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High-cell-density Cultivation of *Escherichia coli* in a BIOSTAT® D-DCU 10-3 Stainless Steel Bioreactor

Application Note

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turning science into solutions

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1. Introduction

Generally, stainless steel bioreactor systems are used for microbial fermentations because their design principles are well-known. Consequently, there is a lot of experience and knowledge for these bioreactors resulting in their use over several decades. Furthermore, such bioreactors are available in a scalable range from laboratory to production scale, which enables easy process transfer and scale-up. These systems have already been successfully used for microbial high cell density cultivations according to earlier reports (Riesenberg et al., 1991, Lee 1996, Horn et al., 1996).

BIOSTAT® D-DCU is a bioreactor for both microbial and cell culture applications, offering the capability of sterilization in place (SIP). It is available with vessels designed for working volumes of 10L, 20L, 30L, 50L, 100L and 200L and in a choice of single- or twin–vessel configurations.

Typical application areas include process development, scale-up and small scale production of biopharmaceuticals and vaccines in the pharmaceutical industry and at contract manufacturing organizations (CMOs).
The BIOSTAT® D-DCU is also a superior process development tool for other biotech products including applications in the food and beverage industry.
The modular design of the BIOSTAT® D-DCU enables flexible configuration of the bioreactor system to meet individual customer needs. Available options range from a basic batch setup to sophisticated configurations supporting advanced gassing and feeding strategies and cleaning in place (CIP).
The bioreactor enables automatic transfer of seed cultures to a larger bioreactor or sterile harvesting into another vessel (stainless steel or single-use).

Every BIOSTAT® D-DCU is comprised of three subsystems:
- A control tower with an integrated gas mixing and pump module,
- A culture vessel with a bottom agitation system,
- A supply unit designed as an open-frame, compact stainless steel piping module.

The control tower features best-in-class control capabilities utilizing proven industrial hardware. It is operated by an easy-to-use, intuitive 19'' touch screen, which minimizes the need for staff training.
The compact design of the stainless steel housing reduces the footprint of the equipment, saving valuable space.

The jacketed stainless steel culture vessel, which has spiral baffles for efficient and homogeneous heat transfer, is available in a choice of aspect ratio (height-to-diameter) of 3:1 or 2:1.
The gear-free bottom drive agitation system is designed for long-term operation with minimal noise. In addition, the sanitary radial diaphragm harvesting and sampling valves feature zero dead volume, providing fresh samples without any residues. Both types of valves are easy to clean and maintain.

The supply unit includes all process piping for temperature control, as well as the exhaust and gas inlet lines. Its open-frame design enables direct access for operation, ensuring easy maintenance. Furthermore, minimal floor contact points allow convenient, thorough cleaning even underneath the skids.

Figure 1: BIOSTAT® D-DCU with a 10 L culture vessel

In this study, the microbial suitability of the BIOSTAT® D-DCU 10-3 was evaluated. Two process engineering parameters, mixing time and volumetric mass transfer coefficient (k_La-value), were determined. Afterwards, a high high cell density *Escherichia coli* cultivation was performed.

2. Material and Methods

2.1.1 Bacterial strain

The strain *Escherichia coli* BL21 (DE3) (Novagen, Merck, Darmstadt, Germany) was used for cell cultivation.

2.1.2 Cultivation media

The medium for the first seed culture was prepared by solving 20 g/L LB-medium powder (Roth). For the subsequent cultivation steps, a chemically defined medium was used (Riesenberg et al., 1991). It contained KH_2PO_4 (13.3 g/L), $(NH_4)_2HPO_4$ (4.0 g/L) and $C_6H_8O_7 \cdot H_20$ (1.7 g/L). The medium was sterilized in the BIOSTAT® D-DCU 10-3 at 121°C for 20 minutes. After sterilization, glucose was added to a final concentration of 10 g/L and $MgSO_4 \cdot 7H_2O$ to 1.2 g/L, respectively. Furthermore, a trace element solution was added to supply the medium with $CoCl_2 \cdot 6H_20$ (2.5 mg/L), $CuCl_2 \cdot 2H_20$ (1.5 mg/L), H_3BO_3 (3 mg/L), $Na_2MoO_4 \cdot 2H_20$ (2.5 mg/L), $Zn(CH_3COO)_2 \cdot 2H_20$ (8 mg/L), Titriplex III (8.4 mg/L), Fe(III)citrate (60 mg/L) and $MnCl_2 \cdot 2H_20$ (12.3 mg/L), where all quantities are specified as final concentrations.

During cultivation, a feed solution containing 770 g/L glucose was supplied to the bioreactor vessel. A 400 g/L $MgSO_4 \cdot 7H_20$ stock solution was added to the feed to achieve a concentration of 19.7 g/L. This stock solution was filtered through a sterile filter (Sartolab, Sartorius Stedim Biotech) with a pore size of 0.2 µm that was attached to the inlet port to maintain sterility.

2.2 Cultivation steps

The first seed culture was prepared in 100 mL Erlenmeyer flasks filled with 20 mL LB medium (see Chapter 2.1.2). For the second seed culture, an 1 L Erlenmeyer flask filled with 200 mL chemically defined medium (see Chapter 2.1.2) was used. The first and second seed cultures were incubated inside a temperature-controlled benchtop shaking incubator (CERTOMAT® IS, Sartorius Stedim Biotech) at 37°C and 150 rpm (orbit diameter = 50 mm).

The main culture was performed in a BIOSTAT® D-DCU with a 10 L working volume and an aspect ratio of 3:1. The stirrer shaft was equipped with 2x6-blade disk impellers (Rushton impeller). A ring sparger was used for the aeration. Four baffles were mounted inside the cultivation chamber to avoid vortexing effects. Depending on the specific oxygen requirements, the agitation rate was varied between 500 – 1,500 rpm (tip speed u = 2 – 5.8 m/s) and the gas flow rate between 2 – 10 Lpm; pure oxygen was added on demand.
The initial volume was 6.5 L (chemically defined medium), and 2.5 L of feed solution (see Chapter 2.1.2) were supplied during cultivation. The temperature was controlled to 37°C and the pO_2 to 20%. The pH was adjusted and controlled to 6.8 by the addition of 20% ammonia solution (Roth). The pH and the pO_2 were measured using conventional Clark cell electrodes (Hamilton).

2.3 Fed-batch cultivation

The fermentation was performed in a substrate-limiting, fed-batch mode with an exponentially increasing feed rate. After the initial glucose in the medium had been consumed, the feed solution (see Chapter 2.1.2) was supplied to the bioreactor vessel.
The supplied amount of feed solution was calculated based on equation 1. The specific growth rate was controlled to 0.125 h^{-1}.

$$F(t) = \frac{\mu_{Set} \cdot X_0 \cdot V_0}{Y_{X/S} \cdot S_{Feed}} \exp(\mu_{Set} t) \qquad (1)$$

$F(t)$	Flow rate of the feed solution [L/h]
μ_{Set}	Fixed value of the specific growth rate [h^{-1}]
$Y_{X/S}$	Yield coefficient [$g_{DCW}/g_{glucose}$]
X_0	Initial cell density at the feed start [g_{DCW}/L]
V_0	Initial volume [L]
S_{Feed}	Concentration of the feed solution [$g_{glucose}$/L]
t	time [h]

3. Results

3.1 Process engineering characterization

To ensure that nutrients are uniformly distributed during a fermentation, efficient mixing is necessary. Therefore, the mixing times for the impeller configurations 2x6-blade disk and 3x6-blade disk impeller were determined and compared with each other by using the neutralisation method. For the measurements, impeller tip speeds between 3 and 6 m/s were used, which is a typical range for microbial fermentation (Stanbury et al. 1995).

At a stirrer speed of 800 rpm (u = 3.1 m/s), the mixing time for the 2x6-blade disk impeller configuration + 4 baffles was 11 s and for the 3x6-blade disk impeller configuration + 4 baffles 10 s, respectively. The mixing time for both configurations was within the measurement error tolerance range of ± 1 s, which is calculated by the response time of the pH probe. For both configurations the mixing time was 7 s at 1500 rpm (u = 5.8 m/s). Mixing times below 10 s are regarded as suitable for microbial fermentation (Lara et al. 2006), therefore it can be assumed that both configurations provide the required mixing efficiency.

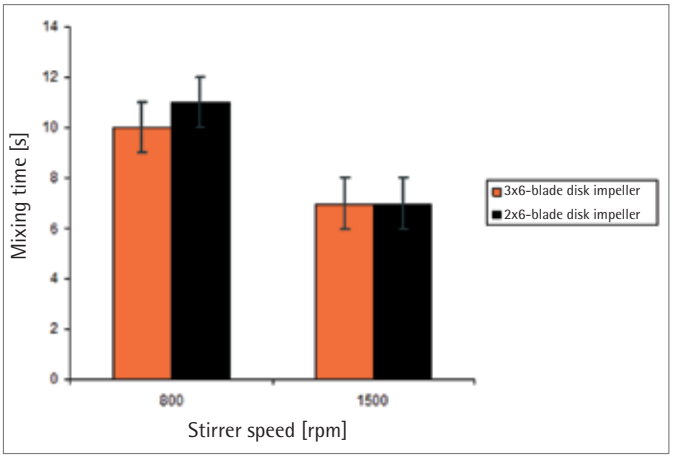

Figure 2: Mixing times for the two 2x6-blade and 3x6-blade disk impeller configurations and 4 baffles at different stirrer speeds.

Another important factor for microbial cultivation is the oxygen transfer capability. To obtain quantitative information about the oxygen transfer rate, the k_La-value was determined by the gassing-out method (Wise 1951). Figure 3 shows the k_La-value characteristics for the two impeller configurations, the 2x6-blade disk impeller + 4 baffles and the 3x6-blade disk impeller + 4 baffles, as a function of stirrer speed. For the measurements the vessel was filled to its maximal filling volume with 1×PBS-buffer. The temperature was adjusted to 37°C and the gas flow rate was set to 1 vvm. For both configurations the k_La-values increase with the stirrer speed. For the 2x6-blade disk impeller, a k_La-value of 308 h^{-1} was obtained. This corresponds to a maximum oxygen transfer rate (OTR) of 350 mmol/(L·h) (OTR = $k_La \cdot \Delta c$, with Δc = 1.13 mmol/L (Truesdale et al. 1955) if pure oxygen is used for gassing.

The 3x6-blade disk impeller configuration had a maximal k_La-value of 236 h^{-1} (OTR_{max} = 270 mmol/[L·h]). Therefore, the 2x6-blade disk impeller configuration had a significantly higher OTR. Because the oxygen transfer rate is the most critical factor for fast growing microorganisms, this configuration was selected for subsequent *E. coli* fermentation.

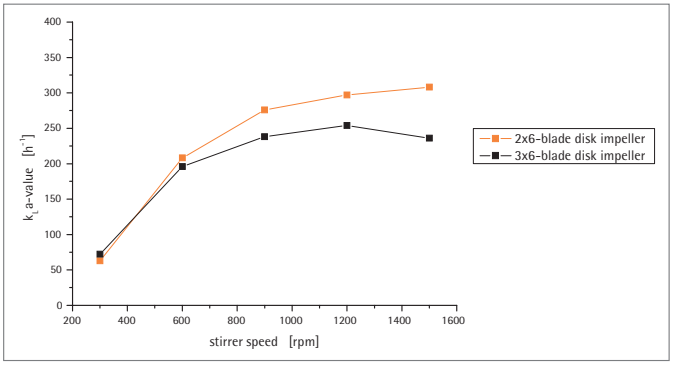

Figure 3: Volumetric mass transfer coefficient for the 2x6-blade disk and 3x6-blade disk impeller configurations and baffles as a function of stirrer speed. The gas flow rate was set to 1 vvm.

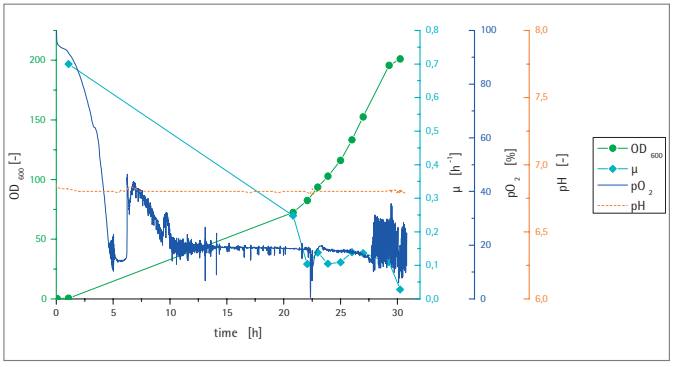

Figure 4: Cultivation conditions and growth behavior during *Escherichia coli* cultivation in BIOSTAT® D-DCU 10-3. The curves of the OD_{600}, μ, pO_2 and the pH are shown.

3.2 *Escherichia coli* fermentation

To demonstrate the suitability of the BIOSTAT® D-DCU 10-3 for microbial fermentation, a fermentation run was carried out using *Escherichia coli* as a high oxygen-demanding model system. Figure 4 shows the characteristics of OD_{600}, the specific growth rate, the pH and the pO_2. The cell density increased exponentially until t = 29 h. Afterwards, the slope of the OD_{600} decreased. A peak cell density of OD_{600} = 201 (DCW = 76.4 g/L) was attained. Between t = 21 and 29 h, the specific growth rate was controlled to 0.125 h^{-1}. For t = 30 h, a decrease of μ was determined. During cultivation, pO_2 was controlled to 20%. Hence, aerobic conditions were ensured. The pH was consistently controlled to maintain a value of 6.8.

Figure 5 shows the pO_2 control parameters. The initial stirrer speed was 500 rpm (u = 2 m/s). The stirrer speed increased exponentially, proportional to the cell density. After t = 25.5 h, a maximum stirrer speed of 1,500 rpm (u = 5.8 m/s) was reached. The initial gas flow rate was 2 Lpm. Similar to the increase of the stirrer speed, the aeration rate was raised to a final value of 10 Lpm. This was done to avoid flooding effects, which have a detrimental effect on the oxygen transfer rate. After 25.5 h, pure oxygen was supplied to the system to further increase the OTR. The final oxygen flow rate was 0.9 Lpm (O_2 ratio = 9%).

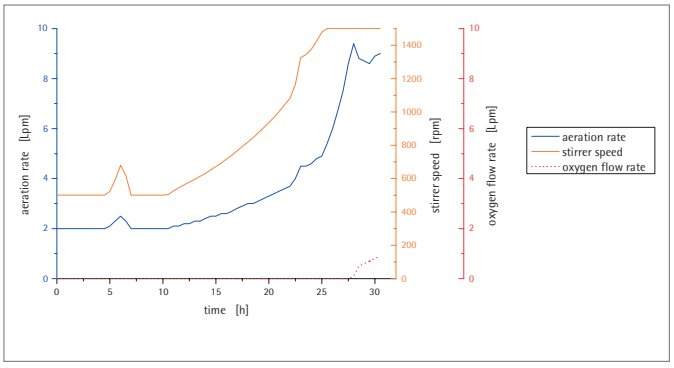

Figure 5: Curves of the pO_2 control parameters during the *E. coli* cultivation. The characteristics of the stirrer speed, aeration rate and oxygen flow rate are shown.

Microorganisms produce large amounts of heat, which makes efficient heat removal necessary to ensure an optimal cultivation temperature. In the BIOSTAT® D-DCU 10-3, temperature is controlled by a double wall (jacket). Figure 6 shows the temperatures of the cultivation process and the jacket.

The temperature was controlled to 37°C, and the lowest jacket temperature was 32.6°C. The liquid inside the jacket can be cooled down to 10°C, which demonstrates the high efficiency of the bioreactor's temperature control system.

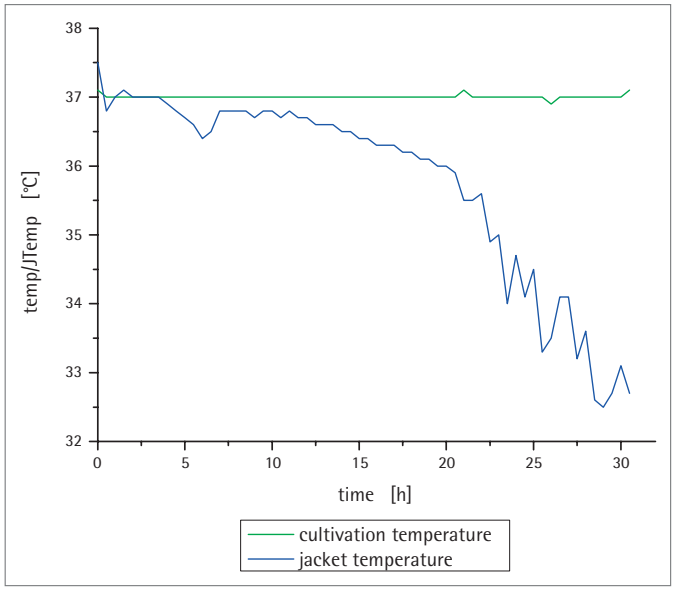

Figure 6: Temperature control during the *E. coli* cultivation. The characteristic temperature curves of the cultivation process and the jacket are shown.

4. Conclusion

Based on the 10 L culture vessel used in this study, we demonstrated that the BIOSTAT® D-DCU 10-3 meets the requirements for high cell density fermentation of *Escherichia coli*. Critical process engineering parameters, such as mixing time and k_La-value, were determined. A mixing time of 7 s was measured; mixing times below 10 s are regarded as suitable for microbial fermentation (Lara et al. 2006).

A maximal k_La-value of 308 h^{-1} was determined. This corresponds to a maximum OTR of 350 mmol/(L·h). The literature describes OTR values above 300 mmol/(L·h) as suitable for microbial high-cell-density fermentation (Ochoa et al., 2009). Furthermore, the pressure and aeration rates of the BIOSTAT® D-DCU could be increased, which significantly increase the OTR (data not shown).

In the fed-batch cultivation performed with *Escherichia coli*, a peak cell density of OD_{600} = 201 (DCW = 76.4 g/L) was achieved. This is comparable to other high-cell-density cultivation processes in stirred tank stainless steel fermenters (Lee 1996), and underlines the suitability of the BIOSTAT® D-DCU for microbial high-cell-density cultivation.

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