Biotechnol Lett (2010) 32:73–78
DOI 10.1007/s10529-009-0124-0

ORIGINAL RESEARCH PAPER

Fed-batch operation of an industrial cell culture process in shaken microwells

N. J. Silk · S. Denby · G. Lewis · M. Kuiper · D. Hatton · R. Field · F. Baganz · Gary J. Lye

Received: 7 July 2009 / Revised: 14 August 2009 / Accepted: 18 August 2009 / Published online: 17 September 2009

Abstract Recently we have demonstrated batch suspension culture of mammalian cells in microwell plates. Here we describe a method for fed-batch culture of an industrially relevant GS-CHO (Glutamine Synthetase-Chinese Hamster Ovary) cell line in shaken 24-standard round well (24-SRW) plates. Use of a commercially available 'sandwich lid' and appropriate dilution of the bolus feeds counteracted liquid evaporation from the wells resulting in similar cell growth and antibody formation kinetics in both 24-SRW plates (800 µl) and shaken flasks (50 ml). Peak viable cell densities obtained were $8 \pm 0.5 \times 10^6$ and $9 \pm 1.3 \times 10^6$ ml^{-1}, respectively, while comparable final titres of a whole IgG of approximately 1.5 g l^{-1} were recorded. Use of microwells provides at least a 50-fold reduction in medium requirements compared to shake-flask and other culture devices currently used in early stage cell culture process development. The ability to run multiple wells in parallel and to automate culture operation also offers considerable enhancements in experimental throughput.

Keywords GS-CHO cells · Fed-batch culture · High throughput · Microwells

N. J. Silk · F. Baganz · G. J. Lye (✉)
The Advanced Centre for Biochemical Engineering, Department of Biochemical Engineering, University College London, Torrington Place, London WC1E 7JE, UK
e-mail: g.lye@ucl.ac.uk

S. Denby · G. Lewis · M. Kuiper · D. Hatton · R. Field
MedImmune Ltd, Milstein Building, Granta Park, Cambridge CB21 6GH, UK

Introduction

The increasing number of candidate cell lines requiring early stage process characterisation puts considerable pressure on industrial biopharmaceutical development groups (Micheletti and Lye 2006; Betts and Baganz 2006). Shake-flask cultures are a standard tool in cell line selection and early stage process development. However, this system has limited throughput. Use of microwell plate cultures will enable parallel cultivation while the reduction in scale will ensure process information is acquired more rapidly and at a fraction of the material and labour costs involved in manual bench scale approaches (Li et al. 2006). Ultimately this will mean that the new therapeutic agents produced by the cells will reach the market, and the patients that need them, more quickly.

Recently we described the batch cultivation of a model hybridoma cell line in 24-standard round well (24-SRW) plates (Micheletti et al. 2006). Subsequent reports have shown the feasibility of on-line monitoring of key process parameters in miniaturised cell

culture bioreactors (Ge et al. 2006), and the introduction of temperature, pH and dissolved O_2 control (Chen et al. 2009). In an attempt to better understand how the culture conditions in shaken microwells compare to shaken flasks and stirred bioreactors we have also examined their engineering characteristics in terms of liquid mixing and energy dissipation (Barrett et al. 2009) and gas–liquid mass transfer (Doig et al. 2005).

The majority of industrial cell culture processes use fed-batch or perfusion protocols rather than simple batch culture to maximise viable cell density and antibody productivity (Hu and Aunins 1997). Fed-batch operation at the microscale presents challenges in accurate liquid handling and implementation of continuous feeding. In addition liquid evaporation from wells becomes more significant over extended culture periods and may lead to increases in culture osmolality. The extended culture time also makes maintenance of sterility more difficult, especially as moisture in the humidified incubators used is conducive to microbial wall growth (Duetz and Witholt 2001). The need for repeated manual intervention for feed addition in the absence of appropriate microwell automation also increases the risk of contamination (Lye et al. 2003). In this work we describe a methodology for the successful fed-batch culture of GS-CHO (Glutamine Synthetase-Chinese Hamster Ovary) cells in shaken microwell plates with cell growth rates, viabilities and antibody productivities that match those in conventional shaken flasks.

Materials and methods

Cell culture

An industrial GS-CHO cell line producing an IgG (supplied by MedImmune Ltd., Cambridge) was routinely subcultured by dilution at 4 day intervals using a seed density of 2×10^5 viable cells ml^{-1}. The culture medium used was CD-CHO (Invitrogen) supplemented with 25 μM methyl sulphoxamine (MSX).

Batch 24-Standard Round Well (24-SRW) and shake-flask cultures were inoculated at a seed density of 2×10^5 viable cells ml^{-1} and placed in a shaking incubator at 36.5°C. The initial fill volumes were 50 ml medium in a 250 ml shake-flask (fitted with vent caps) and 800 μl medium in 24-SRW plates (Ultra Low Attachment plates, Corning Life Sciences). CD-CHO medium was used but without addition of MSX. All cultures were shaken with a 25 mm orbital diameter. Relative humidity in the incubator was controlled at 70% and CO_2 at 5%. Microwell plates were sealed using either a Breath-Easy membrane (Diversified Biotech) or a 'sandwich lid' system (Duetz et al. 2000) in conjunction with a metal clamp (Enzyscreen BV). The sandwich lids were sterilised by autoclaving and subsequently oven dried before use.

Fed-batch protocols

Feeding of the cultures commenced on day four, and involved addition of bolus feeds of a proprietary medium (containing glucose and other nutrients) immediately followed by a shot of bicarbonate solution for pH control (0.75 M Na_2CO_3 and 0.5 M $NaHCO_3$). Addition volumes were fixed at 2 and 0.25% (v/v) of the initial culture volume for medium and bicarbonate additions respectively and bolus feeding was continued for 5 consecutive days. A second feeding strategy employing diluted feeds was also used such that liquid additions were 4 and 2.5% (v/v) of the inoculum volume respectively (corresponding to 2- and 10-fold dilutions of the feed and bicarbonate solutions).

Sampling and analysis

Approx. 0.8 ml medium was aseptically removed from shake-flask cultures while a sacrificial well approach was used with parallel 24-SRW cultures (Micheletti et al. 2006). Control experiments showed there was no well-to-well variation in culture kinetics across the 24-SRW plate (data not shown). All viable cell counts were performed using a Vi-Cell XR automated viability analyzer, with samples diluted using Dulbecco's PBS as necessary. Samples were also analysed for osmolality using a Gonotec Osmomat 030-D. Any remaining sample was centrifuged, and the supernatant stored frozen before quantification of IgG titre by Protein G HPLC (column from Applied Biosystems). All assays were performed at least in duplicate apart from the IgG and osmolarity measurements due to the limited well volumes. Gravimetric analysis was used

to monitor the rate of liquid evaporation from 24-SRW plates and shake-flasks.

Results and discussion

Maintenance of sterility and minimisation of evaporation

The maintenance of sterility and control of evaporation are key to successful operation of fed-batch microwell cultures. Both become more challenging in longer-term fed-batch cultures. Two sterile closures for 24-SRW plates were initially tested in single experiments for their ability to support batch growth of a GS-CHO cell line. One was a standard Breatheasy membrane and the other a 'sandwich lid' system (Duetz et al. 2000). The latter consists of a stainless steel cover containing 24 holes, below which a further three layers including a microfibre filter combine to minimise the rate of water loss from the wells while allowing for adequate gas exchange between the microwell headspace and the air. Both closures provided effective sterile barriers since not one occurrence of microbial culture contamination was observed over multiple parallel microwell cultures.

In terms of GS-CHO growth in batch cultures, use of the 'sandwich lid' resulted in a significantly higher viable cell density than the Breatheasy membrane (Fig. 1). This is attributed to the lower liquid evaporation rate measured through the 'sandwich lid' of 24 compared to 50 µl well^{-1} day^{-1} for the Breatheasy membrane. In the latter case this could lead to a doubling in the medium osmolarity due to liquid losses over the course of the batch culture duration.

Measured evaporation rates from the shake-flask and microwell systems, expressed both in absolute terms and as a proportion of the initial liquid volume, are shown in Table 1. In the case of simple batch cultures, while the absolute liquid evaporation rate from the shake-flask is considerably higher than from the microwells, this is actually around five times lower when expressed as a proportion of the initial liquid volume. Consequently in the microwells the medium osmolarity is increased which results in the lower GS-CHO growth rate and viable cell concentration seen in Fig. 1.

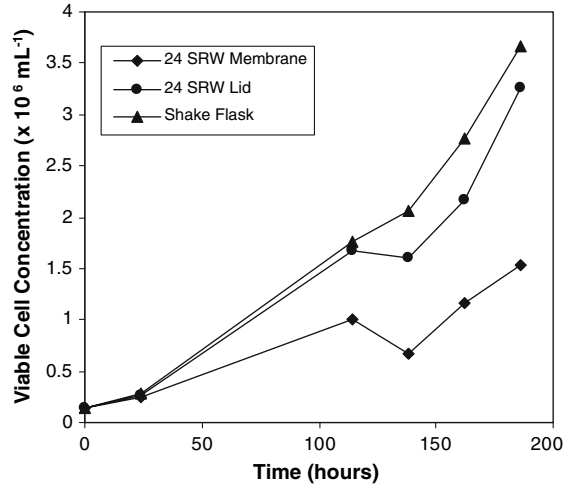

Fig. 1 Typical GS-CHO batch culture kinetics for shaken 24-Standard Round Well (24-SRW) plates with two different sterile seals and a conventional shake-flask: (*filled diamond*) 24-SRW plate with a Breatheasy membrane; (*filled circle*) 24-SRW plate with sandwich lid; (*filled triangle*) 250 ml shake-flask with vent seal. Experiments performed at 36.5°C with 5% CO_2 at 70% relative humidity and shaken with a 25 mm orbital diameter

Fed-batch GS-CHO culture (concentrated bolus feeds)

Microwell fed-batch cultures are compared here to shake-flask cultures as the latter represent the most commonly used culture devices in early stage industrial cell culture process development. Microwell feeding was performed using an already establish bolus feeding method for shake-flasks. This involved five bolus feeds on consecutive days. Each feed comprised addition of nutrient medium followed by a shot of bicarbonate solution for pH control. Since the evaporation rates from the flasks and wells were approximately 0.6 and 3% (v/v) of the initial liquid volume per day respectively (Table 1), it was found that this water loss combined with addition of concentrated feeds led to an average final osmolality of 746 mOsm kg^{-1} in the 24-SRW plates compared to 464 mOsm kg^{-1} in the shake-flasks. This had a detrimental impact on culture performance, as shown in Fig. 2a where the respective peak viable cell densities for shake-flask and microwell systems are 6×10^6 and 3×10^6 viable cells ml^{-1} respectively. The viability of the cells in the microwell culture is also seen to fall rapidly after 140 h. The resultant final antibody titre in the microwell system was found

Table 1 Comparison of absolute and relative evaporation rates from batch and fed-batch microwell and shake-flask GS-CHO cultures

Culture vessel	Sterile seal	Evaporation rates			
		Batch cultures		Fed-batch cultures	
		µl day^{-1}	% (v/v[a]) day^{-1}	µl day^{-1}	% (v/v[a]) day^{-1}
24-SRW[b] plate	Breatheasy membrane	50	−6.3	30	−3.9
24-SRW[b] plate	Sandwich lid	24	−3.0	5	−0.7
250 ml Shake-flask	Corning vent seal	310	−0.6	90	+0.2

Fed-batch relative evaporation rate calculation accounts for liquid replacement by feeding over entire duration of culture. Negative values indicate a net fluid loss from the culture vessel due to evaporation while positive values indicate a net fluid gain due to feeding

[a] Based on initial culture volume (excluding feed); [b] 24-standard round well

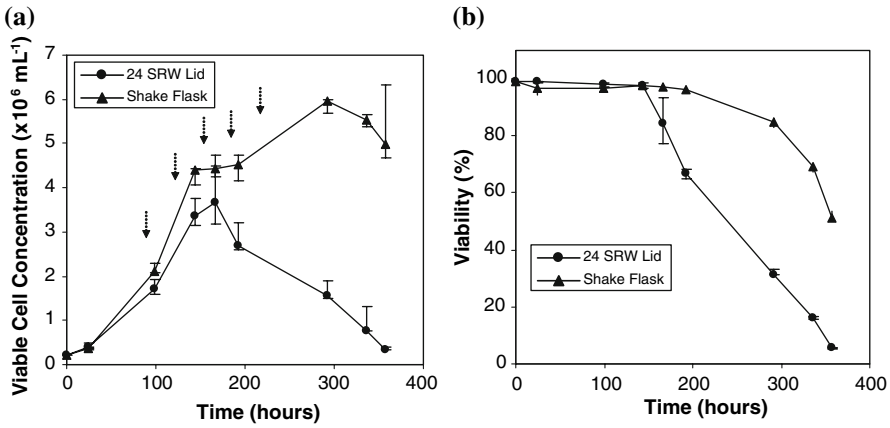

Fig. 2 Initial fed-batch cultivation using concentrated feeds. **a** Fed-batch GS-CHO culture kinetics with a 2% (v/v) daily bolus feed followed by a 0.25% (v/v) shot of bicarbonate solution for 5 consecutive days and **b** cell viability: (*filled circle*) 24-SRW plate with sandwich lid; (*filled triangle*) 250 ml shake-flasks with vent seal. The proprietary bolus feed was added daily as indicated by the arrows, immediately followed by a shot of bicarbonate for pH balance. Culture conditions as described in Fig. 1. Error bars represent the range of the data about the mean ($n \geq 2$)

to be lower at 0.73 compared to 1.3 g l^{-1} in the shake-flask. Takagi et al. (2000) found that productivity of suspended CHO cells was adversely affected at osmolalities in excess of 450 mOsm kg^{-1}, a value that was exceeded by a considerable margin in these initial fed-batch cultures.

Fed-batch GS-CHO culture (diluted bolus feeds)

To further minimise evaporation and changes in medium osmolarity the microwell feed was diluted by a factor of 2. The quantity of nutrients per bolus shot was unchanged but the liquid volume added was doubled. Figure 3 shows growth, viability, product and osmolality data respectively with this diluted feed for the shake-flask and 24-SRW culture systems. The cell growth rates and peak viable cell levels achieved are similar in both cases while the cell viabilities remain high at around 90% after 300 h culture. The kinetics of antibody formation are also similar with final IgG titres for both shaken systems being around 1.5 g l^{-1}. The slightly higher IgG titre measured in the microwells is thought to be a consequence of the slightly raised final culture osmolality of 506 mOsm kg^{-1} compared to 438 for the shake-flasks. Increased specific antibody productivity at slightly raised culture osmolalities is well documented for both CHO and hybridoma cell cultures (Takagi et al. 2000; Yang et al. 1996). The measured pH profiles in both the microwell and shake-flask experiments were identical, with final values around pH 6.7.

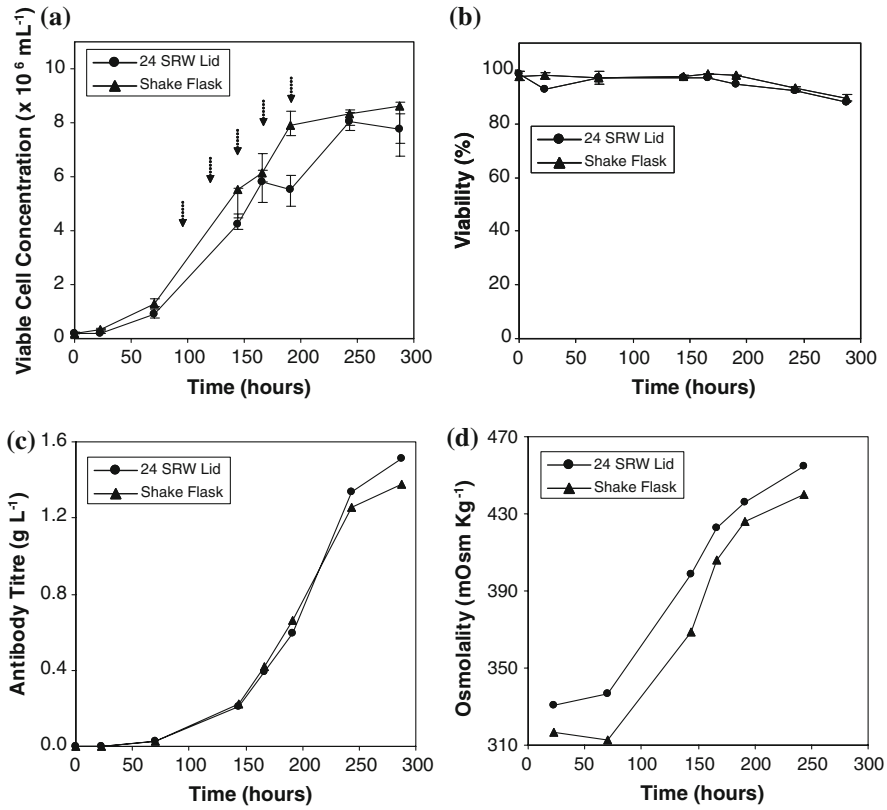

Fig. 3 Fed-batch cultivation using diluted feeds. **a** Fed-batch GS-CHO culture kinetics in which the feed and bicarbonate additions to 24-SRW plates were diluted to counteract evaporation to 4 and 2.5% (v/v) respectively, **b** cell viability, **c** IgG production and **d** medium osmolality: (*filled circle*) 24-SRW plate with sandwich lid; (*filled triangle*) 250 ml shake-flask with vent seal. Culture conditions as described in Fig. 1 and feeding/pH adjustment as in Fig. 2. Error bars, where shown, represent the range of the data about the mean ($n \geq 2$)

Based on the data in Fig. 3 fed-batch microwell cultures with diluted bolus feeds accurately reproduce the performance of conventional shake-flask systems. As shown in Table 1 one of the main reasons for this is that the combination of feeding and use of the 'sandwich lid' results in the overall liquid losses due to evaporation being very small (just 0.6% v/v) and virtually identical to those measured in shake-flasks (0.7% v/v). Methods to further reduce evaporation rates, in the microwells would be advantageous in removing the need for use of diluted feeds. The use of thicker or less permeable sealing films, however, would likely result in a reduction in gas–liquid mass transfer rates (Zimmermann et al. 2003).

Conclusions

The methods and data presented in this work demonstrate the feasibility of performing industrial fed-batch mammalian cell cultures in shaken microwell formats. The results for fed-batch culture of GS-CHO cells indicate comparable cell growth kinetics, viability and antibody titre with conventionally used shaken flasks. This represents a significant reduction in medium requirements and experimental costs compared to shake-flasks.

The use of simple, off-the-shelf microwell culture plates enables the method to be used with the types of shaking platforms commonly used for shake-flask cultures. For high throughput applications the culture process can also be readily automated on standard robotic platforms and commercially available automated CO_2 incubators (Lye et al. 2003). While other miniaturised culture systems are available that enable online monitoring and/or control of key culture parameters they are more costly solutions to the problem of increasing throughput (Chen et al. 2009) and less easy to automate. Ultimately the choice of microwell culture device will depend on the particular application, such as cell line characterisation or medium development and the stage at which they are used in cell culture process development.

Acknowledgements Financial support for this research was from the Engineering and Physical Sciences Research Council

(EPSRC) through the Innovative Manufacturing Programme. The research programme is managed out of the UCL Advanced Centre for Biochemical Engineering in collaboration with other academic departments and with leading industrial partners (http://www.ucl.ac.uk/biochemeng/industry/imrc.htm). The support and advice gained from collaborators is gratefully acknowledged. NJS would also like to thank EPSRC and MedImmune for financial support via an EngD studentship. The GS-CHO cell line used for these studies was constructed using the GS system from Lonza Biologics.

References

Barrett TA, Wu A, Zhang H, Levy MS, Lye GJ (2009) Microwell engineering characterisation for mammalian cell culture process development. Biotechnol Bioeng (in press)

Betts JI, Baganz F (2006) Miniature bioreactors: current practices and future opportunities. Microb Cell Fact 5:21

Chen A, Chitta R, Chang D, Amanullah A (2009) Twenty-four well plate miniature bioreactor system as a scale-down model for cell culture process development. Biotechnol Bioeng 102:148–160

Doig SD, Pickering SCR, Lye GJ, Baganz F (2005) Modelling surface aeration in shaken microtitre plates using dimensionless groups. Chem Eng Sci 60:2741–2750

Duetz WA, Witholt B (2001) Effectiveness of orbital shaking for the aeration of suspended bacterial cultures in square-deepwell microtiter plates. Biochem Eng J 7:113–115

Duetz WA, Rüedi L, Hermann R, O'Connor K, Büchs J, Witholt B (2000) Methods for intense aeration, growth, storage, and replication of bacterial strains in microtiter plates. Appl Environ Microbiol 66:2641–2646

Ge X, Hansom N, Shen H, Kostov Y, Brorson KA, Frey DD, Moreira AR, Rao G (2006) Validation of an optical sensor-based high-throughput bioreactor system for mammalian cell culture. J Biotechnol 122:293–306

Hu W, Aunins JG (1997) Large-scale mammalian cell culture. Curr Opin Biotechnol 8:148–153

Li F, Hashimura Y, Pendleton R, Harms J, Collins E, Lee B (2006) A systematic approach for scale-down model development and characterization of commercial cell culture processes. Biotechnol Prog 22:696–703

Lye GJ, Ayazi-Shamlou P, Baganz F, Dalby PA, Woodley JM (2003) Accelerated design of bioconversion processes using automated microscale processing techniques. Trends Biotechnol 21:29–37

Micheletti M, Lye GJ (2006) Microscale bioprocess optimisation. Curr Opin Biotechnol 17:611–618

Micheletti M, Barrett T, Doig SD, Baganz F, Levy MS, Woodley JM, Lye GJ (2006) Fluid mixing in shaken bioreactors: implications for scale-up predictions from microlitre scale microbial and mammalian cell cultures. Chem Eng Sci 61:2939–2949

Takagi M, Hayashi H, Yoshida T (2000) The effect of osmolarity on metabolism and morphology in adhesion and suspension chinese hamster ovary cells producing tissue plasminogen activator. Cytotechnology 32:171–179

Yang X, Oehlert GW, Flickinger MC (1996) Specific protein secretion rate: application to monoclonal antibody secretion rate kinetics in response to osmotic stress. Biotechnol Bioeng 50:184–196

Zimmermann HF, John GT, Trauthwein H, Dingerdissen U, Huthmacher K (2003) Rapid evaluation of oxygen and water permeation through microplate sealing tapes. Biotechnol Prog 19:1061–1063