E. coli growth and domain antibody (Dab) expression in single-use and stainless steel fermentors

Intellectual Property Notice: The Biopharma business of GE Healthcare was acquired by Danaher on 31 March 2020 and now operates under the Cytiva™ brand. Certain collateral materials (such as application notes, scientific posters, and white papers) were created prior to the Danaher acquisition and contain various GE owned trademarks and font designs. In order to maintain the familiarity of those materials for long-serving customers and to preserve the integrity of those scientific documents, those GE owned trademarks and font designs remain in place, it being specifically acknowledged by Danaher and the Cytiva business that GE owns such GE trademarks and font designs.

cytiva.com

CY13560-19May20-AN

E. coli growth and domain antibody (Dab) expression in single-use and stainless steel fermentors

This application note describes the performance of the Xcellerex™ single-use XDR-50 MO stirred-tank fermentor in an *E. coli* domain antibody (Dab) production process developed for a stainless steel fermentor. Cell growth, cell density, and Dab expression levels were assessed and compared between the two production vessels. The results show that the performance of the XDR-50 MO fermentor is comparable with that of the reference stainless steel fermentor.

Introduction

Single-use technology is increasingly adopted by biomanufacturers to maximize the flexibility and utilization of a facility. However, single-use bioreactors for mammalian cell cultures fall short of meeting the requirements of microbial fermentations in terms of, for example, oxygen transfer capacity and temperature control. Historically, biomanufacturers using microbial processes have had to use conventional stainless steel equipment with the associated need for costly and time-consuming procedures such as cleaning and cleaning validation.

The XDR-50 MO fermentor system is purpose-built to overcome the mammalian single-use bioreactor limitations in fulfilling the needs of a microbial process. The purpose of this study was to assess the performance of an XDR-50 MO fermentor using an existing fermentation process regularly run in a conventional stainless steel fermentor.

Materials and methods

Organism

An *E. coli* RV308 strain, with a pBR322-derived plasmid coding for a domain antibody (Dab) and kanamycin antibiotic resistance, was used in the fermentations. The Dab production is under the control of an inducible promoter. The protein is secreted into the periplasm.

Seed preparation

Two 2.5 L shaker flasks were prepared with 200 mL of Luria broth (LB) medium, containing 50 µg/mL kanamycin (Duchefa Biokemie, Holland) and two drops of sterile Adecanol LG-109 (Asahi Denka Co, Japan), and inoculated with 200 µL glycerol stock of the Dab-expressing *E. coli* RV308 strain. The flasks were incubated at 37°C under 180 rpm shaking for 6.5 to 7 h. Culture OD at 600 nm was determined at the end of the cultivation and it was expected to be in the range of 3 to 5 absorbance units. The seed volume for the bioreactor cultures was calculated to correspond to a final OD of 0.01 in the fermentor after inoculation.

Medium preparation

For medium preparation, $(NH_4)_2SO_4$ (60 g), KH_2PO_4 (92.7 g), K_2HPO_4 (300 g), $C_6H_5O_7$-3Na-2H_2O (24 g), NH_4Cl (12 g), Na_2SO_4 (48 g), and glycerol (160 g 85%) were dissolved sequentially in a mixer bag filled with 22 L distilled water and mixed at 150 rpm agitation in an Xcellerex XDM-100 single-use mixer unit.

Yeast extract (150 g) and Difco™ Select Phytone™ peptone (300 g) were dissolved separately in 2 L distilled water. To facilitate solubilization, the liquid was heated to 45°C to 50°C. The dissolved solution was added to the other medium components in the mixer bag and the final volume was adjusted to 30 L with distilled water.

The XDM mixer bag was connected to the feed port of a presterilized ULTA™ Pure HC filter through a 9.6 mm i.d. pump tube with a disposable SciPres™ pressure sensor (SciLog Inc, Madison, WI, USA). The ULTA filter with a C-Flex® tube on the filtrate side was fused to a large bore size feed line of the presteralized, single-use fermentor bag using the Sterile Tube Fuser. The mixed components were filtered into the bag using a WM520u peristaltic pump (Watson-Marlow, Wilmington, MA, USA). Maximum pump speed (220 rpm) was applied during the process, while the filter inlet pressure was

monitored at all times with a SciPres pressure monitoring unit (SciLog, Madison, WI, USA). After completion of the process, the ULTA filter capsule was sealed off from the fermentor bag, using the Hot Lips Tube Sealer™ device, and disconnected.

Sterile solutions of magnesium sulfate (1 g/L final concentration), thiamine (0.06 g/L final concentration), antifoam agent (0.5 g/L final concentration), kanamycin (50 mg/L final concentration), trace elements and vitamins were added separately to the fermentor through addition bottles fused to one of the small i.d. feed lines of the disposable fermentor bag.

Installation of the XDR-50 MO fermentor bag

The pH and dissolved oxygen (DO) probes were calibrated and sterilized by autoclaving at 122°C for 20 min. The fermentor bag was unpacked, removed from its protective pouch, and examined for damage before installed into the fermentor vessel. The baffles were inserted into the empty XDR-50 MO vessel before the fermentor bag was installed by lowering it into the vessel with the probe ports facing to the front and carefully arranged into its correct operating position. The exhaust filter was inserted into the filter heater and the condenser bag part of the fermentor arranged into the condenser unit. The sparge gas line was connected to the inlet air filter. The bag was partially filled with air (10 L/min). During inflation, the bag was manually adjusted for best fit in the vessel and to remove major creases. After inflation, the sparge gas inlet and the exhaust gas outlet were clamped to maintain partial bag inflation. Partial inflation aids handling during installation. The signal cable was connected to the bag pressure sensor, and the sterile pH and DO electrodes and the temperature sensor were inserted into the bag. The gas inlet was kept clamped until the bag was filled with culture medium.

Culture procedure

Initial process parameters were set and the vessel was equilibrated one hour before the inoculation. Parameter set points are listed in Table 1. The pH was adjusted by addition of either 1 M H_3PO_4 or 25% NH_3 to the culture. DO was cascade-controlled through stirrer speed, air flow, and oxygen supplementation.

Table 1. XDR-50 MO process parameters

Process parameters	Fermentor set points
Temp. before/after shift (°C)	37/30
Initial stirrer speed (rpm)	250
Air flow (vvm*)	0.5
pH	7.2
DO (%)	30

* Gas volume flow per unit of liquid volume per minute (volume per volume per minute)

When the fermentor was equilibrated, 100% DO calibration as well as pH off-set calibration were performed. Batch data logging was started prior to inoculation of the fermentor. The seed culture was added to the medium through the sterile feed line on the fermentor vessel.

The cultures were fed with 60% w/w glycerol solution according to the predetermined feed profile shown in Figure 1. Substrate addition was performed with the WM520u peristaltic pump using a 3.2 mm i.d. pump tube.

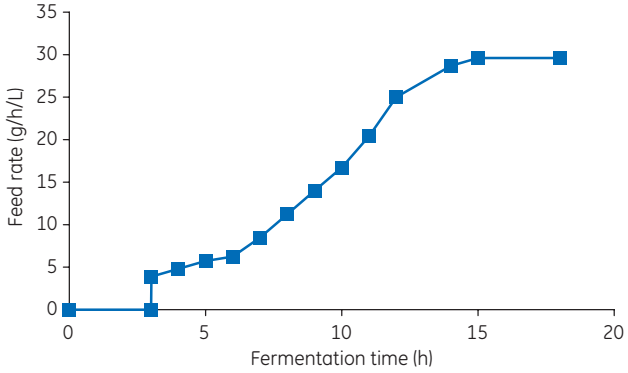

Fig 1. Glycerol (60% w/w) feed profile.

Expression of Dab was triggered with a two-step induction strategy: the first induction was performed by addition of IPTG to the culture to a final concentration of 0.5 mM when OD was in the range of 70 to 90, followed by a second IPTG addition to the same final concentration 3 h later. Prior to the first induction event, a shift in cultivation temperature from 37°C to 30°C was applied. The cultivation was finished after a 5 h induction period and the fermentor system prepared for heat treatment of the cells.

Sampling and off-line analyses

Regular samples were taken during the culture period for off-line analyses. Optical density, measured at 600 nm, and culture dry weight were determined directly, while samples for determination of acetate content and Dab concentration were stored at -20°C prior to analysis.

Measurement of culture dry weight

Samples (6 mL) were centrifuged at 2200 × g for 10 min and the supernatants were removed. The pellets were washed with 6 mL phosphate buffered saline (PBS) and centrifuged as above. The pellets were resuspended in distilled water and final volume was adjusted to 6 mL. Dry weight was determined using a MT HB43 Moister analyzer (Mettler-Toledo, Greifensee, Switzerland).

Culture acetate content

Samples (2 mL) were centrifuged at 16 000 × g for 5 min and the supernatants were collected. Acetate content in the supernatants was determined using an acetic acid assay kit (K-ACET, Megazyme Int., Ireland) according to the manufacturer's recommendation.

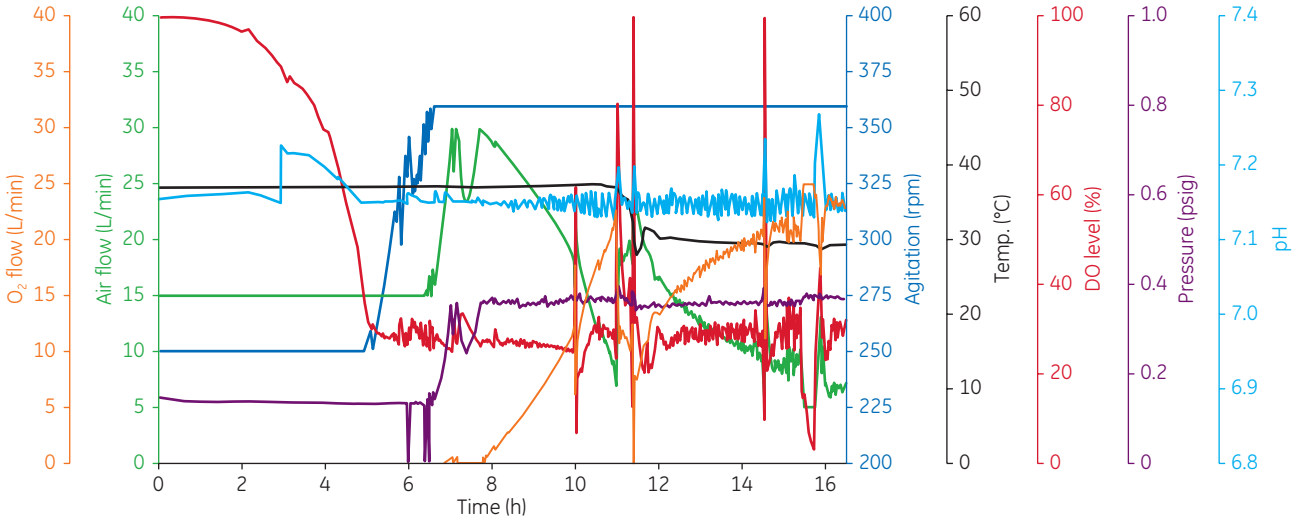

Fig 2. Process parameters plotted against cultivation time.

Dab concentration

Dab concentration in the collected samples was analyzed on a 1 mL Protein L HiTrap™ chromatography column using the ÄKTA™explorer 10 system. Dab concentrations were calculated from the area under the curve (AUC) for the elution peak using known Dab concentrations as reference.

Heat treatment of harvested cells

After termination of the run, the culture was subjected to heat treatment in the XDR-50 MO fermentor to facilitate release of product from the cell periplasm to the culture medium. The agitation in the bioreactor was set to 200 rpm. The heat treatment was performed at 48°C with 3 h hold time. The pressure in the fermentor bag was monitored continuously and excess pressure was released when required to keep the value between 0.2 and 0.5 psig.

After completion, the heat-treated culture was cooled down in the fermentor to 8°C and kept at this temperature until filtered through a hollow fiber cross flow filter (750 000 nominal molecular weight cut-off) using a ReadyCircuit™ single-use filtration assembly (comprising cross flow filter cartridge, pump and pump tubing, pressure sensors, and bags).

In-process samples were analyzed to follow Dab expression level of the culture as a function of induction time. These samples were collected in 2 mL microcentrifuge tubes and a heater block with matching well sizes was used for the heat treatment. Similarly to the large-scale process, the small-scale heat treatment was performed at 48°C for 3 h, followed by centrifugation of the culture and filtration of the supernatant using a 0.45 µm syringe filter disk.

Results and discussion

Fermentation process

Two consecutive *E. coli* cultures were performed using the XDR-50 MO fermentor system. During the cultivations, critical process parameters were monitored. Figure 2 gives a process overview of the fermentation plotted against cultivation time. Temperature was controlled with ± 0.5°C accuracy, stirrer speed with ± 1 rpm accuracy, gas flow with ± 0.01 L/min accuracy, DO with ± 5% accuracy, and pH was controlled with an accuracy of ± 0.1 pH units.

Dissolved oxygen (DO)

DO was automatically controlled through a three-stage cascade comprising stirrer speed, air flow rate, and mixing of pure O_2 into the air flow. Initially, the stirrer speed was gradually increased to its maximum allowed level. Continued oxygen demand was met by increasing the air flow rate up to 1 VVM. Finally, pure oxygen was mixed into the air flow and the total gas flow was maintained at 1 VVM until the end of the cultivation. In Figure 3, the culture DO over the fermentation is displayed in red.

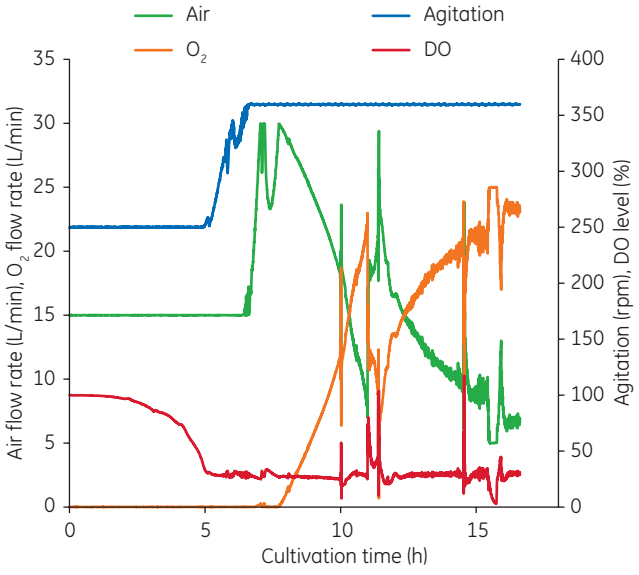

Fig 3. DO in the XDR-50 MO fermentor culture plotted against culture time.

Cell growth

In the XDR-50 MO fermentations, a linear correlation between OD and culture dry weight (DW) was observed. The OD-to-DW ratio was about 4 throughout the culture period.

When comparing growth in the XDR-50 MO fermentor cultures with data from the same process performed at 15 L working volume in a 20 L stainless steel fermentor (B. Braun Biotech GmbH, Germany), similar culture growth was observed in both bioreactor types (Fig 4).

When the cell-specific growth rate in the XDR-50 MO cultures was calculated based on measured OD values and plotted against culture time, it was observed that the cell-specific growth rate decreased with increasing culture time. This shows that the substrate feed is the limiting factor, not providing sufficient nutrition to the culture to maintain a constant growth rate.

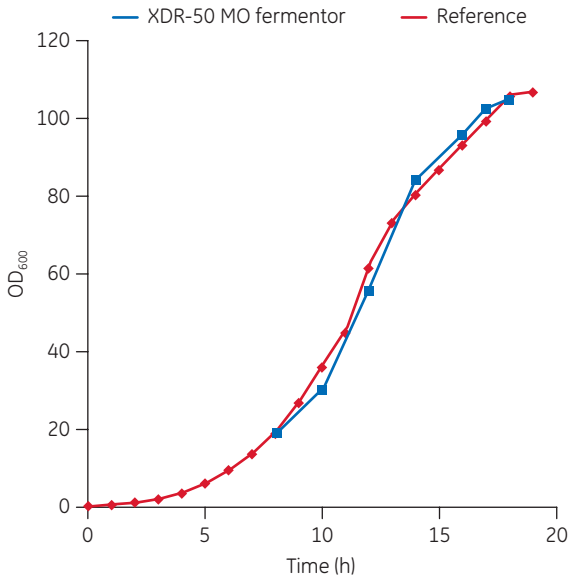

Fig 4. Comparison of culture growth in XDR-50 MO single-use fermentor and stainless steel (SS) fermentor.

Acetate formation

E. coli cultures produce acetic acid as an extracellular product of aerobic fermentation (Crabtree effect). Acetic acid can have a negative effect on growth and product formation. Acetate formation was monitored by regular sampling of the cultures to get more insights into the culture process. The acetate concentration in the cultures remained below 0.3 g/L throughout the culture period and no difference was observed when comparing results from the single-use XDR-50 MO fermentor and the stainless steel system. At such a low acetic acid concentration, the fermentations were not negatively affected.

Heat treatment of XDR-50 MO cultures

For Dab release, the cultures were subjected to heat treatments after completed cultivations. The cultures were heated from 26°C to 48°C during 44 min after which the temperature was maintained at 48°C in the fermentor bag for 3 h (Fig 5). After heat treatment, the cultures were cooled down from 48°C to 10°C during 1 h and 40 min. The accuracy of the temperature control was very similar to what is achieved with the stainless steel fermentor.

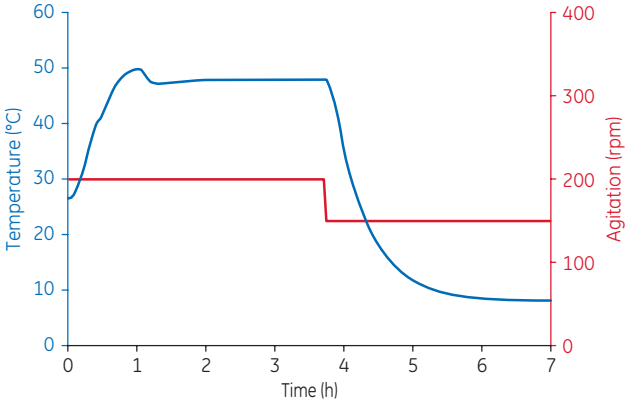

Fig 5. *E. coli* heat treatment procedure for Dab release.

Dab expression

Dab expression was determined after heat treatment of the cultures. In the first XDR-50 MO fermentor culture, the Dab yield was determined to be 1.3 g/L. The result is in the lower part of the the range of what is considered normal yields based on results from previous stainless steel fermentations. The reason for this somewhat low yield was investigated and the medium preparation process was given special attention as this unit operation differs between stainless steel and single-use fermentation processes. In a stainless steel fermentor, all medium components except the heat-sensitive are autoclaved together inside the vessel before fermentation start.

In the single-use fermentor, the medium components are premixed in a separate mixer vessel and the solution sterile-filtered into the single-use fermentor bag prior to culture start. Hence, it was hypothesized that certain medium, components critical for good product yield in this Dab process, might have precipitated during the mixing step prior to the first single-use fermentation. The precipitated components might subsequently have been removed in the sterile filtration process. Therefore, in the second single-use fermentation run, precipitation-prone components were excluded from the mixing step and instead sterile-filtered separately directly into the fermentor bag.

The product yield in the second run was much higher (2.2 g/L) compared with in the first run. This result is within the upper part of the range of what is considered normal for this Dab process and supports the precipitation hypothesis for the first run.

Based on the observed precipitation in the first run, it was hypothesized that this might also occur in the medium preparation process for the stainless steel fermentation, but never observed as the medium remains inside the vessel at all times. This hypothesis remains to be confirmed but was deemed to be out of scope for this study.

In Figure 6, the product yields from the XDR-50 MO cultures are compared with historical data from the process run in a stainless steel system. As reflected in the results, the production process was not optimized for the single-use XDR-50 MO fermentor. Nevertheless, Dab expression levels achieved in the single-use system were comparable with historical levels from processes run in the stainless steel fermentor.

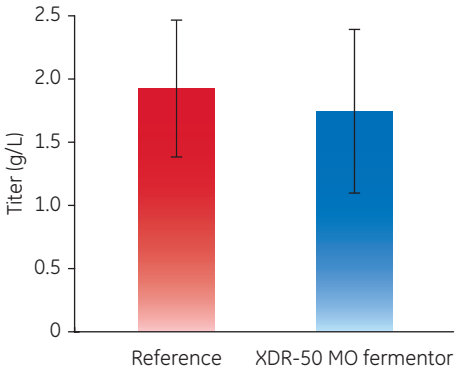

Fig 6. Comparison of Dab expression in the XDR-50 MO fermentor processes with historical data from the process run in stainless steel (SS) fermentor.

To monitor Dab expression during the induction phase in the XDR-50 MO cultures, samples were taken hourly post induction. The results show a linear increase of expression levels over induction time (Fig 7). The results indicate that a higher target protein level could be achieved by extending the induction time provided that no other parameters were limiting.

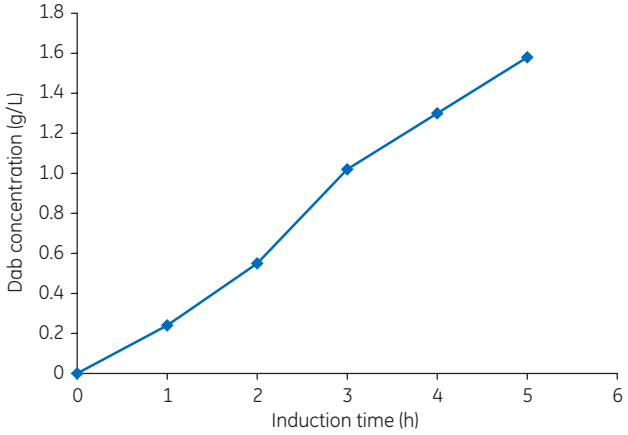

Fig 7. Dab expression level post induction in XDR-50 MO. Culture samples were taken every hour after the first induction and analyzed for Dab content.

Conclusion

This application note describes the performance of the single-use XDR-50 MO fermentor system when used in an *E. coli* Dab production process. An OD-to-DW ratio of 4 was observed throughout the culture period.

The presented data show that cell growth and Dab expression levels from the single-use XDR-50 MO fermentor were comparable with data from cultures conducted in a stainless steel fermentor, for which the process was initially developed. However, the medium sterilization step in single-use fermentation processes differs from the common procedure in stainless steel processes. In a stainless steel process, the majority of the medium components are sterilized by autoclaving inside the fermentor vessel. In a single-use fermentation process, the medium is mixed in a separate mixer vessel and further sterile filtered into the fermentor vessel. To reduce the risk of removal of precipitation-prone components in the filtration process, these components were mixed separately and added to the culture medium after the filtration step.

In comparison with conventional stainless steel fermentors, the XDR-50 MO fermentor system represents a flexible and reliable alternative for biomanufacturers using microbial protein production systems.

Ordering information

Product	Product code
ULTA Pure HC 0,2 um, 10" TCTC	KMP-HC9210TT
Xcellerex XDM-T Quad Jacketed Stainless Steel Mixing System	29054862
Hot Lips Tube Sealer	28411704
Sterile Tube Fuser	28999602

For more information on the XDR-50 MO fermentor system, please contact your local sales representative.

GE Healthcare Bio-Sciences AB
Björkgatan 30
751 84 Uppsala
Sweden

www.gelifesciences.com/upstream

29134111 AA 05/2015