultures consisted of 4.0 g/L (NH₄)₂SO₄, 3.0 g/L KH₂PO₄, 1.7 g/L citric acid, 40 mg/L EDTA, 1 % (v/v) trace elements solution [3], 1.2 g/L MgSO₄·7H₂O, and 20 g/L sucrose. The pH was adjusted to 6.8 with 5 N KOH. Temperature was controlled at 32°C. The culture was inoculated using 65 mL of the precultures grown on sucrose, with an optical density (OD₆₀₀) of 20. The fermentation protocol started with a batch phase using the sucrose as the sole carbon source. Once the initial sucrose was depleted in each 2 L fermentation run, it entered the fed-batch phase. Each culture was fed with one of four solutions that had different ratios of xylose to glucose (table 1). The New Brunswick BioFlo/CelliGen 115 automatically added 50 mL pulses of feed each time a sudden agitation rate decrease indicated a lack of carbon source. The reduced oxygen uptake rate (OUR) of the culture when sugars are exhausted causes the dissolved oxygen concentration (DO) to rapidly increase. The equipment, set to maintain the DO at 10 % by varying the stirrer speed, would then automatically decrease the speed, resulting in a detectable feed trigger. Up to 0.8 L of feed was added during the course of the entire fermentation run. The concentrations of the sugars in the feed were measured offline using HPLC. The concentration of the resulting PHB was measured by determining (also by HPLC) the crotonic acid produced by acid digestion of the biomass, assuming total hydrolysis of PHB into crotonic acid. In order to scale up effectively, the engineers maintained the same impeller tip speed at each scale. At larger fermentation scales, as the impeller radius increases, the rotational speed in revolutions per second (rps) must be reduced in order to maintain the same tip speed (in m/s) as in the smaller scale. The upper part of Figure 3 shows the relationship between impeller diameter and tip speed.

Figure 1: Biotrend facility in Cantanhede, Portugal.

Optimization of feed control at 10 L and 50 L scale

Several fermentations were carried out in 10 L vessels by adapting the optimized fermentation protocol developed at 2 L volume to the larger scale.

A New Brunswick BioFlo 415 benchtop sterilize-in-place unit was used with a 10 L vessel (4.0 – 10.5 L working volume). All fermentations were initiated with 4 L of broth containing 20 g/L of sucrose and 200 mL of inoculum. Feeding started once the initial sucrose had been consumed, using the same automated feeding strategy. All feeds contained 1.5 g/L of phosphate. Another fermentation was performed with phosphate added to the wheat straw hydrolysate. The lignocellulosic hydrolysates were prepared by Biorefinery.de GmbH (Teltow, Germany) from ground wheat straw, using the ammonia fiber expansion (AFEX) process as pre-treatment, followed by enzymatic hydrolysis of the cellulose and hemicellulose fraction [4]. 4 L of feed was added to the vessel.

For the fed-batch phase, the process engineers targeted optimizing the feed rate control switching from an agitation-based feeding to a DO trigger. To avoid the problem of “false” feed triggers caused by momentary increases of the DO readings (e.g., due to accumulated bubbles), they used an algorithm that calculated a moving average of the DO value. It activated feeding, adding a preset volume, when the
moving average DO exceeded 30%.

They then applied this feeding control mechanism to a 50 L fermentation vessel (16 – 50 L working volume), controlled by a New Brunswick BioFlo 610 (figure 1). They used a simulated hydrolysate containing 472 g/L glucose and 302 g/L xylose as feed and applied the fermentation protocol, using a batch phase with 20 g/L of sucrose as carbon source, inoculated with sucrose-grown precultures. The batch phase volume was 20 L, and a total of 20 L of sterilized feed was added on demand, controlled by the DO trigger.

Scale up to 200 L fermentations
After integrating all the information gathered from the earlier experiments, the engineers devised a 200 L fermentation protocol for use in a New Brunswick BioFlo Pro with 240 L vessel (75.5 – 240 L working volume). The seed train for the fermentation required preparation of a preculture grown in shake flasks. A 6 L inoculum was grown in a 10 L fermentor and transferred to the larger scale production vessel. The working volume during the initial batch phase was 100 L. Increases in the moving average DO concentration beyond the 30 % threshold triggered feeding of the sugar mix (520 g/L glucose and 300 g/L xylose) during fermentation. Each feeding pulse lasted for 15 min, and added about 2.7 kg of sugars to the fermentor, for a total of approximately 90 L of feed during the fermentation run.

Results and Discussion
All 2 L fermentations showed similar dynamics in biomass and PHB formation, and feeding proceeded at almost identical rates (Figure 2). Broth xylose concentrations increased with increasing concentrations of xylose in the feed. Once feeding was stopped at the end of the fermentation, all xylose was consumed. Apparently the accumulated xylose (up to 35 g/L) did not inhibit the metabolic activity of the cells, consistent with previously
reported results. Earlier experiments had shown that xylose concentrations higher than 40 g/L did inhibit growth [5]. The results suppose that the developed fermentation strategy will work without problems with real hydrolysates even if their ratio of xylose to glucose varies.

Figure 3 shows the tip speeds calculated from the rotational stirring speeds obtained in fermentations at 2 L, 10 L, and 50 L scales when controlling the fermentations at the same dissolved oxygen level (10 % of saturation). The oxygen transfer required to maintain a specific dissolved oxygen concentration is highly correlated to the tip speed of the impellers. What is more, the figure clearly indicates that the sudden decrease of stirring speed that occurs at the 2 L scale in response to DO increases is less dramatic at the larger scales.

When transferring the 2 L protocol to the 10 L scale, the fermentation resulted in a high concentration of biomass, but the PHB content in the cells was relatively low (data not shown). This led to speculate that higher PHB yields might be obtained by lowering the phosphate content of the feed.

In a next step, a DO-based feed mechanism was implemented. At higher fermentation scales, stirring speeds need to be lowered in order to maintain the same tip speed as in the smaller scale. As the impeller diameter increases, the range of stirring speeds during the fermentation will decrease.

When sugar was present during the fed-batch phase of the 10 L fermentation, the average DO value fluctuated between 0 % and 10 %. It increased rapidly to 30 – 40 % when the sugar in the broth was spent. At this point, a pre-set feed volume was added to the fermentation. The strategy worked flawlessly at the 10 L scale, using a glucose/xylose mixture as feed, providing a reliable means for feed control. In fact, the responsiveness in returning DO levels to the normal range was faster than the agitation-controlled feed used for the 2 L fermentation, with DO-triggered feeding (primary response) responding within 3 – 4 minutes while the agitation trigger (secondary response) took 5 – 10 minutes (data not shown).

The next fermentation, at 50 L scale, accumulated amounts of biomass and PHB equivalent to those produced
at smaller scales (table 2). The dynamics of biomass and PHB levels were similar to those at smaller scales, showing similar glucose and xylose profiles (data not shown). The DO increase in response to exhaustion of glucose diminished towards the end of the fermentation, and the DO trigger ceased to function. At this point, a suboptimal constant feed rate was imposed. It resulted in a decrease of the amount of accumulated xylose in the broth and a decrease in biomass and PHB yields. In the fermentation trials carried out, it was observed that whenever a period of a couple of hours was allowed to elapse between the exhaustion of glucose and the beginning of the feed—forcing the strain to grow exclusively on xylose—the metabolic activity of the culture never fully recovered. Hence, the time between glucose exhaustion and onset of feeding should be minimized.

The concentrations of biomass and PHB obtained in the 200 L fermentation were comparable and even in the higher range of those obtained at lower scale. Indeed, the concentration of PHB was the highest obtained, as was the PHB content of the cells (Table 2).

Figure 4 compares the productivities that were attained at the different scales. The highest productivity was obtained at 2 L scale, but the low feed volume (0.8 L) vs. initial batch volume (0.8 L) did not allow to feed the fermentation during long periods of time. As such the productivity dropped after it reached the maximum. Interestingly, the productivity of the 200 L fermentation started very similarly to the one of the 2 L fermentation, while the 10 L and 50 L fermentations showed time lags. This was caused by the delayed feed described above. The short inflexion of the 200 L productivity curve at about 1 g/(L·h) at 200 L scale suggests that there is further room for optimization of the feeding. But the long productivity plateau at the maximum value of about 1.7 g/(L·h) indicates that during that time the system kept producing PHB in such quantities that it allowed the productivity (PHB produced per unit of volume and unit of time) to stay constant at its maximum value. This is especially remarkable, because the volume of the fermentation broth was continuously increasing, suggesting that the product concentration would drop. Maintaining high productivity is a primary goal when optimizing a bioprocess.

Conclusion

The results confirm that the process engineers at Biotrend achieved a successful fermentation scale up and validated the scalability of the process. Despite the challenges of scaling up bioprocesses, fine-tuning the fermentation protocol in smaller scale transitions resulted in robust and scalable processes. Scale up based on maintaining a constant tip speed is a common strategy, but adjustments may be necessary to manage a scalable yield. The careful, rational, and step-by-step approach, using 5-fold scale increases worked well. The sequence of fermentation trials at successively larger scales was crucial to gathering enough information to successfully deploy a productive process at the largest scale on its first attempt. Overall, the scientists achieved a 100-fold scale increase. Though further improvements are possible, and some opportunities for further optimization have been identified, they have shown that the process is low risk and robust as it scales. It suggests that the same protocol would work at even higher scales in commercial production.

Literature

Ordering information

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<td>New Brunswick™ BioFlo® 415</td>
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<td>New Brunswick™ BioFlo® 610</td>
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<tr>
<td>New Brunswick™ BioFlo® Pro</td>
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This work has received funding from the European Union 7th Framework Programme (FP7/2007–2013) under Grant Agreement number 246449 'BUGWORKERS'.

The authors would like to acknowledge biorefinery.de GmbH (Germany) for providing the hydrolysates.

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Simulating Process Limitations in Microbial Cultivation: A Parallel Two-Compartment Scale-Down Approach

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Abstract

In large-scale industrial bioprocesses, the presence of gradients in critical process parameters, such as dissolved oxygen (DO), pH, and substrate concentration, can be observed. They result in inhomogeneous growth conditions within the bioreactor/fermentor and can affect cell yield and/or productivity. Scale-down approaches at the laboratory scale are a tool to analyze the effects of the inhomogeneity.

Scientists at the Forschungszentrum Jülich have been using a cultivation set-up consisting of two connected stirred-tank reactors (STRs) to simulate inhomogeneous cultivation conditions as they can occur in production scale. In this application note, we describe the effects of oscillating DO and substrate concentration.

This study exemplifies the benefits of flexible bench-scale bioreactor solutions and advanced bioprocess control software. Individual control of parallel operated bioreactors allowed different vessel sizes, individual parameter set-points, and multiple cultivation phases to be implemented easily.

Introduction

Corynebacterium glutamicum is an established host used to produce amino acids for the food and feed industries. The research activities of the Bioprocesses and Bioanalytics group at the Forschungszentrum Jülich are focused on the optimization of microbial bioprocesses. As part of this, the scientists perform high-throughput bioprocess development and characterization, including quantitative microbial phenotyping and next-generation omics technologies.

The project focus was to set up a two-compartment scale-down device composed of two interconnected stirred-tank reactors to mimic inhomogeneous cultivation conditions. The cell suspension was supposed to oscillate between an aerobic and a micro-aerobic compartment with different residence times. Such challenging cultivation conditions can occur in the industrial production scale due to insufficient mixing quality [1 – 3]. Inhomogeneous cultivation conditions potentially result in performance losses and the associated increase of production costs, which can imperil the competitiveness of biotechnological production.

For setting up such a scale-down device, the group was looking for a cultivation platform incorporating four or more fermentors to implement two or more parallel STR-STR set-ups. By combining different vessel sizes, fluid levels, and sensor equipment, the volume ratio between the two compartments as well as the online analytics should be adapted to a broad scope of applications. Additionally, the possibility to monitor and control the conditions in each compartment should enable the observation of separated or combined effects of oscillating pH, temperature, DO, and substrate concentration. In the experiments described in this application note, the effects of oscillatory DO and substrate concentrations on a fed-batch cultivation of C. glutamicum were analyzed.
Material and Methods

**DASGIP® Parallel Bioreactor System**

The STR-STR parallel set-up was implemented using an Eppendorf DASGIP Parallel Bioreactor System and consisted of two interconnected fermentation vessels \([4]\): STR1 (max. working volume = 1.5 L) and STR2 (max. working volume = 300 mL) (Figure 1). The combined working volume of both vessels was 1 L. The volumetric distribution between the compartments was 78 % in the STR1 and 22 % in the additional STR2. Included in the STR2 volume were the connecting tubing (length 1 m; inner diameter 4.8 mm), representing 2 % of the culture volume.

Each vessel was equipped with an optical DO sensor (VisiFerm™ DO 225; Hamilton®, USA) and a pH sensor (405-DPAS-SC-K8S pH probe, sensor length 225 and 120 mm, respectively; Mettler-Toledo®, USA). A DASGIP PH4PO4 monitoring module for pH and DO was used. Titration and substrate feed were realized with a DASGIP MP8 multi pump module. Exhaust was analyzed with a DASGIP GA4. The aerobic compartment (STR1) was aerated by a DASGIP MF4 gassing module and temperature and agitation were controlled by a DASGIP TC4SC4 module. To ensure a constant volumetric distribution between the compartments, a dip tube was implemented in the STR2 at the desired height. Two peristaltic pumps (505U, Watson-Marlow® GmbH, Germany) ensured medium flow between the two vessels: A master pump from STR1 to STR2 was set to a dedicated flow rate and a utility pump enabled flow from

<table>
<thead>
<tr>
<th>Vessel (max. working volume)</th>
<th>STR1</th>
<th>STR2</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.3 L</td>
</tr>
<tr>
<td>pH control</td>
<td>7; acid and base</td>
<td>7; acid and base</td>
</tr>
<tr>
<td>DO control</td>
<td>30 %; Stirrer cascade</td>
<td>0 %; 0.25 vvm N(_2) (non-controlled)</td>
</tr>
<tr>
<td>Agitation</td>
<td>Determined through cascade</td>
<td>1,200 rpm</td>
</tr>
<tr>
<td>Temperature</td>
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<td>30 °C</td>
</tr>
<tr>
<td>Feeding</td>
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![Figure 1: Scale-down setup](image1.png)

**Figure 1: Scale-down setup.**

**A.** Illustration of the STR-STR two-compartment cultivation setup. The device is composed of two interconnected stirred-tank reactors. During cultivation the cell suspension circulates between the aerobic STR1 (78 % of the working volume) and the non-aerated STR2 (22 % of the working volume). The master pump controls the flow rate while the utility pump follows with a 30 % higher pump rate. Two sets of the STR-STR devices were implemented in one cultivation platform.

**B.** DASGIP Parallel Bioreactor System. A 4-fold system for microbial applications is shown. From left to right: The DASGIP Bioblock provides independent temperature control of up to four vessels. DASGIP bioprocess analyzer modules deliver accurate monitoring and control of key process parameters. The DASware® control software features parallel process control with individual control of each vessel.
STR2 back to STR1. The latter one was set to rotate with a 30% higher speed compared to the master pump.

The cultivation runs were performed as a duplicate experiment comprising four vessels in total (2x STR1, 2x STR2). This parallel set-up was controlled by a single PC operating DASware control 5. The software is capable of controlling up to 16 parallel vessels individually and offers process automation features, intelligent recipe management, and integrated reporting.

Three-phased cultivation of *C. glutamicum*
Table 1 summarizes the experimental set-up. The DO concentration in the aerated compartments (STR1) was maintained at 30% by a stirrer cascade. The DO concentration in the smaller compartments (STR2) was supposed to be near 0%, however not controlled. To maintain this micro-aerobic environment, a flow of 3 sL/h N₂ (0.25 vvm) was set through the STR2. The stirrer speed in the smaller STR2 was set to remain constantly at 1,200 rpm. pH in the STR1 and STR 2 was maintained at 7 using 30% (v/v) phosphoric acid and 18% (v/v) ammonia. The non-aerated compartments were programmed to be fed with an exponential feed rate (Figure 2) while STR1 was not fed. The initial batch medium used was a CGXII minimal medium [5] with 5 g/L glucose. To prevent foaming, 1 mL 50% (v/v) Antifoam 204 (Sigma-Aldrich®, USA) aqueous emulsion was added. A CGXII minimal medium without protocatechuic acid (PCA) containing 300 g/L glucose was used as feed medium.

The cultivations were divided into three separate phases. The first phase was a batch cultivation in two parallel fermentors (STR1) incorporating a starting volume of 1 L before adding the additional smaller STR2 compartments. This initial phase was characterized by well-supplied cultivation conditions with glucose and oxygen in excess. The second cultivation phase started when the non-aerated compartments were added, initiating the oscillations between well-aerated and oxygen-limited cultivation conditions. This switch in process conditions has been declared as time point t = 0. The third cultivation phase started 1.7 h after adding the STR2 compartments by starting the exponential feed into the non-aerated vessels. A desired growth rate of 0.2 h⁻¹ was expected for the whole working volume. This last phase was characterized by glucose excess but O₂ depletion in the STR2 vessels and glucose limitation but sufficient O₂ supply in the larger STR1 compartments.

**Results and Discussion**

After inoculation, the dissolved oxygen concentration in STR1 compartments decreased in both parallel runs until a saturation level of 30% was reached (Figure 3). In the time frame of -4.7 h to -4.3 h, the stirrer cascade automatically started in both vessels to maintain a DO of 30%. At the point of time when the additional compartments were added (t = 0), the DO levels in the STR1 vessels increased due to the volume reduction in the STR1. The DO control adapted quickly to the changed conditions and controlled the DO accurately at 30% until the process ended. DO levels in the oxygen-limited STR2 dropped immediately to 0% after oscillation start thanks to aeration with N₂. To ensure that only oscillating O₂ and substrate levels influenced the scale-down cultivation, the pH in both compartment types was controlled at 7.

Offline HPLC analysis revealed that organic acids were produced during oxygen limitation in the STR2 and reassimilated in the aerobic STR1 due to the limited glucose availability. Despite this compartment-specific acid formation, the individual pH control of the vessels was maintained between 7.1 and 6.9 in both compartments, without deregulating the control. The bacterial growth was not significantly affected by pumping the cells back and forth between different cultivation conditions. During the feeding phase, a mean growth rate of 0.19 h⁻¹ was observed, which strongly correlates with the expected growth rate of 0.2 h⁻¹ given by the exponential feed profile.
Conclusion

Using the described scale-down device, the researchers were able to simulate parameter gradients that can occur in the production scale. Metabolic effects of the oscillating DO and substrate concentrations were evaluated by offline analysis. Two identical scale-down processes could be realized at the same time with highly reproducible results.

The independent control of parameters in each vessel was essential to ensure the aerobic conditions in STR1 and micro-aerobic conditions in STR2. An accurate pH control, despite different vessel sizes and environments, made it possible to separate the O₂-limited conditions from a potential effect of acidic by-product-induced pH drop.

The scripting function of the software enabled the implementation of an exponential feed profile that resulted in the desired growth rate. Once the set-up was defined, the comprehensive recipe management allowed the adaptation and applications of the stored templates to any future cultivations. Like this, the researchers will be able to further investigate the relation between the cellular metabolism and inhomogeneities in the fermentor.

DASGIP Parallel Bioreactor Systems and DASware control have proven to be a powerful platform for scale-down experiments. A flexible design of both hardware and software allow even uncommon process set-ups and enable process automation.
Literature


## Ordering information

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The Eppendorf BioFlo® 320 Bioprocess Control Station: An Advanced System for High Density *Escherichia coli* Fermentation

Bin Li, Khandaker Siddiquee, and Ma Sha
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Abstract

In this application note, an *Escherichia coli* (*E. coli*) fermentation run was conducted using the new Eppendorf BioFlo 320 bioprocess control station. High cell density was achieved at 12 h as determined by a maximum optical density (OD$_{600}$) measurement of 215.2. The wet cell weight (WCW) and dry cell weight (DCW) were also measured and presented.

Introduction

The newest offering in the Eppendorf bioprocess portfolio, the BioFlo 320 seamlessly combines form and function into one all-inclusive package. The BioFlo 320 is an advanced bioprocess system developed for both microbial fermentation and cell culture applications. A new industrial design, flexibility between autoclavable and single-use vessels, intelligent sensors, Ethernet connectivity, and an improved software package are only a few of the features that set it apart from the competition.

In a previous application note using the BioFlo/CelliGen® 115 benchtop system [1], we successfully cultured *E. coli* under aerobic conditions with a maximum OD$_{600}$ value of 140. In this application note, *E. coli* cultivation achieved an even higher OD$_{600}$ value of 215.2 using the new BioFlo 320 bioprocess control station (Figure 1). Furthermore, the WCW and DCW were also measured and presented.

Figure 1: BioFlo 320 bioprocess control station with water-jacketed (left) and stainless steel dished-bottom (right) vessels
Materials and Methods

Equipment
Fermentation was performed using an Eppendorf BioFlo 320 bioprocess control station with the configuration outlined in Table 1. The *E. coli* K12 strain (ATCC®, 10798™) was grown in a 1 L (working volume) stainless steel dished-bottom BioFlo 320 glass vessel, as shown in Figure 1. Glucose concentrations were measured using a Cedex® Bio Analyzer (Roche®). The OD₆₀₀ was measured with a spectrophotometer. An Eppendorf MiniSpin® plus was used to pellet the cells. A pH sensor (InPro® 3253i/SG/225) and an optical Dissolved Oxygen (DO) sensor (InPro 6860i), both incorporating Intelligent Sensor Management (ISM®) technology from Mettler Toledo®, were used in this experiment. A laboratory oven (LAB-LINE®, L-C series) was used to dry the cell pellets for DCW measurements.

**Parameter Configuration**

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<td>Automatic gas mix</td>
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<tr>
<td>Motor</td>
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<tr>
<td>Impeller</td>
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<tr>
<td>Sparger</td>
<td>Ring sparger (Macrosparger)</td>
</tr>
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**Table 1: BioFlo 320 hardware configuration**

Medium
*E. coli* was cultured in chemically defined medium, pH 6.8.

The initial fermentation medium was prepared as follows: 150 mL 10 x phosphate/citric acid buffer (133 g/L KH₂PO₄, 40 g/L (NH₄)₂HPO₄, 17 g/L citric acid) and 1.35 L deionized (DI) water were added to the vessel for sterilization at 121 °C for 20 min. After the medium was cooled to room temperature, the following sterile components were added aseptically to make the complete fermentation medium: 15 mL of 240 g/L MgSO₄, 0.34 mL of 20 g/L thiamine, 15 mL of 100 x trace element solution, and 22 mL of 70 % glucose solution. The 100 x trace element solution contained: 10 g/L iron (III) citrate, 0.25 g/L CoCl₂ 6H₂O, 1.5 g/L MnCl₂ 4H₂O, 0.15 g/L CuCl₂ 6H₂O, 0.3 g/L H₂BO₃, 0.25 g/L Na₂MoO₄ 2H₂O, 1.3 g/L zinc acetate 2H₂O, 0.84 g/L EDTA [2, 3].

An additional concentrated feeding medium was prepared separately in a 1 L glass bottle. 67.5 mL of 240 g/L MgSO₄, 2.49 mL of 20 g/L thiamine solution, 22.5 mL of 100 x trace element solution, and 70 % glucose solution were added to a final volume of 750 mL.

**Inoculum preparation and fermentation**

The inoculum was grown in Terrific Broth (TB) medium, prepared as described previously [4]. Two 500 mL baffled shake flasks (VWR®, 30623-210) each containing 100 mL of TB medium were inoculated from a frozen vial of *E. coli* and incubated at 30 °C, 200 rpm overnight in a New Brunswick™ Innova® 40 benchtop incubator shaker. Cell growth was monitored by offline measurement of the OD₆₀₀ value. The vessel was inoculated with 75 mL of inoculum (5 % of the initial fermentation medium volume).

Antifoam 204 (Sigma-Aldrich®, A6426) was added only when needed, since it may reduce the oxygen transfer rate (OTR) and possibly lower the final cell density. About 5 mL of 5 g/L antifoam was added between 7 – 12 h of fermentation, as foam accumulation warranted.

Pump 3 was assigned as the feeding pump. The feeding strategy included increasing or decreasing the feeding pump speed based on the glucose concentration. This strategy was designed to maintain glucose concentration below 2 g/L.

Table 2 and Figure 2 illustrate the adjustments made to the pump speed over the course of the fermentation. Although a similar feed program can be used for repeated fermentation runs, the feed start time must be adjusted each time according to the growth dynamics of each fermentation.

Cell growth and glucose concentration were monitored offline using 5 mL samples taken according to the following schedule. For OD₆₀₀ readings, samples were taken every hour and diluted appropriately for accurate measurement. For the determination of glucose concentration, samples were taken every hour before the initiation of feeding, and then every ~30 min after the feeding began. The specific growth rate (μ) was calculated from the fitted OD₆₀₀ value in Microsoft® Excel®.

**pH calibration and control**

pH calibration was performed outside the vessel using a two-point calibration method and standard buffers. Buffer pH 7.0 was used to set ZERO and pH 4.0 for the SPAN. The pH sensor was calibrated prior to autoclaving the vessel.

**Table 2: Pump speed at different EFT during the fed-batch fermentation**

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<td>5:15</td>
<td>0.3</td>
</tr>
<tr>
<td>6:15</td>
<td>0.5</td>
</tr>
<tr>
<td>6:16</td>
<td>0.2</td>
</tr>
<tr>
<td>7:00</td>
<td>0.5</td>
</tr>
<tr>
<td>7:01</td>
<td>0.9</td>
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<td>4.5</td>
</tr>
<tr>
<td>11:30</td>
<td>5.3</td>
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</table>
Figure 2: EFT-based feeding program as displayed on the enhanced software platform

The pH was automatically maintained at 6.8 by adding 25 % (v/v) NH₄OH via front mounted peristaltic pump (assigned as “base”). The deadband for pH control was 0.02.

DO sensor calibration and gassing control
Since the BioFlo 320 is compatible with multiple types of DO sensors, an optical sensor was chosen for DO control instead of the traditional polarographic DO sensor. One of the advantages of the optical DO sensor is that it does not require the 6 h polarization time of the polarographic DO sensor, which reduces the turnaround time between fermentation runs. Calibration was performed using a standard two-point calibration method: 0 % (set ZERO) was obtained by running 1200 rpm agitation and 3 SLPM N₂ flow until the DO value stabilized. 100 % (set SPAN) was obtained by running 1,200 rpm agitation and 3 SLPM air flow until the DO value stabilized at maximum.

The BioFlo 320 software offers a selection of automatic gassing control cascades that are dependent upon the configuration of the unit. The BioFlo 320 used in this study included the automatic gas mix and four TMFCs with a flow range of 0 – 20 SLPM (Table 1). User-defined DO cascade settings utilizing agitation, air, and oxygen in sequential manner are shown in Figure 3. The DO setpoint was 30 %.

WCW and DCW measurement
Samples were taken to measure the WCW and DCW. 1 mL of culture sample was added into an Eppendorf microcentrifuge tube and pelleted at 7,500 rpm for 5 min. The supernatant was carefully removed using an Eppendorf Research® pipette and the WCW was measured by calculating the difference in weight between the tube before and after sample addition. Furthermore, the tube was kept in a heating oven and maintained at 70 – 80 °C until the cell pellet was dry and the DCW measured similarly.

Results and Discussion
Samples were taken periodically to monitor the cell growth (OD₆₀₀ value) and glucose concentration as described above. Feeding was initiated when the glucose concentration dropped below 2 g/L, which occurred at 5.25 h of cultivation. After starting the feed, the pump rate was adjusted according to the current glucose concentration with the end goal of keeping it at or below 2 g/L (Table 2). As shown in Figure 4A, the OD₆₀₀ value reached 215.2 within 12 h. The growth curve was also plotted on a log scale to calculate the specific growth rate (μ = 0.54 h⁻¹, Figure 4B).

WCW and DCW were also measured during the fermentation. The results are shown in Table 3 and Figure 5. During the cultivation, both WCWs and DCWs increased proportionally with the increase in the OD₆₀₀ value.
Conclusions

The Eppendorf BioFlo 320 bioprocess control station was able to support high density *E. coli* growth using a fed-batch fermentation method. An \( \text{OD}_{600} \) optical density of 215.2 was reached at 12 h. The wet/dry cell weights were measured at various time points, which were used as records of cell growth along with \( \text{OD}_{600} \) values. Although efforts were made to maintain a glucose concentration below 2 g/L, the fermentation was not optimized for medium, growth conditions, or any product yield.

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<th>WCW (g/L)</th>
<th>DCW (g/L)</th>
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<tr>
<td>15.6</td>
<td>67.5 ± 4.7</td>
<td>15.2 ± 2.7</td>
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<tr>
<td>30.3</td>
<td>117.2 ± 4.1</td>
<td>24.6 ± 2.0</td>
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<tr>
<td>58.0</td>
<td>214.1 ± 16.3</td>
<td>41.7 ± 2.0</td>
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<tr>
<td>89.8</td>
<td>334.4 ± 19.3</td>
<td>64.6 ± 2.0</td>
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<tr>
<td>111.2</td>
<td>386.5 ± 8.8</td>
<td>72.4 ± 1.7</td>
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<tr>
<td>215.2</td>
<td>453 ± 13.0</td>
<td>110.7 ± 2.4</td>
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Table 3: WCW and DCW; average was calculated from 5 samples with mean +/- one standard deviation

![Figure 5: Correlation between OD\(_{600}\) value and cell weight](image)

![Figure 4: Fermentation growth curve and glucose concentration](image)
Literature


### Ordering Information

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High Cell Density Fermentation of *Escherichia coli* Using the New Brunswick™ BioFlo® 115

Bin Li, Stacey Willard, and Ma Sha
Eppendorf, Inc., Enfield, CT, USA
Corresponding author: sha.m@eppendorf.com

**Abstract**

This application note presents a successful example of a high density fermentation of *Escherichia coli* (*E. coli*) using the New Brunswick BioFlo 115 benchtop, autoclavable fermentor from Eppendorf. The highest optical density (OD$_{600}$) achieved in this study was 140 at 11 hours (h) without optimized fermentation medium and conditions.

**Introduction**

*E. coli* is a Gram-negative bacterium that has had a long history in the world of laboratory and industrial processes due to its ease of manipulation and well understood genome. It is widely cultivated under aerobic conditions. High cell density fermentation of *E. coli* is a powerful technique for the production of recombinant proteins. In this application note, *E. coli* cultivation achieved a high OD$_{600}$ value of 140 at 11 h using fed-batch fermentation with the New Brunswick BioFlo 115 benchtop fermentor (Figure 1).

**Materials and Methods**

**Equipment**

Fermentation was performed in a New Brunswick BioFlo 115 benchtop fermentor (Eppendorf) with the configuration outlined in Table 1. The *E. coli* K12 strain (ATCC®, 10798™) was grown in a 2 L working volume New Brunswick BioFlo 115 heat-blanketed glass vessel (Eppendorf). Glucose concentrations were measured using a Cedex® Bio Analyzer (Roche®). The OD$_{600}$ was measured with a spectrophotometer.

**Medium**

The initial fermentation medium was prepared as follows: 150 mL 10 X phosphate/citric acid buffer [133 g/L KH$_2$PO$_4$, 40 g/L (NH$_4$)$_2$HPO$_4$, 17 g/L citric acid] and 1.35 L deionized (DI) water were added to the vessel before sterilization at 121 °C for 20 min. After the solution was cooled to room temperature,
the following sterile components were added to make the complete fermentation medium: 15 mL of 240 g/L MgSO₄, 0.34 mL of 20 g/L thiamine, 15 mL of 100 X trace element solution, and 22 mL of 70 % glucose solution. The 100 X trace element solution contained: 10 g/L iron (III) citrate, 0.25 g/L CoCl₂•6H₂O, 1.5 g/L MnCl₂•4H₂O, 0.15 g/L CuCl₂•6H₂O, 0.3 g/L H₃BO₃, 0.25 g/L Na₂MoO₄•2H₂O, 1.3 g/L zinc acetate•2H₂O, 0.84 g/L EDTA [1, 2].

An additional concentrated feeding medium was prepared separately in a 1 L glass bottle. 45 mL of 240 g/L MgSO₄, 1.66 mL of 20 g/L thiamine solution, 15 mL of 100 X trace element solution, and 70 % glucose solution were added to a final volume of 500 mL.

**Inoculum and Fermentation**

The inoculum was grown in Terrific Broth (TB) medium, prepared as described previously [3]. Two 500 mL baffled shake flasks (VWR®, 30623-210) each containing 100 mL of TB medium were inoculated from a frozen vial of *E. coli* and incubated at 30 °C, 200 rpm overnight in a New Brunswick Innova® 40 benchtop incubator shaker (Eppendorf). After the overnight culture, the OD₆₀₀ value was ~9. The vessel was inoculated with 75 mL of inoculum (5 % of the initial working volume).

The initial fermentation temperature was set to 30 °C. Antifoam 204 (Sigma-Aldrich®, A6426) was added only when needed, since it may reduce the oxygen transfer rate (OTR) and possibly lower the final cell density. In this experiment, ~100 µL of 5 g/L antifoam 204 was added at the beginning of the run to prevent foaming and ~4 mL was added between 8 – 11 h of fermentation, as foam accumulation warranted.

Pump 3 was assigned as the feeding pump (maximum speed is 24 mL/min with 0.188 in inner diameter tubing). The feeding strategy included increasing or decreasing the feeding pump speed accordingly, based on the glucose concentration. To achieve high cell density, the target glucose concentration was ≤ 2 g/L. Excessively high glucose concentrations may alter *E. coli* metabolism and reduce peak cell density. Table 2 illustrates the adjustments made to the pump speed over the course of the fermentation while trying to maintain glucose concentration at or below 2 g/L.

Cell growth and glucose concentration were monitored offline using 5 mL samples taken according to the following schedule. For OD₆₀₀ readings, samples were taken every hour and diluted appropriately for accurate measurement. For the determination of glucose concentration, samples were taken every hour before the initiation of feeding, and then every ~30 min after the feeding began. The specific growth rate (µ) was calculated from the fitted OD₆₀₀ value in Microsoft® Excel®.

**pH Calibration and Control**

pH calibration was done outside the vessel using a two-point calibration method and standard buffers. Buffer pH 7.0 was used to set “ZERO” and pH 4.0 for the “SPAN.” The pH sensor was calibrated prior to autoclaving the vessel. The pH was automatically maintained at 6.8 by adding 25 % (v/v) NH₄OH. The deadband for pH control was set to 0.02.

**Dissolved oxygen (DO) sensor calibration and gassing control**

DO sensor calibration was performed using a standard two-point calibration method: 0 % (set “ZERO”) was obtained by disconnecting the sensor from the cabinet and allowing the raw value to stabilize; 100 % (set “SPAN”) was obtained by running 1200 rpm agitation and 3 SLPM air flow until the DO value stabilized at maximum.

The New Brunswick BioFlo 115 Reactor Process Controller (RPC) software offers a selection of automatic gassing control cascades that are dependent upon the configuration of the unit. The New Brunswick BioFlo 115 used in this study included the automatic gas mix option and one TMFC with a flow range of 0 – 20 SLPM (Table 1). Operating in fermentation mode, the automatic DO cascade “Agit/GasFlo/O2” was selected with a DO setpoint of 30 %. User-defined minimum and maximum limits were populated in the cascade screen and are listed in Table 3.

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<td>O2 mix Casc Low Limit</td>
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<td>O2 mix Casc High Limit</td>
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Table 3: DO cascade low and high limits
Results and Discussion

Samples were taken periodically to monitor the cell growth (OD\textsubscript{600} value) and glucose concentration. Feeding was initiated when the glucose concentration dropped below 2 g/L, which occurred at 5 h of cultivation. After starting the feed, the pump rate was adjusted according to the current glucose concentration with the end goal of keeping it at or below 2 g/L (Table 2). As shown in Figure 2A, within 11 h, the OD\textsubscript{600} value reached 140. The growth curve was also plotted on a log scale to calculate the specific growth rate ($\mu = 0.58h^{-1}$, Figure 2B).

Conclusion

High density E. coli growth in the New Brunswick BioFlo 115 was achieved using a fed-batch fermentation method. An optical density of 140 was reached at 11 h. Although efforts were made to maintain a glucose concentration below 2 g/L, the fermentation was not optimized for medium, growth conditions, or any product yield.

Figure 2: Fermentation growth curve and growth rate calculation
A: The OD\textsubscript{600} and glucose concentration over the course of the 11 h fermentation
B: The growth curve plotted on a log scale; a linear trend line was applied in Microsoft Excel, the slope of which is equivalent to the specific growth rate, $\mu$ (h\textsuperscript{-1})

References

## Ordering information

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Introduction

The CEPA LE Model, a tubular-bowl continuous centrifuge, is one of a family of separation instruments characterized by their ability to process many times the capacity of their bowl total volume without interruption. This characteristic results from a design that allows continuous feeding of a solid-liquid mixture, while simultaneously expelling the liquid component. The solids, in this application, are the cell mass, and are retained in the bowl.

Clarified liquid is obtained from an exit port while the machine is running. Cell mass is taken from the tubular bowl after the machine is stopped. A removable plastic bowl liner is often used to simplify cell paste removal.

Materials and Methods

Fermentation

A five liter fermentation was carried out in a New Brunswick BioFlo® benchtop fermentor for the purpose of evaluating the CEPA LE High Speed Centrifuge in a typical E. coli separation. The fermentation broth was determined to contain approximately 6 % wet solids by volume by spinning down a small sample in a laboratory batch centrifuge operated at 2500 rpm for 10 minutes. Dry cell weight was 11.48 grams per liter.

Abstract

In this bioprocess laboratory application, 4.2 liters of E. coli fermentation broth containing 6 % solids by volume were separated by CEPA LE in 22 minutes. The cellular paste that was collected amounted to 193 grams. Supernatant clarity was excellent, with all samples containing less than our visual detection limit of 0.1 % solids.

Setup and Operation

When the fermentation was completed, a Watson-Marlow® peristaltic pump was used to transfer the broth from the fermentor to the centrifuge. A length of silicone flexible tubing was attached to a dip tube in the fermentor vessel, fed through the pump head, and connected to the centrifuge’s inlet nozzle. A second length of tubing was run from the centrifuge’s supernatant outlet port into a collection vessel.

The fermentor was set to maintain temperature at 19 °C. After starting the centrifuge and waiting for it to attain full speed, broth was pumped to the CEPA LE at a rate of 190 mL/min (11.4 L/hr). This value was arbitrarily selected and is near the low end of the system’s range — the CEPA LE has throughput capability up to 30 L/hr. The fermentor agitation was set to a low speed during the transfer to prevent settling and to help maintain temperature uniformity.

Figure 1: The CEPA LE is a benchtop laboratory centrifuge, featuring variable speed control as standard and a wide array of optional bowls for research, scaleup experiments, and small volume production. The LE is typically used with 2 to 15 liter cultures. Maximum throughput is 30 liters/hour.
The centrifuge was configured with a type clarifying cylinder, and a 2 mm inlet nozzle. It was operating at full speed (40,000 rpm) which produces a radial acceleration or G-force of 45,000. The centrifuge and pump operation continued until the liquid in the fermentor fell below the dip tube level.

Clarity Measurements
Six 10 mL samples of supernatant were taken at 4 minute intervals during the separation process, and the 600 nanometer optical density was measured off-line. The 10 mL samples were spun down in a laboratory centrifuge for 10 minutes at 2500 rpm to get a visual measure of residual cell mass.

Results and Discussion
A total volume of 4.2 L was processed through the centrifuge in 22 minutes yielding 193 grams of wet cellular paste in the CEPA bowl. The 250 mL bowl was approximately 75 % full of paste at the point the processing was complete.

<table>
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<th>Visual</th>
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<tr>
<td>1</td>
<td>0.162</td>
<td>&lt; 0.1%</td>
</tr>
<tr>
<td>2</td>
<td>0.203</td>
<td>&lt; 0.1%</td>
</tr>
<tr>
<td>3</td>
<td>0.226</td>
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</tr>
<tr>
<td>4</td>
<td>0.249</td>
<td>&lt; 0.1%</td>
</tr>
<tr>
<td>5</td>
<td>0.300</td>
<td>&lt; 0.1%</td>
</tr>
<tr>
<td>6</td>
<td>0.392</td>
<td>&lt; 0.1%</td>
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</table>

Table 1: Supernatant clarity as indicated by optical density (600 nm) and visual observation of sediment samples taken at four minute intervals.

In addition to separation efficiency, we noted that the time required to carry out the procedures was very short, and handling the system during operation was obvious.

The separation itself took approximately 22 minutes. The entire process from setup through cleaning took less than an hour.

We determined the LE model to be easy to use, as depicted from the short times for setup and clean up. The ease of handling is partly due to its small size, and partly because of its accessible design.

Predictably, the supernatant OD increased as the separation progressed, but even the last sample showed less than 0.1 % wet cell volume. Visually, this was a barely perceptible amount of cells in the supernatant, which could have been reduced further, either by feeding more slowly, or by exchanging the partially filled rotor for an empty one during the harvesting process.

Tests under various operating conditions could be used to develop a protocol that results in the optimum compromise between process time and supernatant clarity for a specific application. Acceptable residual cell mass depends on several factors, including whether the desired product is in the supernatant or the cells, as well as the post-centrifuge filtration and downstream purification processes, if any. Certainly, this particular process could have been run more quickly or more slowly with a change in clarity. Although not explored here, more complex protocols could be established to optimize the process for the user. One example would be to discharge a high feed rate initially, and then decrease it as the bowl fills to take advantage of the initially higher separation efficiency to improve either speed or clarity with no penalty in the harvest process.

This test showed that the smallest CEPA centrifuge efficiently and conveniently harvested and clarified E. coli broth, making it a highly effective instrument for fermentation applications.

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A Comparative Study: Small Scale *E. coli* Cultivation Using BioBLU® Single-Use and Reusable Vessels

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Eppendorf AG Bioprocess Center, Rudolf-Schulten-Str. 5, 52428 Juelich, Germany
*Corresponding author: schlottbom.c@eppendorf.com

**Abstract**

In recent years single-use bioreactors gained more and more importance in animal and human cell culture. With the new line of BioBLU® rigid wall, stirred-tank single-use vessels Eppendorf offers premium solutions for microbial applications.

In the following case study, reproducible process control was achieved with parallel operated BioBLU 0.3f single-use and reusable glass vessels, both used in an Eppendorf DASbox® Mini Bioreactor System. Fermentation of *E. coli* K12 led to very comparable results thus proving the tested single-use vessels to be an appropriate tool to accelerate microbial process development and shorten time-to-market in all industries related to microbial production processes.

**Introduction**

Single-use bioreactors are a suitable tool for time and cost effective bioprocessing. Minimized setup times, eliminated cleaning procedures and therefore reduced labor time can sustainably accelerate bioprocess development.

In all biopharmaceutical industries single-use technologies are widely used in mammalian cell culture. With the new BioBLU® line, specifically designed to meet the needs of fermentation, single-use bioreactors make their way to microbial applications in biopharma, food and cosmetics industries. Microbial applications make specific demands on the bioreactor design and functionality. Fermentation processes need much higher *k* values for proper mass transfer and suitable heating and cooling options as well.

This comparative study investigates the functionality and reliability of a BioBLU 0.3f single-use mini bioreactor and an autoclavable DASbox Mini Bioreactor (figure 2) in a small scale *E. coli* fermentation.

**Figure 1:** DASbox Mini Bioreactor System for microbial applications equipped with BioBLU 0.3f Single-Use Vessels and autoclavable DASbox Mini Bioreactors with Rushton-type impeller.
Materials and Methods

*E. coli* K12 (DSM 498) was cultivated in a fully instrumented Eppendorf BioBLU 0.3f single-use mini bioreactor and compared to fermentations in conventional autoclavable glass bioreactors.

The ready-to-use rigid wall stirred-tank single-use bioreactors, specifically designed for microbial applications, are equipped with a 2x Rushton-type impeller, liquid-free peltier exhaust condensation and direct drive for high performance agitation. The overhead-driven autoclavable DASbox Mini Bioreactors included 2x Rushton-type impeller and liquid free peltier exhaust condensers as well.

A 4-fold parallel DASbox Mini Bioreactor System with active heating and cooling capacities was used with DASGIP® Control* Software for precise process control.

Starting with a working volume of 100 mL each, the cultures were grown for 40 h in PAN medium with an initial glucose concentration of 40 g/L and fed with 50 % glucose solution in the fed batch phase.

The temperature was controlled at 37°C and pH was adjusted to 6.8 via 4 % ammonia solution; the cultures were submerged aerated with a constant rate of 1 vvm (6 L/h or 0.1 L/min). The dissolved oxygen was maintained at 30 % with the stirrer speeds ranging from 600 rpm to 2000 rpm which equals to tip speeds of 0.94 m/s to 3.14 m/s. Exhaust concentrations were measured and corresponding oxygen transfer rates (OTR) were automatically calculated using a DASGIP GA4 exhaust analysis module.

Results and Discussion

Highly reproducible OTR values of up to 250 mmol/L/h were observed in the single-use as well as in the glass bioreactors (figure 3). Supporting $k_La$ values of up to 2500 h$^{-1}$ were determined by static sulfite depletion method (data not shown) demonstrating that the single-use design of the BioBLU 0.3f bioreactors perfectly matches the demands of microbial applications.

The biomass production was determined offline as cell wet weight and revealed comparable growth characteristics in single-use and glass mini bioreactors (figure 4). The maximal biomasses of about 160 g/L achieved in the fermentation are equal to an OD$_{600}$ of about 100 (data not shown).

*DASGIP Control is now DASware® control 5
This case study proves the BioBLU 0.3f single-use bioreactor addresses the specific needs of an *E. coli* fermentation especially in regard to mass and heat transfer. OTR values measured in the single-use vessel process runs as well as the final biomasses reached were comparable to those achieved with the conventional autoclavable DASbox glass bioreactors.

The specifically adapted single-use design of the BioBLU 0.3f mini bioreactor supports the high demands of microbial applications. Used with the Eppendorf DASbox this single-use bioreactor is a premium tool for screening, media optimization and as a scale down model for process development including Design of Experiments (DoE) approaches.

Industry interest in adequate single-use bioreactor solutions for fermentation is steadily increasing. With the BioBLU f line of single-use vessels Eppendorf is offering premium solutions for microbial applications. Users in fermentation can now benefit from advanced process control, accelerated process development, reduced costs and shorter time-to-market.

**Figure 4**: Biomass production. Cell wet weight (cww) of fermentations carried out in BioBLU 0.3f single-use and reusable DASbox Mini Bioreactors, respectively.
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High Cell Density *E. coli* Fermentation using DASGIP® Parallel Bioreactor Systems

Claudia M. Huether, Christiane Schlottbom*, and Ute Ehringer

1 Eppendorf AG Bioprocess Center, Rudolf-Schulten-Str. 5, 52428 Juelich, Germany; 2 BioPharm GmbH, Czernyring 22, 69115 Heidelberg, Germany; * Corresponding author: schlottbom.c@eppendorf.com

**Abstract**

In contract manufacturing one of the most challenging needs is the adaption of proprietary production processes, their optimization as well as process improvements after a transfer from one Contract Manufacturing Organization (CMO) to another. Each single process modification has to be verified with comparable data. The following application note gives an example for such an adapting procedure achieved by the use of the DASGIP Parallel Bioreactor System. The established process of one CMO included a high cell density fed-batch process, controlled by the key process parameters pH, agitation, temperature and growth dependent glucose feeding. This process was adapted and optimized for the use with another CMO.

**Introduction**

Biopharm® GmbH, a GLP certified company offering research and development services for CMOs is using a derivative of the *E. coli* K-12 strain W3110 as their expression platform for prokaryotic production of recombinant, therapeutically proteins. The modified W3110BP strain is a property of Biopharm and is optimized for increased plasmid retention compared to the wild type *E. coli* W3110 as well as other conventional strains like BL21. Additionally, the Biopharm W3110BP strain is outstandingly capable of fermentation with high cell densities.

Aiming to reduce operation time parallel fermentation processes were used. The main item was the comparability of two or more parallel processes to show continuous process development by bridging results from one development round to another one.

The most important process parameter in the Biopharm’s fermentation procedure is the dissolved oxygen (DO) concentration since the DO level determines the set-point from which additional feeding of the culture is needed. Thus, the precise observation and control of the DO level is the crucial factor for efficient fermentation procedures.

Scientists in the Biopharm laboratories are using the Eppendorf DASGIP Parallel Bioreactor System for microbial small-scale process development to get flexibility for their changing needs combined with highest precision and reliability.

**Materials and Methods**

All experiments were carried out using the cytokine producing recombinant strain *E. coli* K-12 W3110BP in complex media supplemented with vitamins, trace elements and other...
additives. Initial small-scale experiments were performed with the DASGIP Parallel Bioreactor System in 250 mL fermentation vessels which were afterwards replaced by 500 mL vessels to increase biomass production. All key process parameters like pH, agitation and temperature were controlled online as well as the critical DO. The online DO level was used as trigger for automated activation of a glucose feeding profile. To proof the scalability properties of the DASGIP Parallel Bioreactor System additional cultivations were run in a 5 L glass bioreactor (3rd party supplier). Temperature, pH and DO were measured online whereas the agitation was manually adapted to the current DO levels. The applied glucose feeding profile was similar to the profile in the 500 mL fermentation approaches. The average cultivation time for all high cell density fermentations described in this application note was approximately 28 h.

Results and Discussion

High cell density fermentation was performed successfully. As shown in table 1 all fermentation results were similar with regard to the final biomass production and product formation. The different working volumes of 500 mL in contrast to 5 L did not influence the course of the process, demonstrating the easy scalability of test results gained with the DASGIP Parallel Bioreactor System. Taking the online measured DO levels into account (figure 2) the vantages of an online controlled agitation as offered by the DASGIP Parallel Bioreactor System are displayed: Constant and precise regulation of the DO by automatically controlled stirring.

<table>
<thead>
<tr>
<th></th>
<th>Unit 1</th>
<th>Unit 2</th>
<th>5 L</th>
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</thead>
<tbody>
<tr>
<td>Final optical density</td>
<td>230</td>
<td>240</td>
<td>268</td>
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<tr>
<td>Final bio dry mass [g/L]</td>
<td>56</td>
<td>54</td>
<td>65</td>
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<tr>
<td>Final bio wet mass [g/L]</td>
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<td>242</td>
<td>275</td>
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<tr>
<td>Final cell number [cells/mL]</td>
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<td>7.0 x 10^{10}</td>
<td>8.8 x 10^{10}</td>
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<td>Final product level [g/L]</td>
<td>2.8</td>
<td>3.0</td>
<td>2.5</td>
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Table 1: Comparison of fermentation results. High cell density fermentations of E. coli K-12 W3110BP were performed in a DASGIP Parallel Bioreactor System (500 mL, units 1 and 2) as well as in a 5 L bioreactor.

All recent improvements which were achieved for the described fermentation processes were successfully implemented into a large scale manufacturing process by a CMO (confidential data, not shown).

The parallel set-up and control of independent fermen-
tations guarantees an easy comparability of different approaches. “When comparing e.g. different bacterial host/plasmid combinations for protein production it is advantageous to use parallel approaches to avoid environmental influences. Thus, the experimental outcomes can be compared directly.”, points out Ute Ehringer, Head of Development at Biopharm.

The Eppendorf DASGIP Parallel Bioreactor System for microbiology was also used for several other projects at Biopharm to accelerate the process development. When searching for suitable host/plasmid combinations for new products, advanced fermentation processes could be established with short development cycles by the time-saving parallel fermentation approaches.
### Ordering information

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<tr>
<th>Description</th>
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<td>16-fold system, benchtop</td>
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</tr>
</tbody>
</table>

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Scalability of Parallel *E. coli* Fermentations in BioBLU® f Single-use Bioreactors

Claudia M. Huether-Franken, Christiane Schlottbom*, Anne Niehus, and Sebastian Kleebank
Eppendorf AG Bioprocess Center, Rudolf-Schulten-Str. 5, 52428 Juelich, Germany
*Corresponding author: schlottbom.c@eppendorf.com

**APPLICATION NOTE** No. 293 | October 2013

**Abstract**

Single-use bioreactor solutions have been successfully established in animal and human cell culture in the last years. Now this technology is going to make its way for microbial applications. In the following case study reproducible process control was achieved with single-use mini bioreactors and 1 L single-use vessels running in parallel. Fermentation of *E. coli* K12 led to highly reproducible results thus proving the tested rigid wall single-use stirred-tank vessels to be an appropriate tool to accelerate microbial process development and shorten time-to-market in biopharmaceutical industry.

**Introduction**

Regardless if in cell culture or in microbial applications, single-use bioreactors provide a couple of advantages for time- and cost-effective bioprocessing. Minimal setup times, no need for cleaning procedures and therefore reduced labor time can accelerate bioprocess development rigorously. Compared to the use of single-use bioreactors in cell culture, microbial applications make specific demands on bioreactor design and functionality. Fermentation processes need much higher $k_a$ values for proper mass transfer as well as suitable heating and cooling options.

**Materials and Methods**

To evaluate the reliability of microbial fermentation processes using single-use technology *E. coli* K12 (DSM 498) was cultivated in a fully instrumented Eppendorf BioBLU 0.3f single-use mini bioreactor and compared to fermentations in BioBLU 1f single-use bioreactors. This rigid wall stirred-tank single-use bioreactors were...
carries baffles as well. Both vessel types include a liquid-free Peltier exhaust condensation and magnetic-coupled overhead drive for high performance agitation.

A 4-fold parallel Eppendorf DASbox® Mini Bioreactor System was used with BioBLU 0.3f Single-use Vessels and the BioBLU 1f fermentations were carried out using a 4-fold DASGIP® Parallel Bioreactor System with DASGIP Bioblock. Both Systems feature active heating and cooling capacities. DASGIP Control* Software was used for precise process control.

The cultures were grown for 24 h in PAN media with an initial glucose concentration of 40 g/L and fed with 50 % glucose solution in the fed batch phase. The processes were started with working volumes of 0.1 L in BioBLU 0.3f and 0.7 L in BioBLU 1f Single-use Vessels, respectively. The temperature was controlled at 37 °C.

When using the BioBLU 0.3f vessels the pH was adjusted to 6.8 via 4 % ammonia solution. The cultures were submerged aerated through dip tubes with a constant rate of 6 sL/h (1vvm). Dissolved oxygen was maintained at 30 % whereas the stirrer speeds ranged from 600 rpm to 2000 rpm which equals to tip speeds of 0.94 m/s to 3.14 m/s. When using the BioBLU 1f vessels the pH was adjusted to 6.8 via 25% ammonia solution. The cultures were submerged aerated through dip tubes with a constant rate of 42 sL/h (1vvm). Dissolved oxygen was maintained at 30 % whereas the stirrer speeds ranged from 600 rpm to 1600 rpm which equals to tip speeds of 1.35 m/s to 3.59 m/s. Oxygen transfer rates (OTR) were automatically calculated via a DASGIP exhaust analyzer GA4.

Results and Discussion

A two-phase cultivation with automatic feed-start was sucessfully carried out. As shown by the dissolved oxygen the utilization of BioBLU single-use vessels in combina-

*DASGIP Control is now DASware® control 5.
tion with Eppendorf DASbox or DASGIP Bioblock allows highly parallel and reproducible fermentation (figure 2). Comparing the BioBLU 0.3f and the BioBLU 1f processes proves the capability for seamless scale-up from single-use mini bioreactors to 1 L single-use vessels. k\textsubscript{a} values of up to 2500 h\textsuperscript{-1} in BioBLU 0.3f and up to 4000 h\textsuperscript{-1} when using BioBLU 1f vessels were determined by static sulfite depletion method (data not shown) and demonstrate that these single-use bioreactor designs perfectly match the demands of microbial applications. The biomass production (figure 3) was determined offline as cell wet weight and revealed comparable growth characteristics in both single-use bioreactors. The maximal biomasses of about 160 g/L gained in the fermentation runs correspond to an OD\textsubscript{600} of about 100 (data not shown).

Conclusion

This case study shows that the BioBLU f single-use bioreactors address the specific needs of *E. coli* fermentations especially in regard to mass and heat transfer. The specifically adapted single-use design, featuring Rushton-type impellers, active heating and cooling, and overhead drive enabling high performance agitation, supports the high demands of microbial applications. Currently, single-use bioreactor technology is mainly used in cell culture. With the introduction of the Eppendorf BioBLU f Single-use Vessels adequate tools to accelerate bioprocess development in microbial applications, even high cell density fermentation, are available now.
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<td><strong>BioBLU® 1f Single-Use Vessel</strong>, microbial, 4 pack, pre-sterilized, 3 impellers</td>
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Amino Acid Fermentation: Evaluation of Scale-Down Capabilities Using DASbox® Mini Bioreactors

Christiane Schlottbom*, Sebastian Kleebank¹, Manuela Hauser¹ and Stephan Hans²
¹ Eppendorf AG Bioprocess Center, Rudolf-Schulten-Str. 5, 52428 Juelich, Germany; ² Evonik Industries AG, Kantstr. 2, 33790 Halle, Germany;
*Corresponding author: schlottbom.c@eppendorf.com

Abstract
Optimization of bioprocesses needs accurate monitoring and control while small working volumes are saving media and other resources. Evonik® has established a 2 L process for production of a nutritionally relevant amino acid in *E. coli*. The following application note describes how this process was adapted to the smaller working volume of an Eppendorf DASbox Mini Bioreactor System.

Multiple runs were performed with close monitoring of all relevant process parameters and comprehensive evaluation of data to prove reliable and reproducible results. The scale-down capabilities of the DASbox system were evaluated by comparing the fermentation results to the data collected at 2 L scale.

Introduction
Rising cost and time pressures in bioprocess development together with rapidly evolving regulatory requirements make process development efforts a special challenge these days. Optimizing every step of the total development workflow is crucial for maintaining a competitive business.

Advanced miniaturized benchtop bioreactor systems can harmonize operations between development and production groups while supporting the aims of Quality by Design (QbD). To meet today’s demands of process development these mini bioreactor systems need to mimic all aspects of large-scale fermentation, and offer comprehensive data and information management tools to support regulatory requirements for both filing support and QbD-driven process development. *In situ* sensors as well as an integrated supervisory control and data acquisition (SCADA) are used to control, monitor, and record critical process parameters such as temperature, pH, dissolved oxygen, and agitation. As in production-scale bioreactors, gassing and feeding proceed according to defined settings. DASGIP® Parallel Bioreactor Systems have the potential to address process...
consistency and harmonization of operations between development and production.

The following application note illustrates how the DASbox Mini Bioreactor System with its working volume range of 60 - 250 mL supports bioprocess development in microbial applications. Scale-down capabilities were investigated by transferring a 2 L fermentation process to a 10x smaller working volume in the DASbox system.

Evonik Industries AG (headquartered in Essen, Germany) is one of the world’s leading specialty chemicals companies. Its Health & Nutrition Business Unit produces and markets essential amino acids, mainly for animal nutrition and for specialties for the pharmaceuticals industry.

Materials and Methods

To evaluate the reproducibility and scale-down capabilities of the DASbox Mini Bioreactor System (Figure 1) experimental series with two different systems were carried out and compared.

Fed-batch fermentation of the amino acid-producing E. coli strain was performed in a standard benchtop bioreactor. The corresponding small-scale approaches were carried out in a DASbox System equipped with four DASGIP Mini Bioreactors.

The E. coli strain was cultivated at 36 °C. During fermentation glucose was added according to predefined feed profiles. Both systems used comparable feeding profiles, the one of the DASbox system being adapted to the smaller working volumes. The DO set-point was maintained by adjusted agitation speed. The bioreactors were equipped with two Rushton impellers each. The pH value was regulated to a constant value throughout the fermentation process.

The critical process parameters were monitored, controlled and visualized online while additionally, optical density (OD\textsubscript{600}) and glucose concentration were entered manually for collective analysis and storage in a joint database. Product concentration was measured at the end of each run.

Results and Discussion

All critical process parameters such as feeding profiles and impeller tip speed as well as pH, DO, and temperature set-points were successfully transferred from the 2 L scale to the DASbox Mini Bioreactor System. The two systems showed similar growth characteristics. Online measured Oxygen Transfer Rates (OTR) resulted in highly comparable curves indicating a successful scale-down (data not shown).

Comparison of parallel fermentation runs performed with the DASbox prove the results to be highly reproducible. OTR values of all four runs again followed highly similar curves. Same was observed for online parameters such as temperature, dissolved oxygen concentration and pH. Fermentation using the DASbox system resulted in product yields comparable to the ones achieved with the larger benchtop system (Figure 2). Again, data obtained from the four individual runs performed with the DASbox Mini

**Figure 2: Cell dry weight (X), product concentration (P) and specific product yield (Y\textsubscript{P/X}), each normalized to the 2 L system.**
Bioreactor System strongly resembled each other and thus prove its reproducibility.

Conclusion

The results presented in this application note give direct evidence to the scale-down capability of the DASbox Mini Bioreactor System. This proves the DASbox to be an excellent tool for microbial process development. With its small working volumes it helps saving resources without cutting back the comprehensive process control of advanced large-scale bioreactor systems. Summarized, the DASbox is a truly parallel mini bioreactor system that provides reliable and reproducible results.
### Ordering information

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Process Development for Silage Inoculants – Optimization of *Lactobacillus* sp. Fermentation with Parallel Bioreactor Systems

August Kreici\(^1\), Florian Strohmayer\(^1\), Claudia M. Huether-Franken\(^2\), and Christiane Schlottbom\(^2*\)

\(^1\) BIOMIN Holding GmbH, BIOMIN Research Center, Technopark 1, 3430 Tulln, Austria; \(^2\) Eppendorf AG Bioprocess Center, Rudolf-Schulten-Str. 5, 52428 Juelich, Germany; *Corresponding author: schlottbom.c@eppendorf.com

**Abstract**

This application note describes the process development of a *Lactobacillus* sp. fermentation for use as silage inoculant at BIOMIN® Research Center. To determine the optimum parameters for maximal yields of active cells an Eppendorf DASGIP® Parallel Bioreactor System was utilized. Sufficient amount of data was generated for statistical evaluations in order to optimize medium composition and growth conditions.

**Introduction**

Silage is an animal feed ingredient produced by controlled fermentation of crops with high moisture content. The main objective of ensiling is the achievement of anaerobic conditions as quickly as possible, which causes the inhibition of undesirable microorganisms such as clostridia and enterobacteria. Thus, the nutritional value of the original crop is preserved. The optimum conditions can only be guaranteed by quick filling and proper sealing of the silo, in order to provide the necessary conditions for the following fermentation process. The fermentation process can be accelerated and improved by the addition of homofermentative and/or heterofermentative lactic acid bacteria (LAB).

The following application note describes the fermentation process development of *Lactobacillus* sp. for the application as silage inoculant using an Eppendorf DASGIP Parallel Bioreactor System. In the experiments eight parallel bioreactors were used simultaneously to test growth parameters. All experiments were carried out at the BIOMIN Research Center in Tulln, Austria. The BIOMIN Holding GmbH is a research-oriented company whose objective is to improve animal health and the economic production of animals.

Its core business is the development and manufacturing of innovative and natural feed additives and preservatives to stabilize feed materials.

The main objective of the process development was to determine the optimum parameters leading to a maximum yield of active cells. These parameters were pH, temperature, agitation, consumption of base, the media components
and their concentrations. The Eppendorf DASGIP Parallel Bioreactor System allows testing of different parameters in parallel fermentations at the same time. Therefore, a sufficient amount of data could be generated for statistical evaluations to optimize medium composition and growth conditions.

In this application note the optimization of temperature and pH is described.

Material and Methods

All experiments were carried out using various LAB strains in media containing for example glucose, yeast extract, peptone and salts. The fermentation time depended on the glucose concentration in the medium.

The experiments were carried out with an 8-fold DASGIP Parallel Bioreactor System with 1.5 L vessels. The initial fermentation volumes were 500 to 1000 mL. Subsequently, scale-up experiments were performed in a 20 L lab fermentor to verify the optimized parameters in pilot scale.

Key parameters during fermentation such as pH, temperature, agitation and most importantly base consumption were controlled online and documented with the software DASGIP Control 4.0*. The same parameters were also measured in pilot-scale fermentation allowing to correlate the results of both systems.

Results and Discussion

Fermentation process development could be performed successfully with the DASGIP Parallel Bioreactor System. All shown figures and data were obtained and analyzed with the Eppendorf software DASGIP Control 4.0*.

Figure 2 exemplarily summarizes the results of a parallel fermentation of *Lactobacillus sp.* for optimizing the growth temperature. Base consumption was used as proportional indicator for the growth-dependent acidification of the fermentation broth. The strain was cultivated at different temperatures ranging from 29 °C to 37 °C and the fermentation was finished after base consumption had stopped. For a detailed insight into the bacterial growth, the colony forming units (CFU) for strains obtained at different temperatures (29 °C, 30 °C, 33 °C, 34 °C and 37 °C) are illustrated in table 1.

Table 1: Evaluation of optimum growth temperature. Colony forming units (CFU) of *Lactobacillus sp.* obtained at different temperatures.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>CFU/mL fermentation broth</th>
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<td>29</td>
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<td>1.59 x 10^10</td>
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<tr>
<td>37</td>
<td>1.74 x 10^10</td>
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</table>

* DASGIP Control is now DASware® control 5. Please refer to ordering information on page 4.
Considering these values, a fermentation temperature of 37 °C turned out to be the most suitable temperature for cultivation of the used *Lactobacillus* sp., verifying the results shown in figure 2.

In the next development step the optimum pH value was determined, again by using eight bioreactors in parallel. Figure 3 shows the base consumption during fermentation at different pH values. After fermentation the CFUs in each bioreactor were determined. The optimum was obtained at a pH value of 5.5. This result is in accordance with the base consumption (figure 3), which was also very high for pH 5.5. In table 2 the numbers of CFUs in the fermentors with different pH values are shown.

Table 2: Evaluation of optimum pH. Colony forming units (CFU) of *Lactobacillus* sp. obtained at different pH values.

<table>
<thead>
<tr>
<th>pH value</th>
<th>CFU/mL fermentation broth</th>
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<td>7.00</td>
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Finally, the process developed with the DASGIP Parallel Bioreactor System was successfully transferred to 20 L pilot-scale fermentation, demonstrating the reliable scalability properties of the system.

**Conclusion**

The Eppendorf DASGIP Parallel Bioreactor System is very suitable for fermentation process development with anaerobic microorganisms. The system allows optimization of fermentation processes very effectively as different levels of certain parameters such as pH, redox potential, oxygen concentration and temperature can be tested at the same time. A particular advantage of the Eppendorf DASGIP Parallel Bioreactor System is the simultaneous calibration of pH, redox sensors and pumps which saves a lot of time during preparation of experiments.

The results obtained in the eight bioreactors are comparable among each other and show good reproducibility between different runs. Additionally, results are comparable to larger scale and therefore can be used for the efficient design of scale-up experiments.

Users at BIOMIN especially liked the attractive design of the DASGIP systems: „The whole set-up is very user-friendly, especially the self-explanatory software. Finally, the support from DASGIP service (since 2013 Eppendorf Bioprocess Center) is always on the spot and helps to handle challenges occurring, for example with installation of software updates.”
Ordering information

**DASGIP® Parallel Bioreactor System for Microbial Applications**, max. 250 sL/h gassing

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**DASware® control**, incl. PC, OS, and licenses

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Fed-Batch Biofuel Production Process Using a New Brunswick™ BioFlo® 115

Rivera, Yamakawa, Garcia, Geraldo, Rossell, Bonomi, Brazilian Bioethanol Science and Technology Laboratory, Campinas, SP, Brazil. Filho, School of Chemical Engineering, State University of Campinas, Campinas, SP, Brazil. Capone, Sierra, Sha, Eppendorf Inc., Enfield, CT, U.S.A.

Abstract

In Brazil, it is common in the biofuel industry to utilize a biochemical process in which glucose, fructose and sucrose (derived from sugar cane juice and sugar cane molasses) are used to produce biofuel through a fed-batch fermentation process. In this experiment, Saccharomyces cerevisiae is used for biofuel production from sugar cane juice. The fermentation process metabolizes glucose into ethanol, and is used to produce many biofuel products in large production volumes. This strain of yeast is also widely used in other industrial applications to manufacture enzymes and proteins for beer, wine and bread. In this application note, we show that Saccharomyces cerevisiae can successfully be cultivated in high densities to convert sugar cane into biofuel using a BioFlo 115.

Introduction

The BioFlo 115 features a versatile and easy-to-use control station with color touchscreen monitor and built-in capability to operate in either fermentation or cell-culture mode. Switching between the operation modes automatically adjusts the control settings. Three fixed-speed pumps, temperature control, agitation control, and a rotameter with choice of gas flow ranges are available in BioFlo 115 systems. Pre-packaged kits for Basic or Advanced Fermentation and Advanced Cell Culture simplify the ordering process. Various kits can include options for direct-drive or magnetic-drive agitation, as well as water-jacketed or heat-blanketed vessels in 1 - 10 L range (approximate working volume). Ancillary equipment such as pH/DO and foam/level sensors are either included in kits, or can be added separately as options.

Materials and methods

Fermentation and cell recycling

For this application, a BioFlo 115 controller with advanced fermentation kit, direct drive and 2 L water jacketed vessel was used (Figure 1). The total fermentation process consisted of two distinct phases: An initial cell propagation and growth phase using complex medium (yeast extract) followed by sugar cane juice (growth medium) under aerobic conditions and the biofuel production phase using sugar cane juice (alcohol fermentation medium) under anaerobic conditions. The initial cell propagation phase utilized 20.0 g/L of medium substrate (dry mass) under aerobic condition, after that, the sugar cane growth media was added. Cells were recovered for use in the biofuel production phase operated under anaerobic conditions. Cell recycling was conducted through centrifugation. The cells were recovered and diluted with 500 mL of sterile water and transferred back to the bioreactor aseptically via an addition port in the headplate. Sugar cane juice feeding was performed over a four-hour period using peristaltic pump 3 (fixed flow of 6.25 mL/min) up to a final volume of 1.5 L and was maintained for two more hours to ensure uptake of accumulated sugar. Cells were recycled for three consecutive alcoholic fermentations. The cell propagation
phase and the biofuel production phase were both operated under fed-batch mode.

**Medium**

The initial cell propagation phase used complex medium as follows (per liter of de-mineralized water):

<table>
<thead>
<tr>
<th>Initial complex medium composition</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>K$_2$SO$_4$</td>
<td>6.60 g/L</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>3.00 g/L</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>0.50 g/L</td>
</tr>
<tr>
<td>CaCl$_2$•2H$_2$O</td>
<td>1.00 g/L</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5.00 g/L</td>
</tr>
</tbody>
</table>

After autoclaving at 121 °C for 15 min, the medium was cooled to room temperature. The carbon source and additional supplements passed through a sterile filter were also added according to the following concentrations:

<table>
<thead>
<tr>
<th>Filter-sterilized elements</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>2.30 g/L</td>
</tr>
<tr>
<td>Thiamine</td>
<td>3.00 g/L</td>
</tr>
<tr>
<td>EDTA</td>
<td>15.00 mg/L</td>
</tr>
<tr>
<td>ZnSO$_4$•7H$_2$O</td>
<td>4.50 mg/L</td>
</tr>
<tr>
<td>CoCl$_2$•6H$_2$O</td>
<td>0.30 mg/L</td>
</tr>
<tr>
<td>MnCl$_2$•4H$_2$O</td>
<td>0.84 mg/L</td>
</tr>
<tr>
<td>CuSO$_4$•5H$_2$O</td>
<td>0.30 mg/L</td>
</tr>
<tr>
<td>FeSO$_4$•7H$_2$O</td>
<td>3.00 mg/L</td>
</tr>
<tr>
<td>NaMoO$_4$•2H$_2$O</td>
<td>0.40 mg/L</td>
</tr>
<tr>
<td>H$_3$BO$_3$</td>
<td>1.00 mg/L</td>
</tr>
<tr>
<td>KI</td>
<td>0.1 mg/L</td>
</tr>
</tbody>
</table>

The growth medium contained 129 g/L of total reducing sugar (TRS). The sugar content is shown in the table below:

<table>
<thead>
<tr>
<th>Sugar cane juice composition</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>102.51 g/L</td>
</tr>
<tr>
<td>Glucose</td>
<td>10.99 g/L</td>
</tr>
<tr>
<td>Fructose</td>
<td>10.01 g/L</td>
</tr>
</tbody>
</table>

The alcoholic fermentation medium contained 171.65 g/L of total reducing sugar (TRS):

<table>
<thead>
<tr>
<th>Sugar cane juice composition</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>133.01 g/L</td>
</tr>
<tr>
<td>Glucose</td>
<td>16.79 g/L</td>
</tr>
<tr>
<td>Fructose</td>
<td>14.85 g/L</td>
</tr>
</tbody>
</table>

**Inoculum**

The *Saccharomyces cerevisiae* strain used in this work was an unnamed strain cultivated at the Brazilian Bioethanol Science and Technology Laboratory. It was originated from the department of Food Engineering, State University of Campinas, Brazil. The strain was maintained on agar plates prepared as follows (per liter of de-mineralized water):

<table>
<thead>
<tr>
<th>Inoculum composition</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract</td>
<td>10.00 g/L</td>
</tr>
<tr>
<td>Peptone</td>
<td>20.00 g/L</td>
</tr>
<tr>
<td>Glucose</td>
<td>20.00 g/L</td>
</tr>
<tr>
<td>Agar</td>
<td>20.00 g/L</td>
</tr>
</tbody>
</table>

Before the inoculation, the strain was transferred from agar plate to a liquid complex medium containing the following (per liter of de-mineralized water):

<table>
<thead>
<tr>
<th>Liquid complex medium composition</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract</td>
<td>10.00 g/L</td>
</tr>
<tr>
<td>Peptone</td>
<td>20.00 g/L</td>
</tr>
<tr>
<td>Glucose</td>
<td>20.00 g/L</td>
</tr>
</tbody>
</table>

The inoculum was cultured in shake flask for 24 hours using a shaker set to 33 °C at 250 rpm.
Fermentor control conditions for cell propagation phase

During the cell propagation phase, the Dissolved Oxygen (DO) was controlled to 60 % using the cascade feature through agitation and air control. Temperature was controlled to 33 °C throughout the run and pH was controlled to 5.0 via the acid and base assigned to peristaltic pumps 1 and 2. These parameters were maintained until the biofuel production phase (alcohol fermentation phase) was initiated. When the cell propagation phase was completed, the medium was transferred to a 2 L flask using an external pump (Watson Marlow®). The medium was then placed inside the laminar flow cabinet and transferred into a centrifuge (Beckman Coulter® centrifuge with JLA-9.100 rotor) and was spun down at 8.000 rpm/4 °C for 10 minutes. Setpoints are listed below:

<table>
<thead>
<tr>
<th>BioFlo® 115 setpoints</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Agitation</td>
<td>Cascaded range at 250 – 600 rpm</td>
</tr>
<tr>
<td>Temperature</td>
<td>33 °C</td>
</tr>
<tr>
<td>pH</td>
<td>5.0</td>
</tr>
<tr>
<td>DO</td>
<td>&gt; 60 %</td>
</tr>
<tr>
<td>Gas (supplied by sparge)</td>
<td>Cascaded range at 0.5 – 1.0 SLPM (Air)</td>
</tr>
</tbody>
</table>

Fermentor control conditions for biofuel production phase

During the biofuel production phase, the process was changed from aerobic to anaerobic fermentation, and DO was monitored between 0 % and 2.3 %, but not controlled. Agitation was set to auto mode and held at 100 rpm. Temperature controlled to 33 °C throughout the run. pH was controlled to 5.0. These parameters were maintained until the alcoholic fermentation/biofuel production phase was initiated. Gas flow was shut-off and the alcohol fermentation medium was introduced so that the *Saccharomyces cerevisiae* could start anaerobic fermentation and produce ethanol. Setpoints are listed below:

<table>
<thead>
<tr>
<th>BioFlo® 115 setpoints</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Agitation</td>
<td>100 rpm</td>
</tr>
<tr>
<td>Temperature</td>
<td>33 °C</td>
</tr>
<tr>
<td>pH</td>
<td>5.0</td>
</tr>
<tr>
<td>DO (monitored only)</td>
<td>0 – 2.3</td>
</tr>
<tr>
<td>Gas</td>
<td>None</td>
</tr>
</tbody>
</table>

DO calibration

The DO sensor was calibrated using a standard two-point calibration method: 0 % (often referred to as the zero point) and 100 % (often referred to as span). The electronic zero method was performed by disconnecting the sensor from the cabinet, allowing the value to stabilize, and then reconnecting the sensor to the cable. The span was achieved by bringing the vessel filled with medium to all of the operational setpoints to a stable value and then spanning the DO sensor. DO should be calibrated post-autoclave as part of the pre-inoculation setup. The sensor was allowed to polarize for a 6 hour period. After calibration, DO should remain around 100 % until after inoculation.

pH calibration

The pH sensor was calibrated prior to the autoclave cycle outside the vessel using a two-point calibration method with two standard pH buffers. The pH 7.0 buffer was used to zero the sensor and the pH 4.0 was used as the span.

pH control

The pH parameters were maintained by adding a sulfuric acid solution (H₂SO₄) via pump 1 (assigned as “acid”) to lower the pH and adding potassium hydroxide solution (KOH) via pump 2 (assigned as “base”) to raise the pH. The dead-band implemented for pH control was 0.02.

Fed-batch

Filter-sterilized media were fed to the vessel through pump 3.

Monitoring

Sucrose, glucose and fructose concentrations were detected by high-performance liquid chromatography (HPLC) using an Agilent® 1260 Infinity with RI detector through an Aminex® column (HPX-87P, 300 mm x 7.8 mm) at 60 °C. EMD Millipore® Milli-Q® water was used (column flow rate 0.5 mL/min) for the eluent phase. Ethanol concentration was determined by HPLC as well, using a Thermo Fisher Scientific® Dionex® UltiMate® 3000 with RI detector (Shodex RI-101) via an Aminex column (HPX-87H 300 mm x 7.8 mm) at 50 °C. Sulfuric acid, 5 mM at a rate of 0.5 mL/min, was used for the eluent phase. Dry weight mass measurements were carried out in triplicate using an analytical balance. Cell propagation was monitored on line through an Aber® capacitance probe as well as by taking samples for optical density measurements at 600 nm using a spectrophotometer.
Results and discussion

Figure 2, right, shows plots of the three key process parameters monitored during the biofuel production phase: total cell mass, X (kg/m³); substrate, S (kg/m³); and ethanol, P (kg/m³). All three are important factors in monitoring ethanol production from metabolized sugar cane juice. The cell concentration profile presented in Figure 2 (A) and the substrate profile presented in Figure 2 (B) illustrate the typical results in a fed-batch configuration. For the first 3 hours of the biofuel production phase, the initial density of yeast cells (44 kg/m³) decreased due to the dilution factor by sugar feeding. In the meantime, the sugar concentration (S) started to accumulate. After necessary sugar feeding, the cell concentration stabilized and the sugar concentration started to decrease and was completely consumed over time. The ethanol, as illustrated in Figure 2 (C), was produced according to available sugars in the medium and the production increased over time to reach a final concentration of approximately 56 kg/m³.

Ordering information

<table>
<thead>
<tr>
<th>Description</th>
<th>N. America Order no.</th>
<th>International Order no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>New Brunswick™ BioFlo® 115 Master Control Station w/Thermal Mass Flow Controller (TMFC)</td>
<td>Call</td>
<td>Call</td>
</tr>
<tr>
<td>Add-a-Vessel Advanced Fermentation Kit 3 L (Water Jacketed)</td>
<td>M1369-1612</td>
<td>M1369-1612</td>
</tr>
</tbody>
</table>

Your local distributor: www.eppendorf.com/contact
Eppendorf AG • 22331 Hamburg • Germany
eppendorf@eppendorf.com

www.eppendorf.com
Abstract

In this study, we used *Saccharomyces cerevisiae* to produce ethanol under anaerobic conditions in a BioFlo 310 fermentor. Initially, the yeast culture was conducted under aerobic conditions; after 24 hours, the culture was switched to anaerobic growth for the production of ethanol. During the fermentation process, redox potential was maintained at -180 mV using a redox sensor and gas flow control. Toward the end of fermentation, a significant amount of ethanol was produced and the cells retained maximum viability. Our study indicated that with a redox sensor, the BioFlo 310 fermentor was capable of maintaining anaerobic conditions, extremely low oxygen level, during the entire fermentation process.

Introduction

Metabolic activity of microorganisms depends on many factors, including oxidation and reduction reactions, or the “redox potential” of the culture environment. Redox reactions govern metabolism of biologically important nutrients such as carbon, hydrogen, oxygen, nitrogen and sulfur. Measuring their redox potential allows the fermentor operator to monitor the addition of reducing or oxidation agents, while ensuring that the potential is in the proper range for cell growth, especially when the DO level is very low.

Since free electrons never exist in any noteworthy concentration, reduction and oxidation reactions are always coupled together, and can be considered a measure of the ease with which a substance either absorbs or releases electrons. The determination of redox potential is a potentiometric measurement, expressed as millivolts (mV). Practically, however, no electrical current flows through the sample solution during this potential measurement.

Redox sensors are most commonly used to maintain anaerobic conditions in a fermentation broth. They can be used to measure trace amounts (< 1 ppm) of dissolved oxygen, at levels that are too low for the DO sensors in various anaerobic fermentation processes. Glucose-containing feed medium can be treated as a reducing source in oxidation-reduction of the culture medium. When the oxidation capacity is increased, the redox potential level will elevate to a higher value. On the flip side, its value will become lower when the culture broth has a higher reducing capacity.

Our study used *Saccharomyces cerevisiae* yeast (ATCC® 20602), because Saccharomyces is widely used in industry (e.g. beer, bread and wine fermentation and ethanol production), as well as in the lab due to its ease of manipulation and growth. Additionally, yeasts are eukaryotic and comparatively similar in structure to human cells. *S. cerevisiae* metabolizes glucose to ethanol primarily.
by way of the Embden-Meyerhof pathway. However, a small concentration of oxygen can be provided to the fermenting yeast, as it is a component in the biosynthesis of polyunsaturated fats and lipids. We used redox potential measurements to maintain these special anaerobic fermentation conditions. A trace of air (oxygen) was introduced as an oxidation agent to raise the redox potential level. A short pulse of air was introduced into the vessel when redox potential fell below -180 mV (Figure 2).

Materials and Methods

*S. cerevisiae* strain ATCC 20602 was grown in a 5 L working volume benchtop Eppendorf New Brunswick BioFlo 310 fermentor. Ethanol production and glucose concentrations were measured with a YSI® 2700 Select™. A Mettler-Toledo® redox sensor was directly connected to the BioFlo 310 controller to track redox potential. A Vi-CELL® XR Cell Viability Analyzer was used to measure cell viability and concentration throughout the entire process.

A seed culture using a 1.0 mL frozen suspension was prepared in a 1 L Erlenmeyer flask containing 250 mL of Becton Dickinson Difco™ YM growth medium. The culture was incubated at 29 °C for 18 hours in an orbital shaker (New Brunswick Innova® 43R) at 240 rpm. The entire inoculum was transferred to the BioFlo 310 fermentor vessel containing 4.75 liters of medium. Medium composition was as follows:

<table>
<thead>
<tr>
<th>Medium Composition</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>10.00 g/L</td>
</tr>
<tr>
<td>MgSO₄•7H₂O</td>
<td>0.60 g/L</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>3.00 g/L</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>10.00 g/L</td>
</tr>
<tr>
<td>CaCl₂•2H₂O</td>
<td>0.14 g/L</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>18.00 g/L</td>
</tr>
<tr>
<td>Soy Peptone</td>
<td>18.00 g/L</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>1.00 g/L</td>
</tr>
<tr>
<td>Thiamine</td>
<td>0.01 g/L</td>
</tr>
<tr>
<td>Trace metal solution</td>
<td>1.00 mL/L</td>
</tr>
<tr>
<td>Antifoam</td>
<td>0.5 – 1.00 mL/L</td>
</tr>
</tbody>
</table>

Setpoints were as follows:

<table>
<thead>
<tr>
<th>Medium Composition</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>30.0 °C</td>
</tr>
<tr>
<td>pH</td>
<td>5.0</td>
</tr>
<tr>
<td>Aeration rate</td>
<td>2.5 L/min</td>
</tr>
<tr>
<td>Agitation speed</td>
<td>0.5 VVM</td>
</tr>
<tr>
<td></td>
<td>200 – 800 rpm</td>
</tr>
</tbody>
</table>

pH was controlled with a 29 % NH₄OH base solution. 50 % glucose was used as feed medium. DO and redox potential were measured during the entire process. DO was cascade-controlled at 30 % via agitation in the growth phase. Glucose feed started at 7 hours of elapsed fermentation time (EFT) after the glucose was close to 1 g/L. New Brunswick BioCommand® software was used to control and log the entire process. The optical density of fermentation broth was measured at 600 nm to monitor cell growth. To
determine concentrations of glucose, ethanol and the dry cell mass, samples were centrifuged and the supernatant and biomass were collected separately. Biomass samples were dried at 80 °C for 48 hours.

Anaerobic Ethanol Production Phase
After 24 hours of cell growth, the fermentation process was switched to an anaerobic condition by exposing culture to two simultaneous perturbations: a rapid depletion of oxygen and glucose feeding regulated by redox potential measurement. Nitrogen, instead of air, was used to sparge the fermentor vessel. Gas flow rate was kept at 0.5 VVM (2.5 L/min). On-line redox potential readings of -180 mV triggered a solenoid valve of air supply to maintain the oxidation-reduction level for the ethanol production (Shown in Figure 1). pH was well controlled at 5.0. Cells remained healthy; OD values were maintained around 80 and viability was 85% at 72 hours EFT. 85 g/L of ethanol were produced in 70 hours as shown in Figure 3.

Conclusion
S. cerevisiae was cultured in an aerobic fermentation, and then switched to an anaerobic process using on-line redox measurements to maintain oxidation-reduction levels for ethanol production. Our study produced 85 g/L of ethanol in 70 hours, while cell viability was maintained at levels as high as 88%. The study provides a new technique for using redox potentials to monitor and control ethanol production from yeast, but also demonstrates the BioFlo 310 fermentor as a versatile fermentor for aerobic and anaerobic fermentations.
Anaerobic Yeast Fermentation for the Production of Ethanol in a New Brunswick™ BioFlo® 310 Fermentor

Yinliang Chen, Jeff Krol, Weimin Huang, Rich Mirro and Vikram Gossain, Eppendorf Inc., Enfield, CT, U.S.A.

Abstract

Whether used for research or production, the versatile New Brunswick BioFlo 310 fermentor from Eppendorf allows growth of a wide variety of aerobic and anaerobic microorganisms, including bacteria, plant, algae, fungi and yeast. Its advanced controller can regulate up to four vessels simultaneously, 120 process loops in all. Here we demonstrate one facet of its versatility—a technique for inducing ethanol production in yeast, by switching from an aerobic growth phase to an anaerobic steady-state culture.

Introduction

Saccharomyces cerevisiae is a model eukaryotic organism, often used in research because it is easy to manipulate and culture, and is comparatively similar in structure to human cells. This yeast is also widely used in industrial applications to manufacture enzymes and proteins for beer, wine and bread, and because it metabolizes glucose to ethanol, is also used to produce many biofuel products. We produced ethanol from a S. cerevisiae (American Type Culture Collection® strain 20602) in a 7.5 liter BioFlo 310 fermentor, to demonstrate the flexibility of this advanced fermentation system. In the first phase, we grew the yeast in an aerobic environment, using a dissolved oxygen cascade control strategy to produce a sufficient cell density. Then we pumped in nitrogen gas to create an anaerobic environment for inducing ethanol production, and used reduction and oxidation (redox) potential measurements to monitor any increase in dissolved oxygen levels, which signaled a slowdown in cell growth. Redox potential activates ethanol production and changes the total soluble protein pattern of S. cerevisiae[1]. We used redox potentials to control the oxidation-reduction level by adding feed medium to the vessel when the redox potential value in the fermentor vessel rose above 130 mV.

Materials and Methods

Advanced Control in a Compact Package

The BioFlo 310 fermentor (Fig. 1) consists of a master control station with built-in controller, 15-inch color touchscreen display, three built-in pumps and 4-gas mixing with a thermal mass flow controller for gas flow control. The
BioFlo 310 is available with choice of four interchangeable autoclavable vessels, 2.5, 5.0, 7.5 and 14.0 liter total volume, and includes a pH probe, dissolved oxygen probe and level or foam probe, as well as hoses, sterile sampler and more. The system can be operated in batch, fed-batch and continuous modes for handling a variety of applications, and it meets current good manufacturing practice (cGMP) requirements. Here we used a 7.5 L vessel and operated the system in fed-batch mode. We added a Mettler-Toledo® redox sensor to the vessel and directly connected it to the fermentor controller’s optional redox input. We used New Brunswick’s optional BioCommand® Plus with OPC Control software to automatically control the process and log data throughout the run. Ethanol production and glucose concentrations were measured off-line using a YSI® 2700 Select™ Biochemistry Analyzer, and we used a Beckman Coulter® Vi-Cell® XR Cell Viability Analyzer to measure the cell viability and concentration during the entire process.

Inoculum Preparation and Growth Phase
We prepared a seed culture in a one-liter Erlenmeyer flask containing 250 mL of Difco™ YM growth medium (Becton Dickinson), using a 1.0 mL frozen suspension. The culture was incubated at 29 °C for 18 hours at 240 rpm in an orbital shaker (New Brunswick model Innova® 43R). Then we transferred the inoculum to the BioFlo 310 vessel containing 4.75 liters of fermentation medium. The fermentor was controlled at 30 °C and pH 5.0. Aeration rate was set at 2.5 L/min[1] (0.5 vessel volumes per minute), and agitation speed was 200 - 800 rpm. We used a 29 % NH4OH base solution to control pH and 50 % glucose as feed medium. Dissolved oxygen and redox potential were measured during the entire process (Fig. 2). Dissolved oxygen was cascade-controlled at 30 % via agitation. The optical density of the fermentation broth was measured at 600 nm to monitor cell growth. Glucose feed started at 7 hours of elapsed fermentation time (EFT), after the glucose was close to 1 g/L[1]. To determine concentrations of glucose, ethanol and the dry cell mass, we centrifuged the samples and collected the supernatant and biomass separately. Biomass samples were dried at 80 °C for 48 hours. The cell concentration reached 3.26 × 10^8 cells/mL[1] in 24 hours, and cell viability remained above 96 %. The feed volume was 1,200 mL in 17 hours of fed-batch growth phase, with the feeding rate ramping from 0 to 16 mL/L/H[1]. Although cells were in an aerated fermentation condition, a small quantity of ethanol (20 g/L[1]) was produced before the beginning of anaerobicity.

Figure 2. Time profiles of dissolved oxygen and redox measurements during S. cerevisiae fermentation. Dissolved oxygen was controlled at 30% during the cell growth phase followed by an anaerobic production phase in nitrogen gas.
Anaerobic Ethanol Production Phase

After 24 hours, we exposed aerobic, glucose-limited cultures grown at a moderate specific growth rate to two simultaneous perturbations: a rapid depletion of oxygen and glucose feeding regulated by redox potential measurement. Nitrogen, instead of air, was used to sparge the fermentor. The gas flow rate was kept at 0.5 VVM (2.5 L/min[1]). The medium feed was controlled by BioCommand OPC software using online redox potential measurement as an on-line input. Redox potential readings of 130 mV triggered a medium feed pump to add carbon source medium to maintain the oxidation-reduction level for the ethanol production (Fig. 3). Cell viability was monitored and measured, validating that cells remained healthy in the anaerobic production phase. Optical density values were maintained around 80 and viability at 88 % at 72 hours elapsed fermentation time. Culture broth pH was well maintained at 5.0.

Conclusion

We cultured yeast in an aerobic fermentation and then switched to an anaerobic process to induce ethanol production, demonstrating how versatile the BioFlo 310 fermentor can be. We produced 85 g/L[1] of ethanol in 70 hours, while cell viability was maintained at levels as high as 88 %, proving this fermentor to be a very powerful and capable research or production instrument.

In addition to being used for a wide variety of fermentation processes, the BioFlo 310 can also be adapted for mammalian or insect culture with the use of optional accessories. Multiple connections are provided for integrating data from ancillary sensors, analyzers, scales or other devices for optimized process control. Eppendorf offers optional validation and training packages, as well as the services of an in-house lab to assist with process development and scale-up. For more information on this system or on our full range of advanced fermentors and bioreactors for research through production, see www.eppendorf.com or write to us at newbrunswick@eppendorf.com.

References

Determination of $k_{La}$ Values of Single-Use Bioreactors

**DECHEMA® Gesellschaft für Chemische Technik und Biotechnologie e.V.** (Society for Chemical Engineering and Biotechnology) brings together experts from a wide range of disciplines, institutions, and generations to stimulate scientific exchange in chemical engineering, process engineering, and biotechnology [1].

Single-use bioreactors are increasingly used in research, process development, and production. Like conventional reusable systems they have to allow the setup of optimal growth conditions. A sufficient supply of oxygen is crucial for aerobic bioprocesses, and the velocity of oxygen entry into the culture medium is often described by the volumetric mass transfer coefficient ($k_{La}$). Because single-use bioreactors offered by different manufacturers vary in parameters critical for oxygen transfer—like mixing, power input, and gassing strategies—they are often not directly comparable to each other and to conventional glass and stainless steel vessels. Even $k_{La}$ values experimentally determined by the manufacturer or users are not necessarily comparable, because the results might differ dependent on the method used.

**DECHEMA published guideline for $k_{La}$-determination**
To help the users to objectively compare the performance of bioreactors in terms of oxygen transfer, the DECHEMA expert group on “Single-Use technology in biopharmaceutical manufacturing” developed a standard operating procedure (SOP) for $k_{La}$ measurements. Using this protocol, different manufacturers experimentally determined the $k_{La}$ values of single-use bioreactors they offer. A DECHEMA report summarizes the results [2], (Figure 1). It also describes in detail the SOP used, to offer scientists a guideline to determine the $k_{La}$ values for any bioreactor of interest.

**$k_{La}$ values of Eppendorf BioBLU® Single-Use Vessels**
Eppendorf determined the $k_{La}$ values of two single-use vessels for microbial applications, namely the Eppendorf BioBLU® 0.3f Single-Use Vessel with a maximum working volume of 250 mL and the Eppendorf BioBLU 1f Single-Use Vessel with a maximum working volume of 1.25 L (Figure 2). For both vessels the Eppendorf application lab measured $k_{La}$ values > 500 h$^{-1}$, which is considerably higher than the values of the other lab-scale fermentors tested (Figure 3).

**Fig. 1:** The DECHEMA report “Recommendations for process engineering characterization of single-use bioreactors and mixing systems by using experimental methods” contains the SOP used for $k_{La}$ determination, results, and background information.

**Fig. 2:** Eppendorf BioBLU 0.3f (left) and 1f (right) Single-Use Vessels.
Outlook
In their report, the DECHEMA group also published guidelines for the experimental determination of specific power input for bioreactors and the mixing time, and they plan to develop further SOPs, such as one for the determination of the volumetric mass coefficient for CO2. These protocols will be valuable guidelines to standardize experiments for the characterization of bioreactor performance and to facilitate their comparison.

References
Software and Automation
A Generic Biomass Soft Sensor and Its Application in Bioprocess Development

Julian Kager¹, Jens Fricke¹, Ulrike Beeken*², and Christoph Herwig**³

¹ Research Area Biochemical Engineering, Institute of Chemical, Environmental and Biological Engineering, TU Wien, Vienna, Austria
² Eppendorf AG Bioprocess Center, Juelich, Germany
* Contact for information on the Eppendorf bioprocess equipment: becken.u@eppendorf.com
** Contact for information on the soft sensor algorithm and implementation: christoph.herwig@tuwien.ac.at

Abstract

Biomass concentration is a key process variable, which is used to identify trends and initiate process events in microbial bioprocesses. Real-time data on the biomass is essential to implement advanced control strategies, including the control of biomass-specific nutrient uptake rates.

Christoph Herwig’s group at Technische Universität Wien (TU Wien) has developed a biomass soft sensor based on readily available measurements and process parameters. They used the soft sensor to control various substrate uptake rates in parallel small-scale bioreactors.

Introduction

In developing a bioprocess, researchers incur significant costs to determine the optimal culture conditions and install the process control technology to maintain them [1, 2, 3]. They routinely monitor and control the temperature, pH, and availability of oxygen in the culture, but these are not the only relevant process parameters. A time-resolved determination of the biomass concentration is also of great importance, enabling them to not only monitor cell growth, but also to establish an optimal supply of substances, like nutrients, co-factors, and inducers. The demand for them changes over time as the culture grows. To manage the availability of nutrients we must know how much of them is consumed. The nutrient supply can be described by the biomass-specific nutrient uptake rate at a given point in time ($q_n$) (Box 1).

There are several methods to determine the biomass concentration in a microbial bioprocess. The choice of the best method for high-cell-density fermentations depends strongly on the demands of the application. At the production stage, robustness and accuracy are the main demands, while cost and transferability are of limited concern [4]. Methods based on light scattering

![Fig. 1](image-url) Fig. 1: In many bioprocesses carbon is metabolized to biomass and CO₂ by oxidative transformation. Out of known or measured amounts of carbon (substrate), O₂ and CO₂ that are introduced to and leave the bioreactor, a soft sensor can calculate the biomass.
and transmittance, like optical density measurement, are widely used. They are inexpensive and easy to implement, but their application range is limited to certain biomass concentrations.

Researchers at TU Wien developed a biomass soft sensor as an alternative method for biomass determination. The term soft sensor combines the words software and sensor. The idea behind it is to use easy-to-determine parameters to calculate variables that are more difficult to obtain by direct measurement. The soft sensor delivers readings like a conventional hardware sensor, but uses a mathematical model to determine the sensor signal based on other measurements [5].

The soft sensor for biomass determination described here uses mass balancing. It is based on the hypothesis that in a bioprocess carbon is metabolized to biomass and CO₂ by oxidative transformation. This applies to the most industrially used bacterial, mammalian, and yeast cell lines. From the amount of carbon that goes into the bioreactor (quantified, for example, by measuring the amount of substrate that is fed in) and leaves the bioreactor (quantified, for example, by measuring the CO₂ concentration in the exhaust), we can estimate the biomass concentration (Fig. 1). Feeds and outflows of the bioreactor are quantified using standard online measurements, and the software calculates the biomass based on a data model.

This application note describes the successful implementation of such a biomass soft sensor in an Eppendorf DASbox® Mini Bioreactor System using the DASware® control software. The biomass estimate was used to evaluate and control the biomass-specific substrate uptake rates of different nutrients in high-density E. coli fermentations. The combination of the biomass soft sensor with an optical density sensor enables full automation of the multi-bioreactor system for bacterial processes.

Material and Methods

The project comprised two steps. First, the researchers implemented the soft sensor. Then they used it to set up control loops to control the biomass-specific uptake rates of different substrates in E. coli high-density fermentation processes (Fig. 3). For example, they show results on the simultaneous control of the biomass-specific uptake rates of glucose and lactose.

**Soft sensor implementation**

**Setup of data model**

As the first step of the soft sensor implementation the

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**Box 1: Biomass-specific nutrient uptake rate qs**

A way to describe the substrate consumption of organisms is to calculate the biomass-specific nutrient uptake rate at a given time point (qs(t)). It indicates how many grams of substrate are consumed per gram of biomass per hour. The amount of substrate consumed by the culture equals the amount of substrate fed in, as long as the nutrients are limited and thus are completely taken up by the culture. The nutrient supply, and thus the biomass specific substrate uptake rate, can be controlled with the feed pump rate [9]. To calculate qs(t), the biomass concentration must be measured.

$$qs(t) = \frac{F(t) C_s}{x(t) V(t)} \text{ [g/(g*h)]}$$

qs(t): Biomass specific substrate uptake rate [g/g] at time point (t)

F(t): Feed flow rate [L/h] at time (t)

C_s: Substrate concentration in feed [g/L]

x(t): Cell dry weight concentration [g/L] at time (t)

V(t): Bioreactor volume [L] at time (t)
researchers set up a data model that describes the oxidative transformation of carbon to biomass and CO₂. The mass balance system is depicted in Figure 2. One can solve the material balances of carbon and free electrons (C balance and DoR balance). The rates of substrate uptake, carbon evolution, oxygen uptake, and biomass formation have to be estimated [6]. Table 1 summarizes the measurements and constants which are used to do so. The substrate uptake rate is determined from the pump flow rate. The rates of carbon evolution and oxygen uptake are calculated from exhaust composition and mass flow.

The additional system redundancy allows for system reconciliation. The residuals ε are assigned to the rates according to their measurement error. A statistical test (h-value) evaluates the system integrity, which applies if the residuals can be explained solely by measurement errors [7].

In this application, the researchers designed the soft sensor to work in strictly C-source-limited fed-batches or

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**Table 1:** Needed measurements and constants for rate estimation.

<table>
<thead>
<tr>
<th>Rate</th>
<th>Description</th>
<th>Measurement</th>
<th>Constant parameter</th>
<th>Assumed error</th>
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<tr>
<td>rₛ [C-mol/h]</td>
<td>Substrate uptake rate</td>
<td>Pump flow rate [L/h]</td>
<td>C-concentration (C-mol/L)</td>
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<td>r_{CO₂} [mol/h]</td>
<td>Carbon evolution rate (CER)</td>
<td>CO₂ and O₂ concentrations (off gas analyzer [%] and mass flow [sL/h])</td>
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<td>rₒ₂ [mol/h]</td>
<td>Oxygen uptake rate (OUR)</td>
<td>Calculated by soft sensor</td>
<td>Elemental composition of biomass (g/C-mol)</td>
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<td>rₓ [C-mol/h]</td>
<td>Biomass formation rate</td>
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<td></td>
<td>≈10 %*</td>
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continuous cultures where the product and possible by-products can be neglected.

**Creation and integration of soft sensor algorithm**

Once they had established the data model, the researchers created the soft sensor algorithm in MATLAB®. They incorporated it into the DASware control software through a Visual Basic control script that accesses the function. The results are stored, and can be visualized directly, within the DASware control software (Fig. 4) without the need for further customization or the establishment of OPC connections. Additional features like automated phase transition from batch to fed batch and induction as well as control options were included.

Figure 3 summarizes how the soft sensor is integrated into the bioprocessing environment.

**Soft sensor calibration**

The bioprocesses were run in two phases. In the initial batch phase the cultures grew to a cell dry weight (CDW) of up to 10 g/L. During this phase, the biomass concentration can be measured accurately with optical density sensors. At the end of the batch phase, the actual biomass concentration measured with the OD sensor was used to start the soft sensor.

**High-density fermentation**

**Culture conditions**

The researchers conducted the fermentations in a DASbox® Mini Bioreactor System (Eppendorf AG, Germany) equipped with glass vessels or BioBLU® 0.3f Single-Use Vessels. Both vessel types have a maximum working volume of 250 mL.

They used a recombinant *E. coli* strain, which bears a green fluorescent protein (GFP) gene under the control of the LAC promoter. The bacteria were cultivated in a chemically defined medium with a limited amount of C-source (10 g/L glucose).

After C-source depletion, glucose and inductor (lactose) were fed to the culture with a DASGIP® MP8 multi pump module (Eppendorf AG, Germany).

The pH was maintained at 7.2 by NH₃ addition, which served as an additional N-source. Air saturation was kept over 30 % by increasing the stirrer speed and oxygen concentration in the inflow air, which was supplied with 2 VVM through an L-sparger. The five M18 ports were occupied by pH, DO, and OD sensors (Eppendorf DASGIP OD4 Module, 880 nm), gas inlet and gas outlet. Three dip tubes were used for feed, base addition, and offline sampling.

The exhausted gas was analyzed by a DASGIP GA4 gas sensor module (Eppendorf AG, Germany) with a ZrO₂ sensor for O₂ and infrared CO₂ sensor technology.

**Offline measurements**

To determine the cell dry weight (CDW) the researchers centrifuged 2 mL of culture broth (4,500 x g, 4°C, 10 min), washed the cell pellet with a 0.1 % NaCl solution, and subsequently dried it at 105°C for 48 h.

Cell-free samples of the cultivation broth were analyzed...
for concentrations of substrates and metabolites using HPLC (Agilent Technologies, USA) with a SUPELCOGEL™ C-610 H ion exchange column (Sigma-Aldrich, USA) and a refractive-index detector (Agilent Technologies, USA). The mobile phase was 0.1 % H₃PO₄ with a constant flow rate of 0.5 mL/min, and the system was run isocratically at 30°C.

Results

To demonstrate the functionality of the system, the researchers carried out experiments with a recombinant E. coli strain bearing a GFP gene under the control of the LAC promoter. They aimed to simultaneously control the availability of lactose and glucose, to equilibrate the bacterial growth, energy maintenance, and productivity.

Lactose was used instead of IPTG to induce the GFP hosting LAC operon. Lactose is metabolized by the host strain, so for continuous protein production it must be continuously added to the culture.

The researchers tested different glucose-to-lactose ratios in four parallel fermentations. Each fermentation run used the same setpoint for the glucose uptake rate \( q_{s\text{Glu}} \) but varied the lactose uptake rate \( q_{s\text{Lac}} \). To control \( q_s \), the biomass concentration was estimated online using the soft sensor. Based on this data, the pump flow rate was automatically adjusted to control the supply of a mixed feed containing lactose and glucose.

Figure 5 shows optical density and the soft sensor-based biomass concentration estimates of one fermentation. The biomass concentration deriving from offline measurements (black crosses), OD correlation (blue line) and soft sensor (orange line) are depicted.

The data derived from the biomass soft sensor showed good alignment with the reference measurements. The biomass soft sensor application achieved an accuracy of < 10%, which is within its working range [5]. The soft sensor application achieved an accuracy of < 10%, which is within its working range [5].

Fig. 5: Biomass concentration estimates.
Crosses: Biomass concentration (cell dry weight, CDW) deriving from offline measurements.
Blue line: OD correlation.
Orange line: Results from soft sensor.
NRMSE: Normalized root mean square error

Fig. 6: Setpoints and time courses of \( q_{s\text{Glu}} \) (solid lines) and \( q_{s\text{Lac}} \) (dashed lines) in the four fermentation runs (bioreactor A-D). spt: setpoint
Conclusion

The researchers implemented a relatively simple and straightforward method for biomass estimation in a small-scale parallel bioreactor system. The soft sensor works for strictly C-source-limited fed-batch and continuous cultures. The researchers used it to simultaneously control the uptake rates of a substrate and an inducer. The results obtained are fully scalable and applicable in industry without the need for expensive additional measuring equipment. Besides manual offline sampling and sample volume reduction, the described fermentations could run fully automatically.

In bioprocess development, the control of biomass-specific substrate uptake rates is a valuable tool to determine the optimal supply of nutrients and/or inducers. The researchers performed similar studies with *Pichia pastoris* strains to tune the pAOX promoter. Repressing glycerol was used as the C-source and methanol as the inducer.

The Eppendorf bioprocess control software DASware control 5 (former version DASGIP control 4.5) offers the interface for the integration of soft sensors. The soft sensor algorithm and process control strategies are developed by the end-user.
Literature


## Ordering information

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A Quality-by-Design Approach to Upstream Bioprocess Interrogation and Intensification

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* Corresponding author: becken.u@eppendorf.com

Abstract

Efficient biopharmaceutical process development relies on the Quality-by-Design (QbD) paradigm. QbD is a scientific, risk-based proactive approach to drug development that aims to have a full understanding of how the process and product are related. This knowledge is gained by process analytical technology (PAT). In this case study the Applied Process Company (APC) integrated external PAT and an APC-developed controller with an Eppendorf DASGIP® Parallel Bioreactor System. Online PAT measurement and control of the identified critical process parameters led to greater understanding and the streamlined optimization of a mammalian cell bioprocess. The study exemplifies the value of flexible bioreactor systems which allow ease of integration of third-party technology.

Introduction

Biopharmaceuticals represent a significant and growing sector of the pharmaceutical industry. The global biopharmaceutical industry is currently worth over 107 billion euros, according to research conducted by BioPlan Associates [1]. However, a lack of understanding of the process and product interdependencies potentially results in manufacturing challenges. Optimization of protein production based on empirical experimentation and quality testing of the end product often results in bioprocesses operating under suboptimal conditions and hence in extended cycle times, excessive raw material and utility requirements, and elevated numbers of process or product failures which together culminate in a high cost of manufacturing.

To ensure consistent drug quality and reduce batch-to-batch variability pharmaceutical companies and regulatory authorities aim to replace the "Quality-by-testing" with a "Quality-by-Design" strategy. In a QbD approach, critical quality attributes of the end product are defined and subsequently a production process is developed which aims at meeting those attributes. This involves identification of a design space, a range of experimental conditions within which variations in process parameters do not affect product quality. Prerequisite is the identification of critical process parameters by PAT that in the course of protein manufacturing are then monitored and, if needed, controlled.
The PAT required to support a QbD product may be simple or complex depending on the nature of the product and process used for its manufacture. Generally speaking, mammalian cell culture processes and the products, which they produce are complex, and hence, there are many challenges to implementing a PAT/QbD strategy. However, the potential benefits coupled with regulatory pressures are driving a strong interest in and an increased level of adoption in the biopharmaceutical industry. Biological processes are known to have a much higher level of variability than their chemical counterparts. PAT and QbD offer an avenue to better understand and control this variability, which has implications for the process economics, control and final product quality. Traditionally, temperature, pH, and dissolved oxygen are the main parameters measured and controlled online due to the availability of traditional, robust sensors. However, as technology advances, other process parameters such as cell density, cell viability, substrate concentrations, product and by-product concentration, dissolved carbon dioxide and biomarkers can now be measured and analyzed in real-time in an automated manner, although not yet routinely. The availability of real-time process information is of particular value in addressing the variability and unpredictability of animal cell cultures as it opens up the possibility of implementing advanced control strategies capable of directly impacting critical process parameters and critical quality attributes.

APC delivers chemical and bioprocess engineering solutions and technologies to enable streamlined development, optimization, and supply of new and existing chemical and biological entities. APC’s process development technology platform (BIOACHIEVE™) combines PAT technology, multivariate data analysis, process modelling, and advanced control strategies [2].

This application note describes the successful integration of external PAT with an Eppendorf DASGIP Parallel Bioreactor System using the Eppendorf’s software DASware analyze. The aim was to optimize the performance of a Chinese hamster ovary (CHO) mammalian cell bioprocess. In a previous study the glucose concentration was identified as a critical process parameter [3]. The objective of the following case study was to improve process performance by optimizing the glucose feed profile. By moving from the traditional bolus fed-batch to a continuous feeding fed-batch strategy, nutrient depletion should be prevented and a stable macro-environment for the cells should be established.

Material and Methods

Cell culture

CHO cells were cultivated in a glucose-free formulation of EX-CELL® CHO DHFR medium (Sigma-Aldrich® Co., LLC, USA) supplemented with 20 mM glucose, 4 mM glutamine, 1 μM methotrexate, 0.1 % (v/v) Pluronic® F-68 (Sigma-Aldrich Co., LLC, USA) and 10 mL/L penicillin-streptomycin (Sigma-Aldrich Co., LCC, USA). Cells were cultivated for
7 to 9 days at 37°C using a four-fold DASGIP Benchtop Bioreactor System for cell culture equipped with 2.5 L (working volume) DASGIP Benchtop Bioreactors (Eppendorf AG, Germany) (figure 1). Cultures were agitated at 120 rpm. The pH was controlled at 7.2 using 1 M NaOH and CO₂ gas, respectively. Air saturation was set to 50% and controlled using air and oxygen supplied via a sparger.

**Offline measurement of process parameters**

Cell density and cell viability were determined offline using a Cedex® HiRes system (Roche Diagnostics® GmbH, Germany). Offline measurements of glucose concentrations were performed using an enzymatic assay kit (Megazyme® International Ireland Ltd.).

**Integration of external PAT and in-house developed controllers**

Figure 2 illustrates the integration of external PAT and an in-house developed controller with an Eppendorf DASGIP Parallel Bioreactor System. Online measurements of glucose concentration were performed by Raman spectroscopy. A RamanRxn2 Multi-channel Raman analyzer (Kaiser Optical Systems, Inc., USA) was integrated with the DASGIP system using DASware analyze software (Eppendorf AG, Germany). DASware analyze utilizes an object linking and embedding for process control (OPC) communication protocol, an industry standard which facilitates the communication of devices from different manufacturers. To translate the Raman spectra into concentration information, chemometric partial least squared calibration models were developed using the SIMCA® multivariate data analysis package (MKS Umetrics AB, Sweden).

To maintain the glucose concentration close to the set-point an APC-developed model predictive controller (MPC) algorithm was integrated via bidirectional OPC communication. The essence of MPC is to optimize forecasts

![Fig. 3: Glucose set-point control via a model predictive controller algorithm resulted in an increase in cell densities.](image)

A, B: Glucose concentration was measured online and offline, respectively. (A) Glucose concentration was adjusted via a bolus fed-batch feeding strategy. (B) The glucose set-point was maintained in a continuous feeding fed-batch strategy using MPC.

C, D: Total cell densities (TCD), viable cell densities (VCD), and viability were determined for the bolus fed-batch culture (C) and the continuous feeding fed-batch culture (D).
of process behavior. This forecasting is accomplished with a process model, and, therefore, the model is an essential element of an MPC controller. Based on online readings of the identified critical process parameters, the model is used to calculate optimal feed rates for set-point maintenance. MPC facilitates to proactively counteract deviations from the set-point and to simultaneously control multiple process parameters and is hence especially suited to ensure constant culture conditions in multivariate bioprocesses.

The MPC actuated a DASGIP MP8 multi pump module (Eppendorf AG, Germany) to execute continuous glucose feeding.

**Bolus fed-batch and continuous fed-batch glucose feeding**

The glucose concentration was adjusted using a feed medium consisting of 653.6 mM glucose, 58.8 mM glutamine and 58.8 g/L soy protein hydrolysate dissolved in glucose-free EX-CELL CHO DHFR medium.

Two fed-batch feeding strategies were investigated in the study. The first was a fed-batch culture manually fed with bolus additions, at 24 h intervals, the volume of which was proportional to offline integral viable cell density measurements for the previous 24 h interval.

The second regime was a continuous feed, the rate of which was determined and adjusted automatically using a model predictive controller and Raman-determined glucose values to keep the glucose concentration in the bioreactor at a set-point of 11 mM throughout the culture.

**Results and Discussion**

Using the DASware analyze software a RamanRxn2 Multi-channel Raman analyzer (Kaiser) and an APC-developed MPC algorithm were successfully integrated with an Eppendorf DASGIP Parallel Bioreactor System. The glucose concentration in the CHO mammalian cell culture was determined online using Raman spectroscopy coupled with chemometric partial least squared modeling. The offline results determined via an enzymatic assay were highly comparable to the results obtained online by Raman spectroscopy (figure 3A, 3B).

Overall, the continuous feeding fed-batch bioprocess resulted in an increase in peak viable cell density and the integral of the viable cell density (figure 3C, 3D) which is directly related to increased titre.

**Conclusion**

The Eppendorf DASGIP Parallel Bioreactor System was a suitable host for APC’s BIOACHIEVE process development technology platform, which APC applies to upstream bioprocesses. The DASGIP system facilitated the ease of integration of external PAT and transfer of this information to and from APC-developed controllers. This allowed optimization of the process performance based on greater process understanding, ultimately delivering improvements in cell growth. This case study exemplifies the value of increased process understanding and control in biopharmaceutical manufacturing. Advancements in sensor technology and data analysis facilitate the ability to control the identified critical process parameters to their respective target levels and thus optimize the critical quality attribute design space.

To apply the QbD approach to upstream bioprocess development, bioreactor systems which allow smooth integration of external sensors and controllers are crucial.
Literature


## Ordering information

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Automated Bioreactor Sampling – Process Trigger Sampling for Enhancing Microbial Strain Characterization

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1 UC Berkeley Energy Biosciences Institute, Berkeley, CA, USA; 2 UC Berkeley Department of Bioengineering, Berkeley, CA, USA; 3 Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA, USA; 4 Flownamics® Analytical Instruments, Inc., Madison, WI, USA; 5 Eppendorf AG Bioprocess Center, Juelich, Germany
* Corresponding authors: mbiksacky@flownamics.com, huether.c@eppendorf.com

Abstract

This application note describes the integration of a Flownamics Seg-Flow® 4800 Automated On-line Sampling System with Eppendorf DASGIP® Parallel Bioreactor Systems as implemented at the Energy Biosciences Institute in Berkeley, California. The automated process trigger sampling technology enabled the researchers to rapidly characterize process events, parameters and stress responses that impact yeast strain gene regulation and, ultimately, biofuel productivity.

Introduction

Scientists at the Energy Biosciences Institute (EBI) conduct research in a variety of areas in bioenergy development. The Quantitative Engineering of Industrial Yeast program at the EBI focuses on a thorough, systems-level understanding of bacterial and yeast metabolism, gene regulation, and stress response for elucidating principles to help rationally engineer bacteria and yeasts for improved biofuel production from lignocellulosic sources [1].

In order to accomplish their goals and objectives, researchers in the Quantitative Engineering of Industrial Yeast program have implemented automated processes, including the use of an integrated parallel bioreactor system and automated bioreactor sampling system, to conduct experiments for optimizing yeast strain characterization and selection.

Materials and Methods

Incorporating tools such as parallel bioreactor systems and automated bioreactor sampling technologies can significantly reduce project timelines and increase the efficiency of the microbial strain characterization and selection process.

DASGIP® Parallel Bioreactor Systems

Eppendorf DASGIP Parallel Bioreactor Systems allow for advanced screening of bacteria, yeasts and/or fungi. The multi-bioreactor/vessel design enables parallel experimentation intended to accelerate process development and increase throughput. Multiple bioreactor vessels are controlled via shared equipment and a single computer system, enabling the experimenter to test multiple conditions side-by-side or by allowing multiple independent
experiments to be run simultaneously using the shared equipment resources. Additionally, the DASGIP Parallel Bioreactor System’s modular design provides ease of setup and maintenance, while offering the same control strategies and precision as larger scale production plants to achieve a reproducible and scalable process (figure 1) [2].

The DASGIP Control* software and associated hardware provides high precision monitoring and control units designed for small working volumes, high information output and easy comparative data analysis. The Eppendorf software DASware® analyze, utilizes the platform-independent Object Linking and Embedding for Process Control (OPC) communication protocol for enabling bidirectional communication between the DASGIP system and third-party analytical devices, including automated bioreactor sampling systems.

Seg-Flow® Automated On-line Sampling System
The Seg-Flow 4800 Automated On-line Sampling System (Seg-Flow System) is a liquid and data management device designed to withdraw samples from up to eight bioreactors and deliver them to up to four analytical instruments and/or fraction collectors. This functionality enables real-time analysis and sample collection from parallel bioreactor systems. The Seg-Flow System’s patented “segmented on-line sampling” technology allows a wide range of sample volumes to be obtained and rapidly delivered to distances up to 7.6 meters (25 feet) from the bioreactor.

The FlowWeb™ software platform, which controls all the Seg-Flow System functions, provides seamless connectivity with various third-party analyzers for enabling real-time analysis of important culture process parameters such as nutrients, metabolites and various cell measurements. Upon completion of the analysis, the Seg-Flow system acquires and processes the analyzer data. The FlowWeb OPC software suite communicates the analyzer data into any OPC-enabled supervisory control and data acquisition (SCADA) system, which expands real-time monitoring capabilities for bioprocess cultures. Figure 2 shows the Seg-Flow configuration used by EBI for conducting automated on-line fraction collection for their microbial strain characterization evaluation.

Process trigger sampling
The Seg-Flow System is capable of performing automated sampling and analysis during planned or unplanned process events in response to an external SCADA or other bioprocess management system such as the DASGIP Control/DASware software platform. This is achieved through OPC connectivity.

The process events used to activate, or trigger, the Seg-Flow System are user-defined. Examples of process events include pH or dissolved oxygen excursions, culture induction, feeding or other in-process control actions. The process events used to trigger the Seg-Flow system require OPC data tag configuration and must be programmed into the host SCADA/bioprocess management system. When the process event is detected by the bioreactor station, the data trigger is communicated to the SCADA system to commence the remote activation of the Seg-Flow system (figure 3).

Once the Seg-Flow system is activated, a sample is

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*DASGIP Control is now DASware control 5. Please refer to ordering information on page 6.
automatically withdrawn from the bioreactor for sample collection and/or analysis. Upon completion of the sample collection or analysis, the data is communicated to the SCADA/bioprocess management system via OPC over the laboratory network. When the sampling functions and data transfer are completed, the Seg-Flow System returns to an idle status. The data retrieved from the Seg-Flow system can then be used for additional process monitoring and control options. This unique remote control function allows the process scientist to conduct “around-the-clock” monitoring and sampling of unique process events that could impact process productivity and/or product quality.

Results and Discussion

Integrating the Seg-Flow® and DASGIP® Parallel Bioreactor System

Prior to implementing the Seg-Flow process trigger sampling technology, process events and environmental states affecting yeast stress responses and biofuel production could not be adequately evaluated or characterized due to the lack of automated sampling triggered in response to changing culture conditions.

Using OPC communication, the Seg-Flow automated on-line sampling system was integrated with the DASGIP Parallel Bioreactor System to allow the process trigger sampling technology to be employed (figure 4). Process event tags, which were used to activate the Seg-Flow system for process trigger sampling, were configured and programmed in the DASGIP Control software. The DASware analyze OPC client facilitated OPC connectivity between the FlowWeb OPC server and the DASGIP Control system.

Process trigger sampling

Two yeast cultures were cultivated over a 2.5 day duration using a continuous-culture process. A turbidostat control loop was employed to maintain a prescribed biomass concentration as measured by an in-situ optical density probe. The DASGIP Control system activated process media feed and removal from the culture vessels in response to optical density measurements, and user-defined values of media feed volume addition were used as the process trigger events for the Seg-Flow sampling system.

When the desired values of media feed volume addition were reached, the process trigger start command was communicated by the DASGIP Control system to the Seg-Flow system via OPC communication (figure 5). Upon activation, the Seg-Flow system withdrew the programmed sample volume from the bioreactor and delivered the sample to the FlowFraction 400 fraction collector. The collected sample was stored in the fraction collector at a prescribed temperature until the sample was analyzed using an off-line HPLC or other analyzer.

Vessel-specific sample collection data included the beginning and end of the Seg-Flow sample collection phase as well as the sample collection vial position. All data were date- and time-stamped in the FlowWeb software, communicated to the DASGIP Control software using the FlowWeb OPC Server and recorded in the DASGIP Control software. This sample collection data was synchronized in real-time with the fermentation process information and the Seg-Flow Activation time (process trigger time), aligning the remotely controlled sample collection with the process event (figure 5). Also, the remote monitoring functions of the Seg-Flow and DASGIP systems eliminated the need for evening shift coverage and manual sampling.
Conclusion

Coupling the DASGIP Parallel Bioreactor and Seg-Flow automated on-line sampling technologies enabled EBI’s Microbial Characterization Facility research staff to implement remote-controlled, automated process trigger sampling as an integral part of its yeast strain characterization activities. By integrating this functionality into their high-throughput screening and selection process, EBI research scientists are better able to rapidly characterize process events, parameters and stress responses that impact yeast strain gene regulation and, ultimately, biofuel productivity.
Literature


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Isobutanol from Renewable Feedstock—Process Optimization by Integration of Mass Spectrometry to two 8-fold DASGIP® Parallel Bioreactor Systems

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Abstract
This application note describes the integration of a Thermo Scientific® Prima® dB Mass Spectrometer (MS) with DASGIP Parallel Bioreactor Control Systems implemented at Gevo®, Inc. in Englewood, Colorado. The availability of real-time MS data will aid in maximizing cell growth and isobutanol production.

Introduction
Isobutanol has broad market applications as a solvent and a gasoline blendstock that can help refiners meet their renewable fuel and clean air obligations. It can also be further processed using well-known chemical processes into jet fuel and feedstocks for the production of synthetic rubber, plastics, and polyesters. Isobutanol is an ideal platform molecule that can be made inexpensively using fermentation. The ability to automate the data analysis would increase production and reduce costs.

Gevo, a leading renewable chemicals and advanced biofuels company is developing biobased alternatives to petroleum-based products using a combination of synthetic biology and chemistry. Gevo plans to produce isobutanol, a versatile platform chemical for the liquid fuels and petrochemical market.

The main objective of implementing OPC communication

Figure 1. Isobutanol - a versatile platform Chemical
between the Thermo Scientific Mass Spectrometer and the DASGIP Control* Software during a fermentation run was to optimize growth and isobutanol production through automation. The system previously in place at Gevo required that manual data calculations had to be performed by merging the bioreactor runtime data with the MS data to assess the fermentation performance.

Results and Discussion

By integrating the Thermo Scientific Prima dB Mass Spectrometer with the DASGIP Parallel Bioreactor System the calculation of key fermentation operating values was successfully automated. This automation streamlined the workflow and allowed for data-driven control decisions using the real-time exhaust based analytical results.

Before Automation:
Without integration of the MS and the DASGIP Parallel Bioreactor System using OPC, calculation of key fermentation operating values was time-consuming and labor-intensive.

Optimized by MS Integration:
Using OPC communication the real-time MS results were sent to the two DASGIP Control systems. Within the control system, the fermentation runtime data and the MS results were charted and transferred to the data historian with synchronized time stamps.
Key fermentation operating values were calculated online from combined fermentation and MS runtime data, charted and sent to the data historian and were then available for data-driven control decisions. Set-up and script calculations were stored in a user-editable recipe.

Conclusion

With its comprehensive data management functions the DASGIP Parallel Bioreactor System allowed the seamless integration of the Thermo Scientific Prima dB Mass Spectrometer.

The most important success criterion was the ability to calculate isobutanol production rates in real-time giving instant feedback on the quality of run. The availability of the exhaust-based analytical results made data-driven control decisions possible. A secondary success criterion was the fermentation runtime data and MS data was logged with synchronized time stamps to allow for post-run analysis if needed.
## Ordering information

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### DASware® control, incl. PC, OS, and licenses

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Efficient bioprocess development

Eppendorf’s bioprocess marketing and communications manager Christiane Schlottbom and product and application specialist Stephan Zelle list the 10 main factors to consider when choosing a bioreactor control software.

Bioprocess development requires software that not only monitors and controls process runs, but also integrates design, execution and evaluation of experiments. In this way, the software supports initiatives to implement QbD (quality by design) principles, ensuring product quality and successful scale-up to manufacturing. There are several points to consider when selecting SCADA (supervisory control and data acquisition) software and associated data and information management.

1. Number of bioreactors
Parallel operation of bioreactors saves valuable time and accelerates process development. Advanced control software should be able to control multiple vessels individually, up to 24, for example, at a time through incorporated batch management. Ideally, it also features parallel cleaning and calibration procedures.

2. Vessel flexibility
Laboratories that use both autoclavable and single-use bioreactors of various sizes call for flexibility in their control software. Advanced packages allow control of different vessels setups in one and the same experimental run.

3. Software knowledge of scientists
An easy-to-use interface can be the difference between good and bad software. In bioprocess control, integrated recipe management with templates for fermentation and cell culture further aid beginners. Experienced users should be able to maximise their processes, through user-defined scripting, for example.

4. Control strategies and levels of automation
Profile and equation-based feedback control provide the ultimate flexibility in bioprocess control. Feed rates based on offline analyser data, oxygen transfer rates (OTR)-based feed control, or temperature shifts that are triggered by cell performance are only a few examples used to automate a process.

5. Third-party laboratory equipment
Real-time analytical data deliver vast insight into the status of a cultivation. Integration of autosampling and analytical devices, such as nutrient analysers, HPLC, mass spectrometry and Raman spectroscopy, enables feedback control and enhanced automation.

6. External alarm notification and remote control
Alarm warnings via e-mail or text message enhance process safety. Progressive bioprocess control systems also enable users to manage process settings remotely via PC or mobile devices. Users can react immediately to changing process conditions, even from outside the laboratory.

7. Statistical approaches
Design of experiments (DoE) is a structured approach for investigating the influence of critical process parameters and how they interact. Parallel bioreactor systems and easy-to-execute DoE setups streamline process development and facilitate regulatory processes. Advanced control integrates with common DoE tools.

8. Reporting and analysis
Analysis of bioprocess data can be time-consuming. Automated Microsoft Excel reporting, which is based on user-defined templates, saves manual workload and makes complex analyses easier. A powerful chart creation tool, with an unlimited number of data tracks and axes and an export function to Excel, is a plus. When it comes to comparison of historical and current runs, users benefit from intuitive queries and editable query templates.

9. Cross-system analysis
If multiple bioreactor systems, or even multiple sites, need to be evaluated and compared, tools are required to go beyond the possibilities of standard SCADA software. Sophisticated solutions enable mixed-system control and cross-platform analysis of runtime data between systems made by different manufacturers.

10. Data storage and management
Documentation demands can require a central database with managed access. If the bioreactor system is integrated with legacy corporate historians, communication via OPC (object linking and embedding for process control) must be installed. This allows company-wide access to all relevant bioprocess data.

Summary
Efficient bioprocess control software facilitates the implementation of QbD principles in pharmaceutical development and offers flexibility. Sophisticated solutions for interconnectivity of benchtop equipment and bioprocess data and information management provide further tools to integrate processes, reduce the manual workload and accelerate process development.
DoE bioprocess development

Eppendorf DASware is the effective route to design of experiments (DoE) in early-stage bioprocess development. Claudia M Huether-Franken and Sebastian Kleebank explain how the design software applies DoE to DASGIP parallel bioreactor systems.

Many different industries apply quality by design (QbD) principles to ensure product quality and efficient manufacturing. For at least the last decade, ever since the US FDA launched its pharmaceutical cGMP initiative in 2004, QbD has been driving the pharmaceutical industry as well. Since the ICH Guidelines Q8–Q11 (2009–2012) were finalised, there has been increasing regulatory emphasis on QbD for pharmaceutical manufacturing processes.

State-of-the-art pharmaceutical development follows the QbD guidelines even in early process development. Risk analysis is used to identify the critical process parameters considered to have an impact on product quality. Design of experiments (DoE) and multivariate analysis (MVA) are structured approaches to the development and optimisation of processes. Compared with a one-factor-at-a-time method, they offer a reliable and meaningful way to determine a proper design space for the manufacturing process. Designed for simultaneous operation of multiple bioreactors, the Eppendorf DASbox and DASGIP parallel bioreactor systems are adequate tools for the easy implementation of DoE in bioprocess development processes.

Design of experiments – the efficient way

DoE is a structured method for investigating the influence of various critical process parameters, and interactions and dependencies of specific values. It increases the efficiency of development processes on the one hand and enables the streamlining of post-approval changes and regulatory processes in later manufacturing processes on the other. In early product development, DoE can be used as a time and cost-effective way for clone and cell-line screening or media optimisation, for example. Parallel cultivation systems fully support seamless DoE approaches. Set points such as pH, dissolved oxygen, temperature, induction time stamps and feed profiles can be automatically varied. Parallel operations save time compared with sequential ones and eliminate reproducibility issues. Using advanced parallel systems reduces manual operations, which are error prone and usually hard to track.

Application to parallel bioreactor systems

DASGIP parallel bioreactor systems ensure defined and controlled process conditions to facilitate the screening of bacteria or cell cultures, and the optimisation of media or substrate quantities on a small scale. For example, the easily extendable modular DASbox system with four mini bioreactors per unit offers controlled and reproducible cultivation results. All processes can be precisely defined, optimised and adapted. In addition, all results are accurately and precisely documented. The comprehensive Eppendorf DASware design software easily applies DoE to DASGIP parallel bioreactor systems.

The following example gives an overview of how easily such an experiment can be set up using DASware design. A full-factorial three-factor (pH, temperature, feed-stock concentration) design was chosen for an E.coli batch fermentation using a single fourfold DASbox mini bioreactor system. The biomass production (OD_{600}) served as response value.

Setting the design space

Upper and lower levels for each factor were defined as values that appreciably differed from the centre point while remaining biologically reasonable. The DASware DoE builder was used to create a full-factorial design chart. Alternatively, the design can be created using common third-party DoE software and later imported into DASware with a single mouse click. Resource mapping automatically compiles individual, process-specific instructions with DoE information and available hardware resources. Using the fourfold DASbox, three parallel runs were needed to carry out the 11 process runs in total. All critical process values were monitored and documented throughout.

Analysis and a consistency check were performed using the comprehensive DASGIP information manager, along with user-friendly chart displays. Via simple exports, the data can also be analysed with renowned third-party DoE tools.

Gathering full process understanding, and tracking any interfering factors and interacting parameters at an early stage of product/process development are the keys to short time to market. The Eppendorf DASware design software and DASGIP line of parallel bioreactor systems ease DoE approaches and support user-friendly and comprehensive documentation, data analysis and information management.
Application support

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