CellMaker Application Note:

Protease expression in a CellMaker™ (single use bioreactor), using high optical density media

Scott AL[1], Shepherd SM[1] and Gray DW[2]

[1]Biological Chemistry and Drug Discovery, School of Life Sciences, University of Dundee, Dundee, DD1 5EH, Scotland, UK
[2]Drug Discovery Unit, School of Life Sciences, University of Dundee, Dundee, DD1 5EH, Scotland, UK

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Summary

In this application note, an *Escherichia coli (E. coli)* fermentation run was conducted using the CellMaker system. A His-tagged protease was expressed and purified showing enhanced expression in the CellMaker compared to a standard shaker flask.

Materials and methods

Cell line and culture media

A plasmid containing His-tagged protease was transformed into *E. coli* cell line BL21 (DE3) pLysS by heat shock method, prior to being plated onto LB agar plates supplemented with 50µg/mL carbenicillin. The plate was incubated at 37°C for 16hr.

Medium

The initial fermentation medium was prepared as follows: 350mL 10× phosphate/citric acid buffer [133g/L KH_2PO_4, 40g/L $(NH_4)_2HPO_4$, 17g/L citric acid] and 3.15L MilliQ water was autoclaved at 121°C in a 5L Duran bottle for 20 min.
After the solution was cooled to room temperature, the following sterile components were added to make the complete fermentation medium: 148mL of 70% glucose solution, 8.4mL of 500g/L MgSO4 solution, 0.8mL of 20g/L Thiamine solution, 1.75mL of 50µg/mL carbenicillin and 35mL of 100× Trace metal solution [1].
The 100× trace element solution contained: 10.0g/L Iron (III) citrate, 0.25g/L Cobalt (II) chloride, 1.50g/L Manganese (II) chloride, 0.15g/L Copper (II) chloride, 0.30g/L Boric acid, 0.25g/L Sodium molybdate, 1.30g/L Zinc acetate, 0.84g/L EDTA.

Bioreactor preparation

Prior to inoculation, the CellMaker bag was set up in a laminar hood. The bag was inserted into enclosure, connected to O_2 optical sensor and Electro Lab FerMac 280 Foam Control Module, and 3.5L media was pumped into the bag before being heated to 37°C.

pH Calibration and Control

pH calibration was done outside the vessel using a two-point calibration in pH 4 and 7 buffers. The pH sensor was calibrated prior to sterilisation. During the run, pH was automatically maintained at 6.8 with 14.5M NH_4OH (Sigma).

Dissolved oxygen (DO) sensor calibration

pO_2 sensors were calibrated using one-point calibration of 100% air obtained by running 4 standard litre per minute (SLPM) air flow until the DO value stabilised at maximum (10min).

Antifoam System

Two probes are linked to an Electro Lab FerMac 280 Foam Control Module, which detects foam when an electrical connection is made between the two probes. This in turn then pumps in a small volume of 10% Antifoam C (Sigma) through one of the top luer locks on the CellMaker bag.

Sampling and analysis

Samples were taken as required from 3-way tap using 10ml syringe. 10ml dead space volume was discarded and sample tested using OD_{600} on spectrometer (Eppendorf BioPhotometer).

Inoculum preparation

A cell scraping was used to create the starter culture in 100mL LB supplemented with 50µg/mL carbenicillin, which was incubated at 37°C, 200rpm shaking for 16hr. A 20mL volume of the starter culture was used to inoculate each litre of citrate/phosphate media, which was supplemented with 50µg/mL carbenicillin.
Cells were subsequently grown at 37°C degrees in either the CellMaker or 1L shaker flask. Shaker flasks were maintained at 200rpm in shaking incubator (Infors, Multitron).

Induction and harvest

Cells were maintained at 37°C until induction, when both the shaker and CellMaker were cooled to 20°C. Cells were grown for 7hr (OD_{600} of 0.6-0.8), 12hr or 19hr, then induced with 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and 4.55mL/L yeast extract. Cells were then grown for a further 12hr before 1L was harvested and centrifuged at 3500g for 30 minutes to pellet cells and stored at -20°C.

Purification

Cells were defrosted and chemically lysed (50mM sodium phosphate buffer pH 8.0, 150mM NaCl, 25mM imidazole, 10% glycerol) supplemented with 10µg/mL DNAse1 and protease inhibitor tablets (Roche). Cell lysis was carried out at 30kPSI using a Constant Cell Disruption System (Constant Systems). The supernatant was separated from the cell debris by centrifugation at 40,000g for 30 minutes at 4°C prior to being filtered through a 0.45µm syringe filter.
The sample was loaded onto a HisTrap HP 5mL column (GE Healthcare) equilibrated against 10 column volumes (CV) of buffer A (50mM sodium phosphate buffer pH 8.0, 150mM NaCl, 25mM imidazole, 10% glycerol) using an AKTA PURE (GE Healthcare).
A linear gradient of buffer B (50mM sodium phosphate buffer pH 8.0, 150mM NaCl, 800mM imidazole, 10% glycerol) was carried out over 20 CV's. The protein was eluted into 1M EDTA and 1M DTT (final concentration 1mM) to prevent precipitation and purity was analysed using an SDS PAGE gel electrophoresis. Samples containing the protease were pooled and concentration determined using A_{280} (DeNovix, spectrophotometer), before being flash frozen in liquid nitrogen and stored at -20°C.

Activity Assay

A His-Protease-ProteinX was expressed in BL21 DE3 and purified using an AKTA PURE on a HisTrap HP 5mL column, before fractions were pooled, flash frozen in liquid nitrogen and stored at -20°C in 1mg/mL aliquots. 100µg protease from previous experiments in an overall volume of 500µl were incubated with 1mg ProteinX for 16hr before being analysed on an SDS-PAGE gel to show cleavage of protease site on ProteinX.

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Experiment

Experiment 1

The process was set up to determine *E. coli* cell growth optical density at various points in the growth cycle: 7hr (early lag phase), 12hr (middle lag phase) or 19hr (end lag phase). Samples were taken periodically to monitor the cell growth and pH.

Culture volume	3.5L
pH value	6.8
Temperature	37°C
Induction temperature	20°C
Dissolved oxygen s.p	40%
Air flow rate	4 SLPM
Max. O2 flow rate	1 SLPM
Start cell concentration	20ml/L
Growth time	7, 12 and 19hr

After the set growth time prior to induction, the culture was induced with 1mM IPTG and allowed to express for a further 12hr at 20°C. Harvested pellets were then lysed, loaded onto the HP column, protein was purified and yield quantified for an activity assay.

Experiment 2

The machine was set up and the process was started using the same process parameters as used for experiment 1, apart from induction time, which was only 12hr. This experiment was repeated three times.

Experiment 3

Analyse protease produced by Experiment 1, using densitometry to view purity of the protein.

Experiment 4

Activity assay of protease produced by Experiment 1, to cleave the protease site on His-protease-ProteinX.

Results

Experiment 1

Experiment 1 showed a marked increase in cell growth in the CellMaker with increased time before induction, whilst there was only a slight increase in OD in the shaker flask [**Figure 1**]. However this did not translate into protein expression [**Table 1**], with 12hr being the optimum time for induction in the CellMaker with both increase in OD and protein expression [**Figure 2**], which was then repeated in **Experiment 2**. In the shaker flask at 19hr, there was almost no protein produced and was optimum at 7hr, which is the standard laboratory protocol.

In **Experiment 3**, the protease produced in **Experiment 1** was analysed using densitometry to assess purity of the protein. **Figure 3** shows that at 2µg protein, all of the protease from **Experiment 1** showed an equal purity of protease at molecular weight (MW) of 28.617kDa apart from the shaker 19hr. The protease does not appear to have been expressed in the shaker at 19hr.

In **Experiment 4**, the activity of the protease produced by **Experiment 1** was tested by cleaving 1mg His-protease-ProteinX with 100µg of the protease. This was then analysed on an SDS-PAGE gel. **Figure 4** shows that the uncleaved MW of ProteinX is 34.639kDa (Lane 2) and cleaved ProteinX is at MW of 32.546kDa, The gel shows that the protease produced from all of the experiments, with exception of Shaker 19hr, cleaved the protease site of ProteinX as the bands all show a decrease in molecular weight compared to the uncleaved sample.

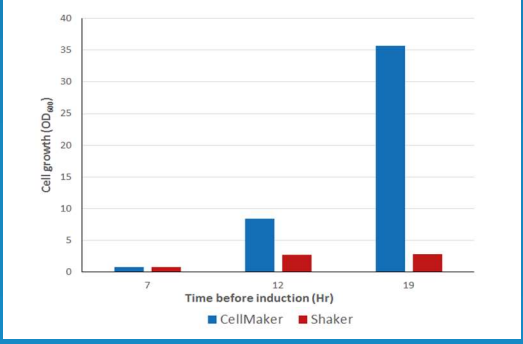

Figure 1
Cell growth of the CellMaker and shaker flask at 7hr (early lag phase), average 12hr (middle lag phase) or 19hr (end lag phase).

Time before induction (hours)	Average Protein Expression in the CellMaker (mg/L)	Average Protein Expression in the Shaker (mg/L)
7	102	45.36
12	491.75	19.25
19	213.75	0.209

Table 1
Average protein expression

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Conclusion

E. coli grows to higher cell density in the CellMaker compared to standard shaker flask at 12 and 19hr before induction.
However, whilst *E. coli* grew to a higher cell density after 19hr in the CellMaker, optimum protein expression of the protease was at 12hr.
In the shaker flask optimum protein expression was at 7hr; although the yield was much lower than the CellMaker.
The CellMaker produced a greater quantity of protease compared to the shaker flask at all time points. The quality and the activity of the protease was equivalent to the shaker flask for all conditions with the exception of the shaker flask at 19hr, which did not express any protease and so did not have any activity.
In conclusion, the CellMaker is an excellent choice of single-use bioreactor delivering a high yield of top-quality protein at an optimum time point for translational research or bioproduction.

References

[1] Korz DJ, Rinas U, Hellmuth K, Sanders EA, Deckwer WD. Simple fedbatch technique for high cell-density cultivation of *Escherichia coli*. J. Biotechnol. 1995;39:59-65.

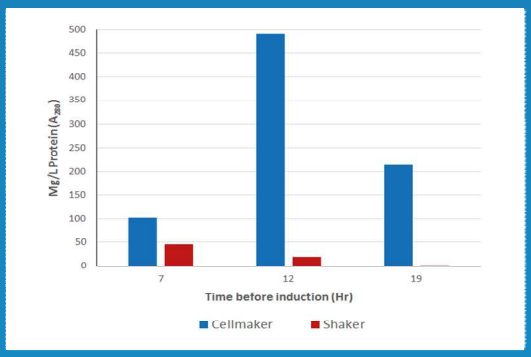

Figure 2
Protein expression of a His-tagged protease in the CellMaker and shaker flask at 7hr (early lag phase), average 12hr (middle lag phase) or 19hr (end lag phase).

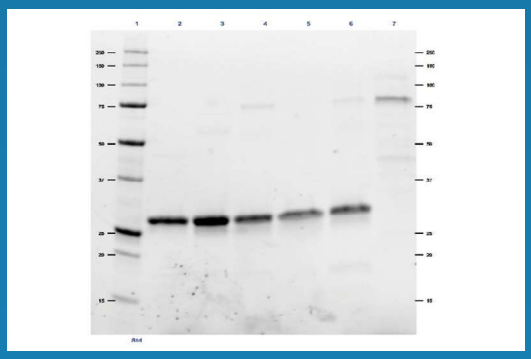

Figure 3
2µg protease on SDS-PAGE gel was analysed using BioRad Image Lab 4.0.
(1) Unstained Marker (BioRad); (2) CellMaker 7hr; (3) Shaker 7hr; (4) CellMaker 12hr; (5) Shaker 12hr; (6) CellMaker 19hr; (7) Shaker 19hr.

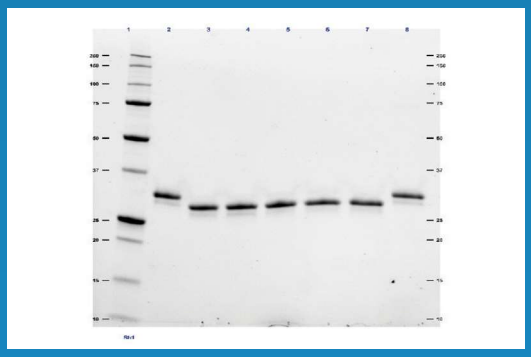

Figure 4
ProteinX on an SDS-PAGE gel was analysed using BioRad Image Lab 4.0.
(1) Unstained Marker (BioRad); (2) Uncleaved ProteinX and then ProteinX cleaved by 100µg : (3) CellMaker 7hr; (4) Shaker 7hr; (5) CellMaker 12hr; (6) Shaker 12hr; (7) CellMaker 19hr; (8) Shaker 19hr.

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Contact details

Headquarters - United Kingdom

Cellexus International Limited

6 Riverside Court, Mayo Avenue
Dundee, DD2 1XD
United Kingdom

Telephone: +44 (0)1382 666357
Email: sales@cellexus.com

For full details of our distributors please see our website.

www.cellexus.com