CellTank SUB in perfusion mode operation at Bioneer

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1. Introduction

A number of different methods exist for cultivation of mammalian cells in suspension. However, most systems are limited to cell density ranging 1-5x10E+06 cell/ml mainly due to mass transport limitations of nutrients and in specific oxygen access. CerCell has designed and manufacture the CellTank Single-Use-Bioreactor (SUB) with a novel flow system which allows near gradient free mass transport therefore fully scalable from lab to pilot and production scale size. The SUB concept is built around an even number of envelopes with permeable walls each envelope harbouring a cell supporting and selectable porous scaffolding. The SUB design permits a forced flow of media in a direction essentially perpendicular to the envelopes hosting the cells. In this particular test the cell supporting matrix consists of numerous small cavities which each harbour mobilised cells in continues suspension. By stacking these cell supporting discs a matrix volume comparable to a fixed-bed bioreactor is obtained, yet no gradients are developed as the matrix bed-height is small. With the CellTank system Bioneer has been able to cultivate suspension CHO cells at densities of 0.6x10E+08 cell/ml matrix under perfusion mode conditions at extended operation time and continues expression of recombinant product.

2. Objective

- Will suspension CHO cells grow at high density and harbored inside a CellTank scaffolding matrix SUB - as perfusion culture.
- The comparison is against a traditional 500 ml batch cultivated in parallel both inoculated with equal amount of cells.
- Inoculation in matrix is predicted to take less than 3 hours
- Do cells survive, multiply to numbers significant higher than batch? May the proliferation be controlled via temperature?
- Will a capacitance based biomass sensor be able to measure the harboured cell number on-line?
- Production of antibody - and efficiency?
- Lactate/glucose values - same as for standard-batch during the first three days?
- May CO2 values in head space be measured on-line?
- Glucose consumption and production of lactate – can it be measured at starting perfusion level (1L/24hr) for the first few days?
- Do the cells need a higher degree of perfusion after several days of culture (more than 1L/24hr)?
- Amount of cells at termination? Target is >50 mio cells / ml matrix for 2 weeks cultivation and several times higher titer.

3. Materials

3.1 Bioreactor

For the trial performed October 2011 at research service company Bioneer A/S in Denmark, www.bioneer.dk the following materials and sensors was used:

- CellTank #2202 SUB with a theoretical volume of 2,000 ml hosting the CellCore reactor body
- Reactor body integrate 2 parallel oriented circular envelopes, each envelope ID20, OD80, height 18 mm for a total matrix volume of 150 ml
- Matrix: PET fibre matrix selected to harbour the suspension cells with 91% porosity
- Process control system: DasGip MP8
- Stirrer table: DasGip M10 dual pole magnetic/inductive
- Bioreactor core body volume: 280 ml

- Media volume added to the reservoir prior to inoculation: 1,250 ml
- Media: CD with 4.5% glucose
- Cell line: CHO suspension producing IgG antibody
- Sparging gas: 3 litre/hour constant variable mixed O2/CO2/nitrogen
- Temperature ranging 33-37°C controlled via 50 Watt heating blanket around the SUB
- Internal re-circulation performed by the build-in centrifugal pump driven magnetically by the DasGip stirrer table, capacity 0-2.4 l/min
- Medium exchange during perfusion 2-4 l/24h equivalent to 1-2 matrix volume/h
- Total perfusion volume spent was 31 litre over 11 days
- On-line in CellTank sensors:
    - Fogale PG13.5x120 biomass sensor mounted inside the matrix
    - Finesse TrupH EFP-K8 PG13.5x120 chemical pH sensor mounted in the reservoir
    - Hamilton optical PG13.5x120 optical oxygen sensor mounted in the reservoir
    - Finesse TruCell Optical Density (OD) sensor mounted in reservoir
    - Trace Analytics Process online glucose analyzer mounted in perfusion harvest line
    - BlueSens BlueI-n-One_ferm CO2 %vol sensor mounted in off-gas stream
    - Integrated temperate: pt1000
    - Integrated re-circulation media mass flow: 0-2.4 l/min
    - Integrated matrix back pressure: 0-600 mm WC (water column)
- Off-line sample measurements:
    - Lactate: YSI 2700 Chemical Analyser
    - Glucose: YSI 2700 Chemical Analyser
    - Cell count and viability: manually from 1 ml samples through NucleoCounter YC-100
    - Product: ELISA based assay

Figure 1 – set-up on DasGip with batch in traditional glass/steel STR on stirrer table 1 and perfusion SUB on stirrer table 2.

3.2 On-line analyzers

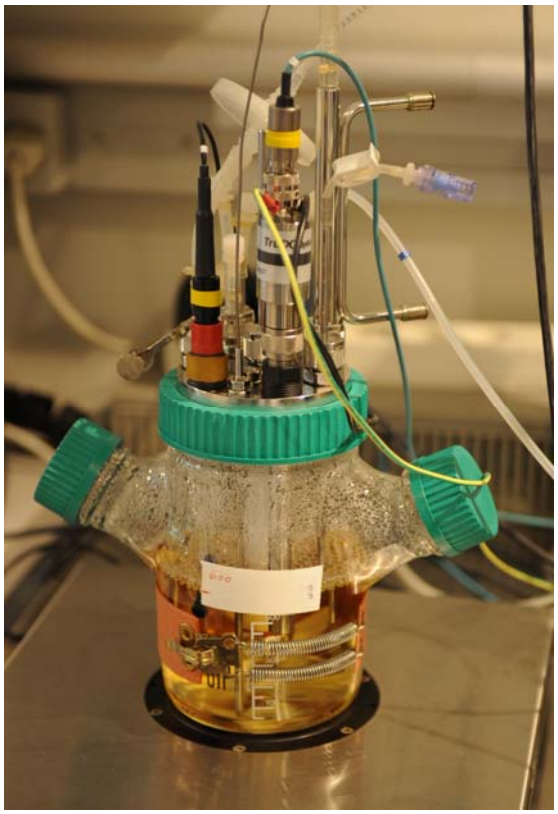
On-line analyzers and sensors were used to monitor the fermentations, and to correlate the different online techniques: Viable Cell Density (VCD), Optical Density (OD), off-gas CO2 and O2 concentration and glucose concentration.

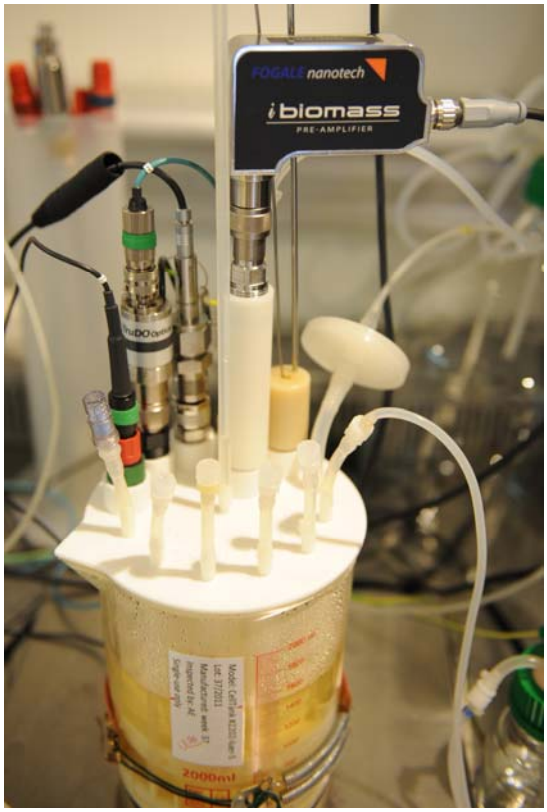
Viable Cell Density, VCD:
This analyzer uses permittivity, which is expressed in picoFarad/cm (pF/cm). Permittivity has been shown to have a linear correlation with the volume of living biomass (ref. D. Voisard 2010).
The Fogale Nanotech biomass system is a multi-frequency capacitance device operating in the range of 0.3-15 MHz.
The sensor uses an alternating electric field, where cells become polarized. Lysed cells, air bubbles and solid particles are not polarizable; therefore, only cells with intact plasma membrane can be detected by dielectric measurements. This makes this technology ideal for use in fibre matrix systems.
VCD using dielectric spectroscopy, several additional parameters can be measured: Δε, fc and cole-cole-Alpha (ref. A Negrete 2007) Δε is the permittivity based on the spectroscopy data.
Assuming constant parameters, the critical frequency fc is correlated inversely to mean cell size.
Employed on cell cultivation, α describes the homogeneity of cell size. A low α is due to a high slope and therefore similar cell size. A higher α indicates difference in the cell sizes.

Optical Density, OD:
This sensor uses a laser with light in the Near-InfraRed (NIR) range, 850nm, to monitor the cell density. All cells and particles will be detected as scattered or absorbed light, not arriving at the detector. Sensor has a filter to remove visible light, enabling the use in transparent bioreactors.
OD measurement is not suitable for measuring cells in a matrix, as it requires a moving liquid. This sensor was mounted in the reservoir, and only measured the cell density of cells outside the matrix. Zero was made on media prior to inoculation, but as optical sensor also monitors the base turbidity of the media, the very low cell density in the reservoir was only seen during inoculation, where later the media becoming less turbid dominated this measurement.
Sensor has a filter to prevent influence from surrounding light. The measurement wavelength is utilized to prevent colour changes of the media to influence the cell density reading, and therefore must be linearized to match the most common off-line instruments measuring OD at 600 or 650 nm.

Off-gas CO2 and O2:
This sensor has a combined measurement of volume % CO2 and O2, with correction for pressure and humidity using build-in sensors.
CO2 measurement indicates both the volume of live cells and the metabolism. O2 measurement must be linked to O2 sparging volume and concentration.
As CO2 sparging is also used for lowering of the pH-value, which was only needed in the short first part of the run, the measurement is only valid for the remainder of the run.

On-line glucose:
This analyzer uses an Enzymatic-amperometric sensor to monitor the glucose level in the harvest line during perfusion. A flow-through sensor was used, which for this run was not the right choice as bubbles got trapped in the sensor. A bubble-trap mounted or a filtration probe would have given a very stable measurement, suitable for controlling perfusion rate to have a constant low glucose level in the harvest line.





4. Methods for matrix bioreactor operation

4.1. The SUB was supplied 4x8kGy E-beam pre-sterilized in dual foil bags and opened inside a laminated-air-flow bench and equipped with sensors and media.

4.2 The CHO cell line was prepared from frozen stage and by cultivation in a 3 litre shaker flask for 7 days in 1 litre CD media in a 5% CO2 Thermo incubator at 37°C. The obtained 1x10E+09 large amount of cells at 99.5% viable cells for inoculation was selected in order to boost the test purpose of a final production phase with high density of cells.

4.3. Day minus one the reactor was equipped with dO2, pH and bio mass sensors and media was added in the LAF including 100U/ml penicillin and 100U/ml Streptomycin (Lonza 100x, 10.000Uml Pen/Strep). The SUB was mounted on the magnetic stirrer table where temperature probe and heating blanket was mounted. The temperature stabilized at 37°C after 1.5 hours. The optical dO2, chemical pH sensors was calibrated and the SUB kept the night over with impeller revolution at 260 rpm with 30% O2/70% N2 gas mix at 3 l/hour flow.

4.4 Day one (Thursday) the SUB was inoculated 3 times each with 111 ml media approximately with 1 hour intervals adding a total of 7.38x10E+08 cells through port 1 equivalent to 4.9x10E+06 cells/ml matrix or 5.9x10E+05 in the total reservoir volume. The re-circulation mass flow was set by 260 rpm impeller speed corresponding 1.0 l/min media re-circulation mass flow equivalent to 10 cm/min flux in order to distribute the cells inside the matrix. First recorded reading on the Fogale i465 display was 70 minutes after last inoculation and showed 4.6 pF/cm indicating cells was being trapped inside the matrix. The added volume of 333 ml was removed and the SUB was set to operate in batch mode. A sample was taken from the reservoir showing 1.5x10E+05 cells/ml.

Third day the mass flow was increased to 2.0 l/min equivalent to 20 cm/m flux and kept for the duration of the cultivation. As to the constantly increasing biomass the media flow dropped 10-20% each day and was re-adjusted to 2.0 l/min by increasing the impeller rpm each morning. The SUB continued operating in batch mode. A sample was taken from the reservoir showing 3.9x10E+04 cells/ml.

Six day the perfusion, exchange of media was started at a rate of 2 l/24h via a on DasGip peristaltic pump from a 2 litre vessel with fresh media. Harvest was taken from port 4 via a 400 ml/h capacity peristaltic pump on the DasGip MP8 unit to a 2 litre harvest vessel which each morning was exchanged. Optimum perfusion inlet is port 2 and harvest from port 3 for largest difference in product content.

Ninths day the exchange of media was increased to a rate of 3 l/24h. Impeller rpm set to 340 and pF/cm value 45.6 read on Fogale i465. The harvested liquid was stored in a freezer for later analyses.

Eleventh day the perfusion flow was increased to 4 l/24h, re-circulation flow kept at 2 l/min now at 360 rpm and the temperature kept at 35°C. Lowest glucose level at 4.9 mMol was seen and Fogale i465 display showed a drop in bio mass volume as to the decreased cell size being a result of the reduced temperature from 37 to 35°C. Further the reduced temperature slowed the cell proliferation down to 25% compared to 37°C operation. Purpose testing if the cells in the reactor could express the product constantly with reduced proliferation. Bio mass estimation was based on measured 58 pF/cm equivalent to 5.8x10E+07 cell/ml matrix – a correction factor of 10% low temperature and 9% matrix volume is perhaps needed.

Twelfth day the perfusion flow was decreased to 3 l/24h, re-circulation flow kept at 2 l/min now at 360 rpm.
Fourteen's day the temperature was reduced to 33°C and kept for 33°C the rest of the cultivation.

Fifthteen´s day the temperature is continuing at 33°C and the bio mass figure at 73 pF/cm slightly increasing. Needed rpm in order to overcome the matrix / biomass backpressure of 295 mm and 2 l/min media re-circulation mass flow was 480. A low balance of temperature and glucose addition has been reached.

Eighteen day (being a Sunday) the rpm was increased to 590 in order to overcome the very high matrix back pressure drop as a result of bio mass measured to be 79 pF/cm. In the evening the traction was lost and the SUB started to loose cell to the reservoir.

Nineteen's day the cultivation was terminated.
After the experiment, the bioreactor core was opened in its parts to evaluate and count the total number of cell and see where the cells were located. The matrix discs were flushed with 35 ml liquid A and 35 ml liquid B from ChemoMetec designed for use with the NucleoCounter and 35 ml media for cell number measuring. It is the experience that the precision of NucleoCounter is ± 5-10%. The cells were located with less than 5% difference axially in the matrix.

Operation of SUB started with traditional batch fermentation after the first 6 days followed by perfusion mode operation the remaining 12 days in total 18 days.

The media reservoir volume and collected bio mass was centrifuges on a Hettich Rotanta 460 in 4x450 ml containers at 300 G for 10 minutes. The total cell number was measured to be 0.93x10E+10 in the 150 ml matrix.

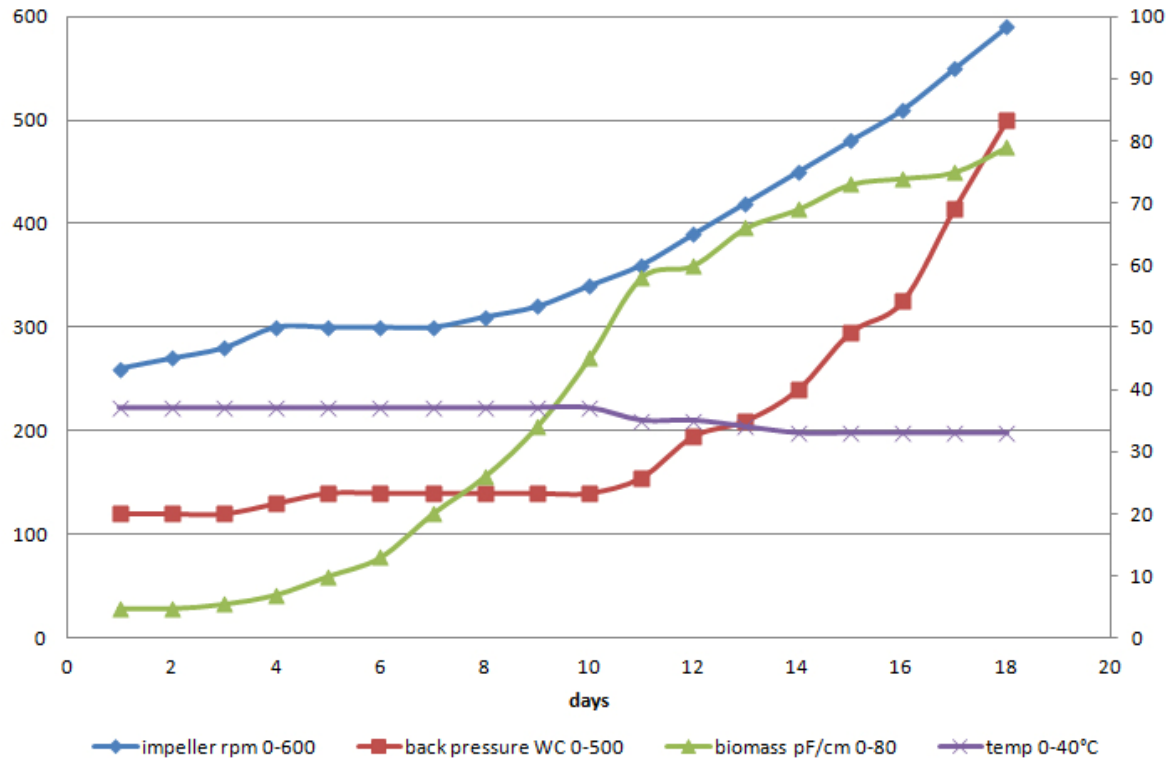


Figure – Relation between data which show the development of increased impeller rpm in order to keep constant flux at 20 cm/min as to the increase in bio mass measured in pF/cm.

5. Methods for STR

In parallel a 500 ml standard glass and steel Stirred-Tank-Reactor, STR was cultivated over the 12 day period. Parameters measured were pH, dO2, glucose, lactate, cell number and viability.

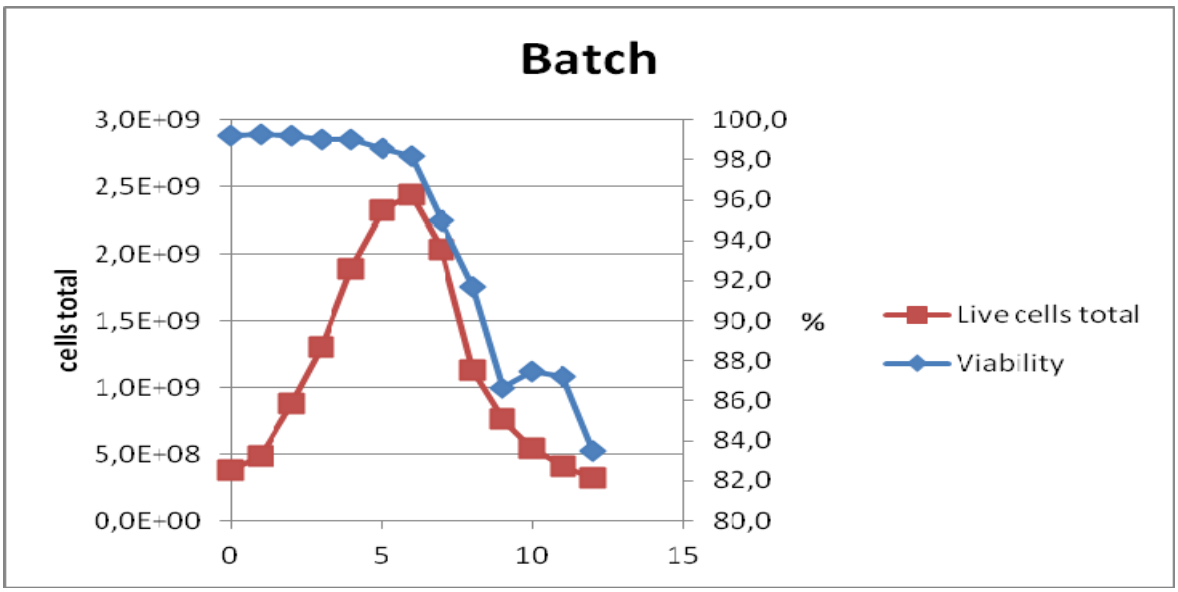


Figure illustrates traditional performance of a batch. Viability dropped as a function of all the Glucose was used and no extra added.

The batch performed as predicted with 30% O2 sparging gas mix and impeller at 60 rpm.

6. Sensors, probes and measures

The optical dO2 sensors were calibrated in air for 100% saturated O2 and N2 for 0% saturated O2 followed by in media calibration at operation temperature.

Integrated re-usable standard Fogale PG13.5x120 biomass sensor was not mounted correctly inside the matrix, but 5 mm above the well bottom in the matrix. It is believed this clearance caused higher than expected cells suspended in the reservoir.

The SUB was monitored for cell viability and number manually each day and measured with the NucleoCounter.

The same cell line from 1 litre batch cultivation in a T-flask was centrifuged down in Hettich Rotanta 460 for 10 minutes at 300 G for separation. The remaining concentrated cells was measured with NucleoCounter and contained 2.7x10E+08 cell/ml tightly packed. Hereby it is revealed that at termination the cell concentration in the CellTank occupy ~30% of the total volume (91% porosity).

7. Results and discussion

7.4x10E+08 cells in 330 ml was inoculated into the reservoir in 3 individual 110 ml injections, the reservoir volume adjusted after each inoculation in order to obtain constant volume. Such changing volume also changes the mass flow of the re-circulation pump slightly.

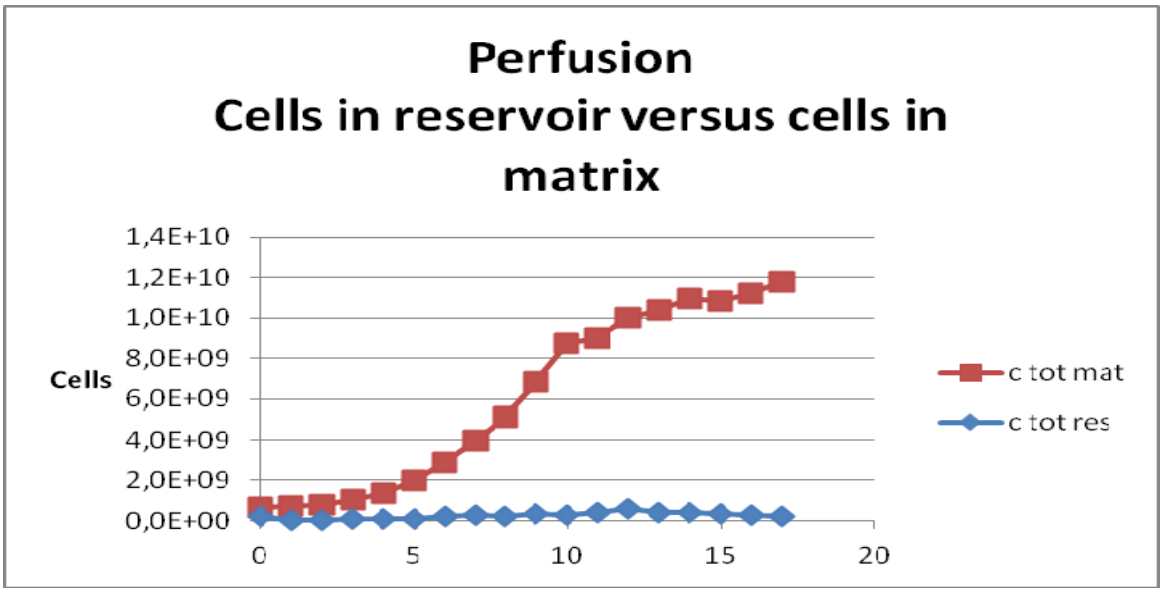


Figure – Free cell concentration in the reservoir is more or less constant at below 4x10E+05/ml (total 4x10E+08) and the difference to cell density in the matrix (total 1.7x10E+10) is increasing over time. Approximately 2% cells were found in the reservoir and 98% in the matrix at the particular flux.

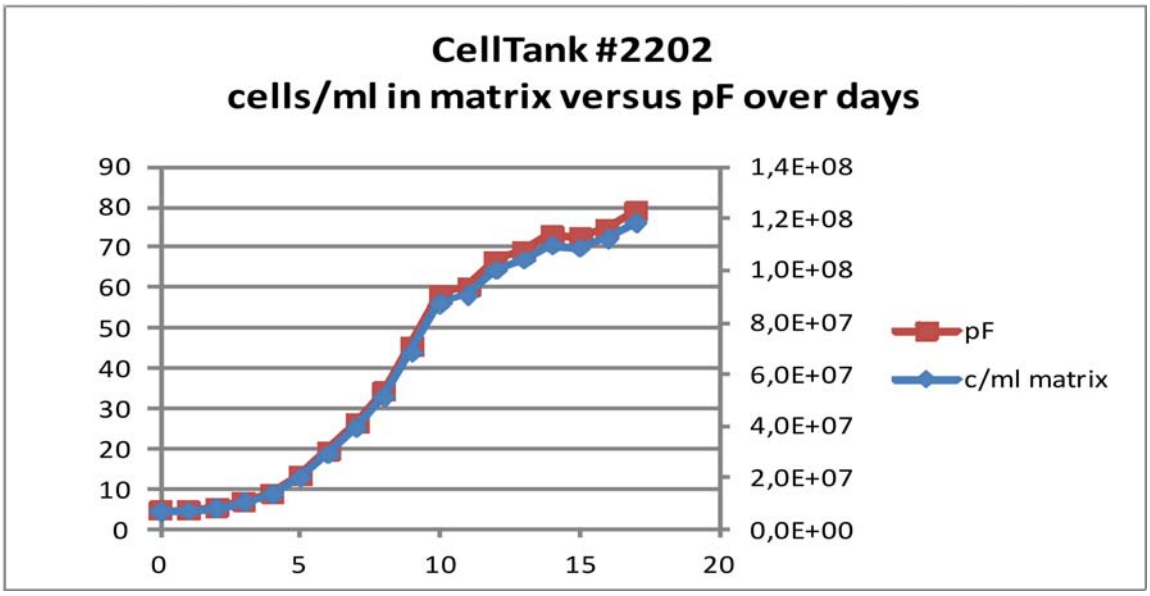


*Figure - Cell proliferation started day one with 4.9 pF/cm and ended at 79 pF/cm. The measured bio mass to actual cell number **correction factor** is 1 pF/cm = one million cells per millilitre matrix volume. The bumps on curves at day 10 and day 14 on the horizontal axis are due to SUB temperature reductions.*

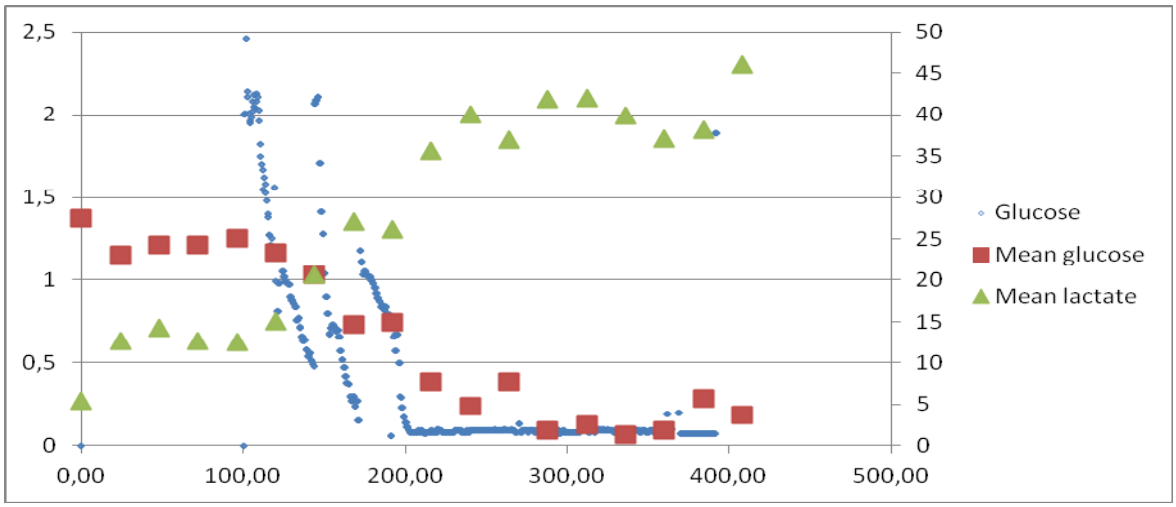


Figure – Glucose and lactate development. Mean samples were analyzed as 1 value on a mix of harvest over the last 24 hours.

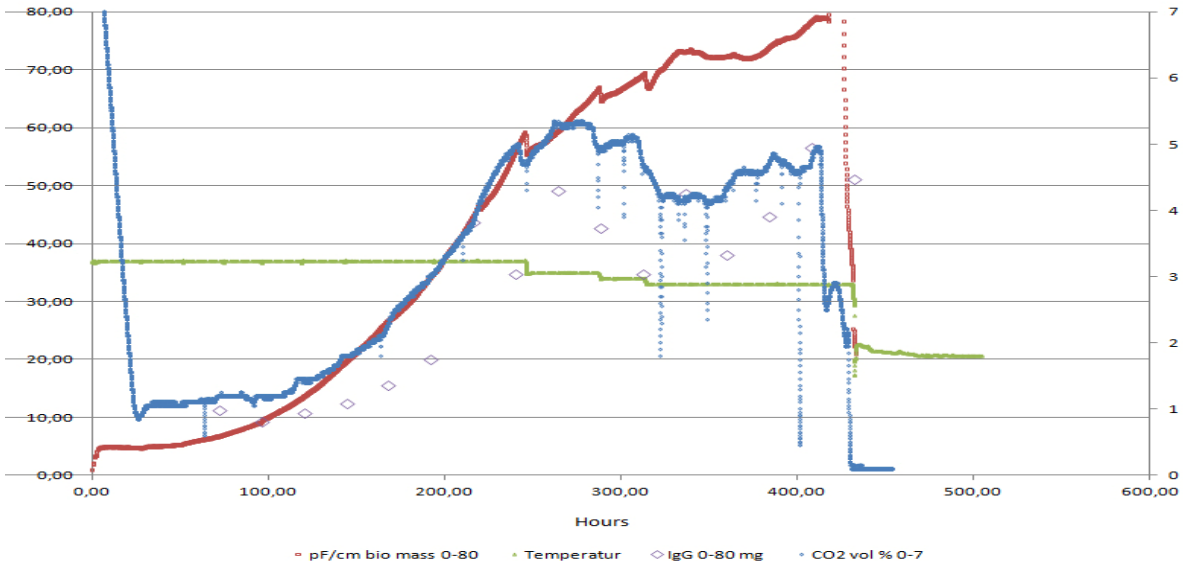


Figure – VCD measurement of bio mass vs. vol % CO2 in off-gas vs. specific productivity of IgG in 10 mg day over hours.

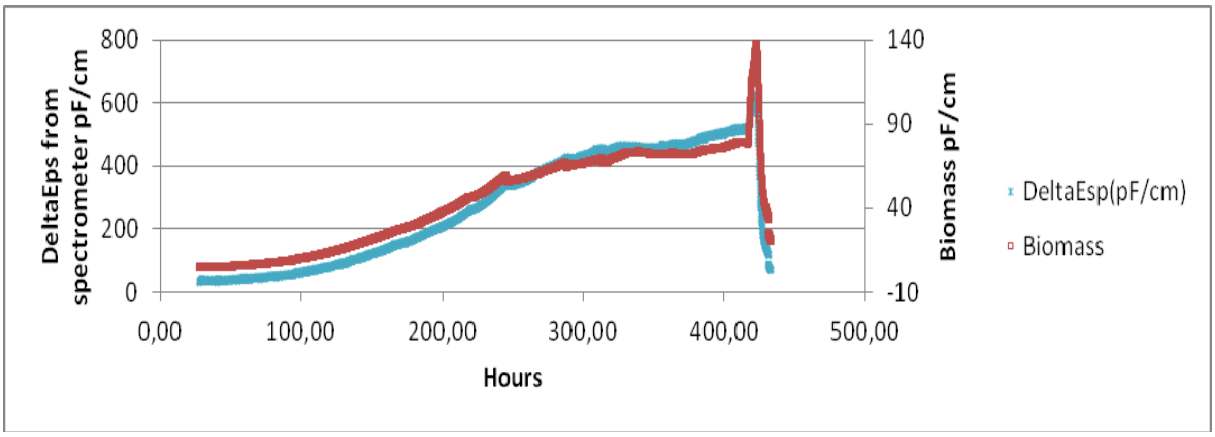


Figure – 2 frequency VCD measurement vs. DeltaEps spectrometer measurement. When these parameters split, this indicates that cells are not spherical anymore, which is from 250 hours, where temperature is dropped from 37 to 35°C.

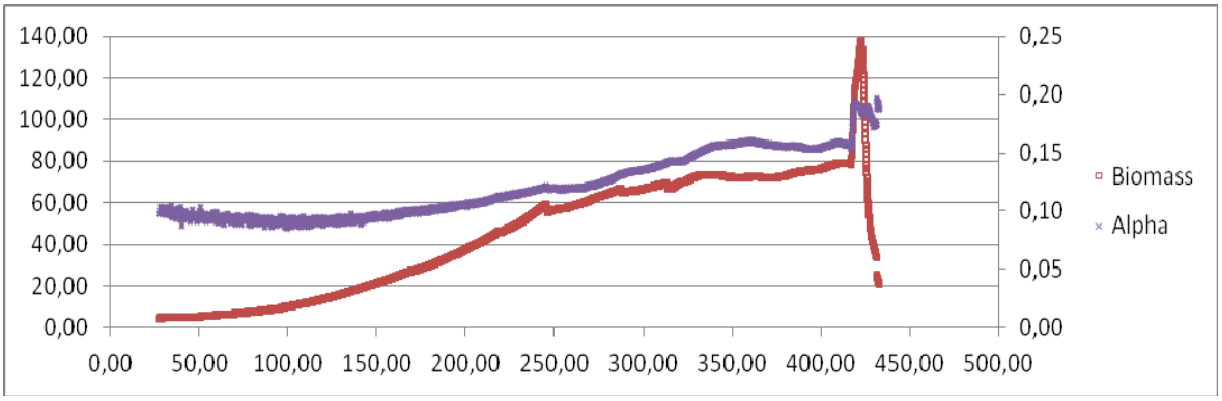


Figure – Alpha indicates that there is a tendency towards less homogeneity in cell size. From 280 hours, alpha indicates that cell size or cell radius's are less homogeneous (non-spherical cells). Bio mass reading increased rapidly the last 12 hours as to the stirrer table traction loss and cultivation loss.

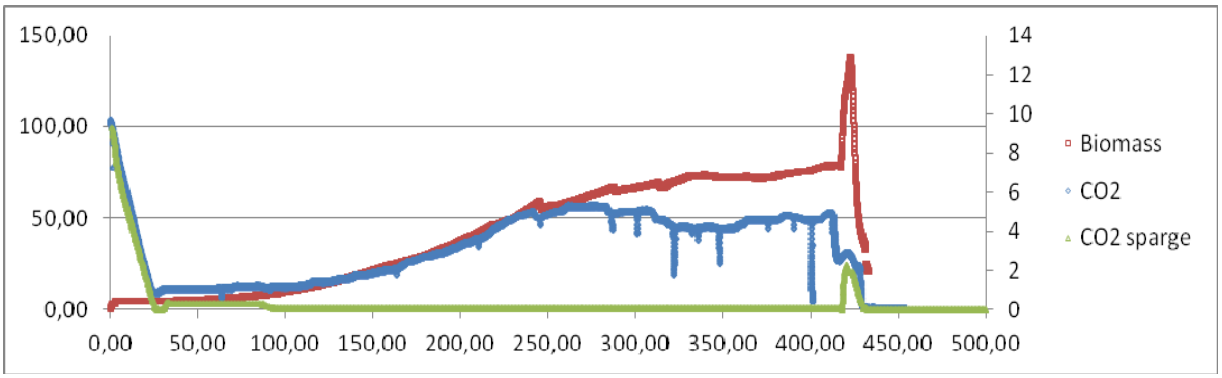


Figure – CO2 in off-gas vs CO2 sparging. When sparging gas is subtracted, the correlation to VCD during log-phase is very good.

Endpoint cell density was measured to be 0.8x10E+08 c/ml/matrix measured by cell counting of the content in the SUB and reduction of the reservoir.

This figure is based on the following:

- Monday the total cell number in the SUB was 0.93x10E+10 cells
- loss of traction from the particular stirrer table which was set to operate at 590 rpm despite the fact that 550 rpm is maximum for the two-pole DasGip equipment. Hereby cultivation stopped Sunday evening and the cells did not see media flow over the 12 hours until the process was stopped Monday. Estimated 20% cell mass was lost during termination and hereby a total of 1.15x10E+10 in the 150 ml matrix is found.

A CellTank correction factor to the pF/cm figure read from bio mass sensor is established. The calculated 1.15x10E+10/matrix / 150 ml = 0.76x10E+08/matrix / 79 pF/cm and hereby ~1 pF/cm is equivalent to 1x10E+08 cells/ml for this particular CHO suspension cell line.

8. Conclusions

The obtained data demonstrate that the CellTank bioreactor is a functionally perfusion system for constant expression of product by a CHO suspension mammalian cell line at high cell density. It appears that reducing temperature from 50 pF/cm and hereby limit the proliferation over several weeks until maximum obtainable bio mass in a viable strategy. Some optimisation is needed in order to find the optimum balance of operation.

The SUB operated over several days at ~70 pF/cm cell mass density at 33°C with app 1 g/liter glucose and ~40 mMol lactate. The CHO cell line was able to express a product at temperature as low as 33°C with limited proliferation. The SUB produced 0.52 gram product over 15 days with 31 litre spent media being 11 times more than the STR batch which produced 0.045 gram antibody based on 0.6 liter media.

PCS comments

The DasGip MP8 kept an impressive stable dO2 concentration in both the STR and the SUB. The M10 dual inductive magnet stirrer table has under earlier specific test show traction loss at 600 rpm and should be operated below 550 rpm. This indicates the max suitable cell density for this particular mechanical set-up being app 0.8x10E+08 cells/ml.

Sensor comments:

- The re-usable bio mass sensor demonstrated this particular sensor principle worked effectively for on-line determination of the actual cell number inside the matrix. Such valuable information is worthy to be used for on-line process variables adjustment.
- On-line glucose, lactate and dCO2 measurement in the reservoir would have given valuable information.

Problems of the test:

- The head space was not flushed with air – which may have caused the dissolved pCO2 content increased dramatically, perhaps flushing with 6 l/h air.
- NaOH addition in large amounts was needed in order to control pH and ammonia.
- The low perfusion flow was not able to remove the produced lactate though the cells seemed to accept the high lactate content.
- Not sufficient media in stock for continuation of the perfusion and flushing out the lactate and ammonia
- Stirrer table not able to transfer power above 590 rpm and the cultivation ended brutally a Sunday evening!

9. END

We would like to thank the *Danish Agency for Science and Innovation* for the funding and Senior Scientist Holger K. Riemann at Bioneer for all the work and Svanholm.com for supplying the instrumentation.

List of abbreviations:

SUB = Single-Use-Bioreactor
SUS = Single-Use-Sensor
RUS = Re-usable-Sensor
RPM = Revolutions per Minute
MAb = Monoclonal Antibody
IgG = Immunoglobulin G = antibody molecules
PBS = Phosphate Buffered Saline
ELISA = Enzyme-linked immunosorbent assay = diagnostics tool
STR = Stirred Tank Reactor
PCS = Process-Control-System
VCD = Viable Cell Density

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Further information is available on www.cercell.com