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Technical report

Use of lactate dehydrogenase release to assess changes in culture viability

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Abstract

This study reports the use of lactate dehydrogenase release to monitor changes in culture viability in flask culture and fixed bed, porosphere bioreactor systems. Lactate dehydrogenase release shows good agreement with increase in non-viable cell numbers and decline in glucose utilisation in flask cultures. Studies with the immobilised system show that lactate dehydrogenase release can detect loss of viability which is not always indicated by a decrease in glucose utilisation. The data show that culture viability in a repeated-feed-and-harvest system is influenced markedly by both a) the medium change regime itself and b) the use of an immobilised bioreactor compared to a flask system for the same medium change regime.

Introduction

Free suspension culture is the preferred process technology for the growth of animal cells as it facilitates scale-up to large volumes, process monitoring and control, and allows a homogeneous environment to be maintained upon scale-up. The health of the culture is readily monitored as the cells are easily sampled. However, free-suspension culture is not always the most suitable technology. Commercially important cell lines do not always grow in suspension, or product may be expressed at a lower level or have altered properties compared to growth in anchorage-dependent cultures (Clarke and Spier, 1980). Such considerations, coupled with demands for greater process intensity, have led to the development of entrapped cell systems (reviewed in Griffiths, 1988). The need in such systems to maximise the surface area available to cells has resulted in the isolation of cells in/on structures which are difficult to access directly. Consequently the cells are not easily sampled for assessment of cell numbers and viability.

A variety of techniques have been developed to assess cell viability (reviewed in Cook and Mitchell, 1989). However, most of these techniques suffer from the disadvantage that they require sampling of the cells for biochemical analysis or invasive microscopic examination. Such techniques are obviously not applicable to a system where cells are entrapped within, for example, an immobilised bed.

Non-invasive metabolic parameters commonly used to assess viable cell numbers are glucose and lactate levels. A decrease in the rates of

utilisation/production, as a result of a decrease in metabolic activity, is taken as an indication of a decline in culture viability. However this has its disadvantages, especially for immobilised cultures: (a) as a trend may take time to become apparent; (b) since glucose is not the only carbon source used by animal cells in culture (Donnelly and Scheffler, 1976; Zielke *et al.*, 1976) it is possible that a decreased rate may result from a switch in metabolism and not from decreased viability; (c) as variation in rates of metabolite production/utilisation also occurs as a result of a change in the growth phase of the culture (Reuveny *et al.*, 1986); (d) because of difficulty in assessing whether changes in volumetric rates are the consequence of cell death or fluctuation of cell numbers.

Analysis of the release of intracellular enzymes can be used to measure loss of cell viability. The release of lactate dehydrogenase (EC. 1.1.1.27.: LDH) is commonly used in cell culture studies (e.g. Petersen *et al.*, 1988). The assumptions are made that intracellular enzymes are only released after damage to the cell membrane and that all activity is rapidly released from damaged cells. Thus, the larger the rate of release of enzyme, the greater the extent of cell death/damage which occurred in that period. The advantage of such methods over measurement of metabolite levels is that the parameter measured varies in response to changes in culture viability and not cell metabolism.

This study reports the use of LDH release to follow changes in culture viability of a hybridoma cell line grown in a fixed bed fermenter and in flask culture. The object was to assess whether LDH could replace metabolic parameters in monitoring and controlling immobilised cells.

Materials and methods

Cell lines

The mouse-mouse hybridoma cell C1E3 (Wright and Balfor, 1983), which produces IgG against *Toxoplasma gondii* antigen, was obtained from Dr. S. Clark (PHLS CAMR). Cells were tested for mycoplasma infection and found to be negative by the Hoechst stain test.

Cell culture

The medium used was RPMI 1640 with 5% heat-inactivated FCS (56°C for 45 min) (Imperial Laboratories, Andover, UK). Glucose was included at 2 g/l.

For both the flask cultures and the fermenter propagations, cell seed was taken from mid-exponential phase cultures (4–5 $\times$ 10^5 cells/ml).

In the flask cultures 100 ml medium in a 175 cm^2 T-flask was inoculated to an initial density of about 10^5 viable cells/ml. Growth was allowed to continue at 37°C, and samples were taken daily. At the points shown on the figures, varying fractions of the conditioned medium were replaced with an equal volume of fresh medium, and growth allowed to continue.

For the fermenter propagations C1E3 cells were grown in Siran porous glass spheres (porospheres) (Schott Glaswerke, Mainz, FRG) in a fixed bed system, whose design and operation have been previously described (Looby and Griffiths, 1988a,b). The large pore size (60–300 μm) and open structure of interconnecting pores causes physical entrapment of cells within the spheres. The physical entrapment of hybridoma cells, in combination with their loose adherence to the sphere itself, allows changes of medium without excessive loss of cells. In this study the system was operated in repeated-feed-and-harvest mode. The times of feeding/harvesting and fraction of the reservoir volume (10 l) replaced are indicated in the figures.

Assays

Cell numbers were determined in an improved Neubauer chamber and the viability by trypan blue exclusion.

Glucose was determined using a Beckman Glucose Analyser 2 (Beckman Instruments Inc., CA, USA).

LDH activity was determined in cell-free medium. LDH was assayed spectrophotometrically at 30°C by following the oxidation of NADH at 340 nm (Vassault, 1983). The reaction was initiated by the addition of pyruvate. The rate was corrected by subtracting the value measured in the absence of pyruvate. Triplicate assays were done on each sample. One unit (U) of activity was defined as 1 μmol NADH consumed per minute.

Results and discussion

Flask cultures

The relationship between LDH release and growth kinetics in a batch, flask culture (designated F1) is shown in Fig. 1. In the period between inoculation and early stationary phase (96 h) the viability of the culture was high (>90%). The LDH activity of the medium was relatively constant (c. 0.11 U/ml) in this period. As the culture progressed through stationary phase, the non-viable fraction of the culture increased – between 96 and 192 h it rose from c. 5 to c. 75%. In this same period the LDH activity of the medium increased by a factor of about 2.5.

Thus the data for the batch, flask culture (F1) show a good correlation between loss of culture viability and an increase in the LDH release rate.

To determine if the correlation between growth and LDH release kinetics holds in a more complex system, the relationship was examined in a repeated-feed-and-harvest flask culture (designated F2). In culture F2, the medium was completely replaced at 72 h, and thereafter on a daily basis until the end of the culture. Growth, glucose utilisation and LDH release data for culture F2 are presented in Fig. 2.

The cell number data show that the cells were in the exponential growth phase between 20–60

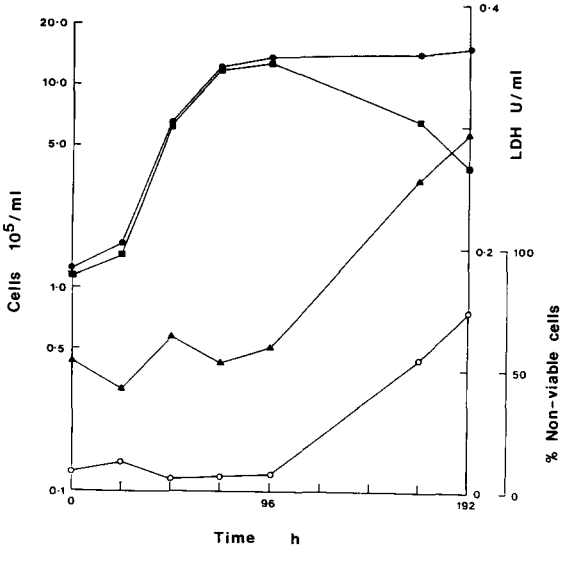

Fig. 1. Relationship of growth and LDH release kinetics in culture F1. (● total cell density; ■ viable cell density; ▲ LDH activity; ○ % non-viable cells).

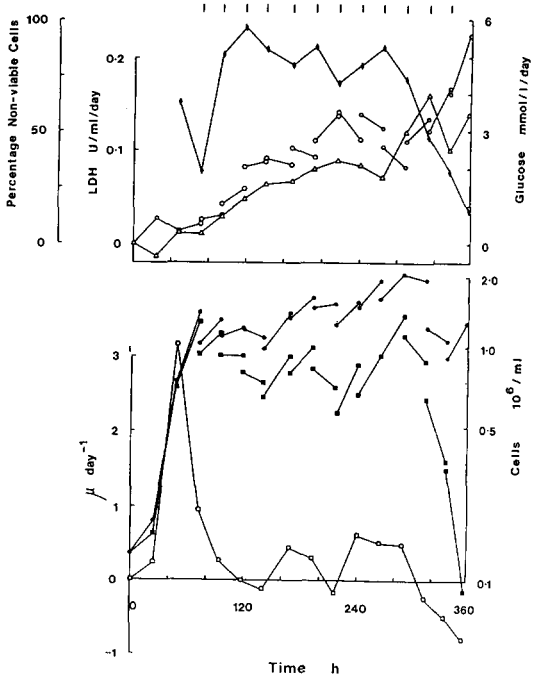

Fig. 2. Relationship of growth, LDH release and glucose uptake kinetics for the culture F2 (Δ volumetric LDH release rate; ❑ specific growth rate; ◆ volumetric glucose uptake rate; ● total cell density; ■ viable cell density; ○ percentage non-viable cells). The times when the medium was changed are indicated by the vertical arrows.

h, before entering the deceleration and stationary phases. This is in agreement with the specific growth rate data which show maximum rates between 20–50 h. After 50 h, μ decreased markedly with the appearance of negative values at about 190 h. The proportion of non-viable cells showed a marked increase after about 90–100 h (the start of stationary phase). At about 290 h the number of non-viable cells started to increase rapidly. Between 290 h and the end of the culture, the percentage of non-viable cells increased from 46 to 93%. In the period 0–72 h, very little LDH activity was released. After the first medium change (72 h), LDH activity started to increase at a relatively constant rate until about 270 h when the rate showed a marked increase until the end of the culture. Data for glucose utilisation show that the utilisation rate was relatively constant in the period 90–290 h. At 290 h there was a marked decrease in the utilisation rate, and corresponding flattening of the cumulative utilisation curve.

The growth and LDH release data in Fig. 2 show that for a viable culture, the rate of LDH release is low and the medium activity does not exhibit marked variation from the value at T = 0 h. Loss of culture viability was accompanied by an increase in the LDH release rate and medium activity higher than at T = 0 h. In culture F2, the marked decrease in culture viability between 290–360 h was accompanied by a further increase in the LDH release rate. On the basis of this data, there would again appear to be good agreement between changes in culture viability and increases in LDH release.

Comparison of the data in Fig. 2 shows good agreement between decrease in glucose consumption and loss of culture viability, measured either as decrease in viable cells or increase in LDH release rate. However, closer examination of the data Fig. 2 shows that the increase in LDH release occurred between 260–290 h whereas the marked decrease in glucose consumption and increase in non-viable cell numbers occurred between 290–310 h. Monitoring of LDH release was able to detect loss of culture viability before it was detectable as a decrease in viable cell numbers or a lower glucose consumption rate.

The data for culture F2 show that, in the period 92–264 h, the glucose utilisation rate fluctuated between 4.5 and 5.7 mmol/l/day with no overall trend. In contrast, over the same period the LDH release rate increased approximately linearly from 0.03 to 0.07 U/ml. The cell number data show that over this period there was a general downward trend in culture viability, the proportion of non-viable cells increased from 17 to 52% of the total cell count. The loss of viability is not as readily observable from the glucose consumption data, because of the apparently random fluctuation in utilisation rates in this period. In contrast, the steady increase in LDH release rate is clearly indicative of a loss of culture viability.

Immobilised porosphere cultures

As part of our studies to optimise growth conditions in fixed bed porosphere reactors, the cultures were grown using different medium change regimes. LDH release and glucose utilisation kinetics were used to monitor changes in culture viability. Culture I1 was grown using a medium change regime (designated regime 1) which increased the percentage of the reservoir medium replaced from 50 to 100%. A second culture (designated I2) was grown using 100% replacement of the reservoir medium (designated regime 2), and is analogous to culture F2.

Data for the glucose utilisation and LDH release by cultures I1 and I2 are presented in Fig. 3. In culture I1 the rate of LDH release was low and relatively constant from the start of the propagation until about 200 h. Subsequently, the release rate increased by about twofold. In the period 260–280 h there was a massive release of LDH activity, accounting for one-third of the total activity released. The glucose utilisation rate remained relatively constant, although there was a slight trend upwards over the period of the propagation. The data in Fig. 3b show that the marked increases in total LDH released between 200–290 h were not accompanied by a similar decrease in the total glucose used. The rate data in Fig. 3a show that the marked increase in LDH release

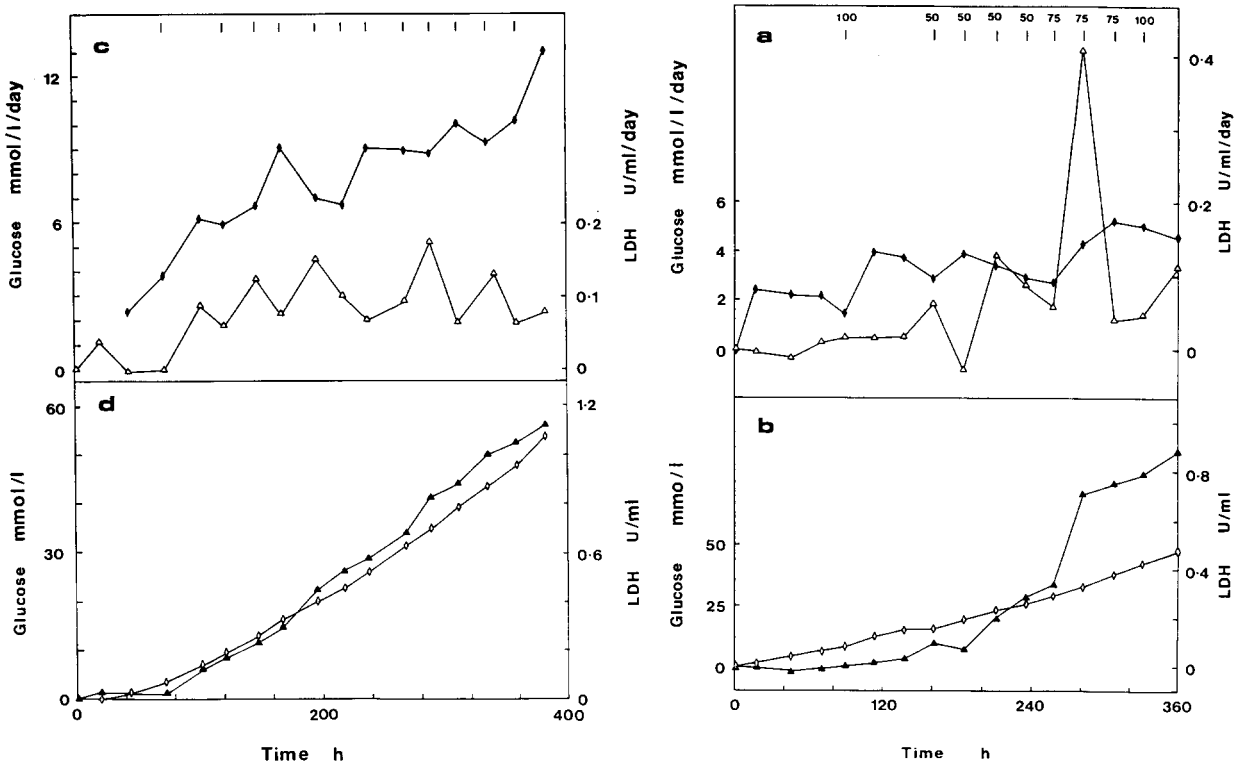

Fig. 3. Relationship of LDH and glucose uptake kinetics for propagations of the cell line C1E3 in porosphere, fixed bed bioreactors using different medium change regimes. a) rate data for culture I1; b) cumulative data for culture I1; c) rate data for culture I2; d) cumulative data for culture I2. Symbols: Δ volumetric LDH release rate; ◆ volumetric glucose uptake rate; ▲ cumulative LDH; ◊ cumulative glucose. The times when the medium was changed are shown by the vertical lines. The percentage of the reservoir medium replaced in culture I1 is indicated at the appropriate points in the culture.

rate between 190–210 h was accompanied by only a slight decrease in the glucose utilisation rate. The large increase in LDH release rate between 260–280 h was accompanied by an increase in the glucose utilisation rate.

LDH release in culture I2 exhibited a broadly biphasic pattern (Fig. 3c and d). After an initial release of activity (possibly due to damage caused by inoculation and commencement of the run), LDH activity in the medium remained constant until the first medium change (72 h). At 72 h the rate of release of LDH activity increased to a level which remained relatively constant until the end of the propagation although fluctuation about this point was observed. The glucose utilisation data show that the utilisation rate increased with time. The glucose utilisation rate increased to a peak at about 160 h, before declining slightly and subsequently increasing. Changes in the glucose utilisation rate did not always mirror the changes in LDH release rate.

Comparison of cultures

The data in Figs. 1 and 2 show good correlation between LDH release and increased numbers of non-viable cells. Figure 2 also shows good agreement between decrease in glucose consumption and increase in LDH release. However, the data in Fig. 3 show that in long-term, repeated-feed-and-harvest bioreactor cultures the agreement between the interpretation of changes in glucose utilisation and LDH release and changes in culture viability does not always hold true. LDH is released from the cell upon loss of membrane

integrity, while glucose utilisation can decline as a result of either loss of cell viability or change in cell metabolism. The data suggest to us that LDH release is a more reliable indicator of changes in culture viability in complex, long-term culture systems than, for example, glucose utilisation.

The LDH release data for culture I1 suggest that the media changes from the second 50% change onwards were no longer able to maintain the cell viability at the level observed in the first 180 h. It is unclear why there should have been a massive release of LDH activity after the first 75% change. It may simply be the result of the cumulative damage caused to the cells by the series of 50% medium changes which were insufficient to maintain the culture viability. Although it is assumed that LDH is released immediately upon cell damage, this is not always true because the primary effect is not always in the cell membrane (see Cook and Mitchell, 1989). This results in a delay between the primary damage and the ability of an observer to detect it.

The observation for culture I2 that the rate of release of LDH activity was generally constant, suggests that the medium change regime was able to maintain the culture viability at a stable level. This contrasts with culture I1, which showed considerable variation in the rate of release. Therefore, regime 2 was better able to maintain the cells in a constant state.

The rate of LDH release from culture I2 in the period 72–200 h was greater than for culture I1, although subsequently the rates were comparable. Part of the explanation is probably, that there were more cells in the bead bed for culture I2: 2.82×10^7 and 3.98×10^7 total cells per ml bed for I1 and I2 respectively. A higher cell density would result in a greater volume activity even if the specific activity was lower.

At the end of the experiments, final cell number and viability determinations were able to be carried out. Culture I2 had a higher percentage of viable cells (63%) compared to I1 (46%). This suggests that regime 1 was causing more damage to the cells than regime 2. This is supported by the observation that the LDH activity released from the beads at the end of the propagation was 0.281 U/10^7 total cells in I1 and 0.314 U/10^7 total cells in I2.

Therefore, it can be concluded that the medium change regime in culture I2 was better able to maintain the culture viability at a constant level and also did less damage to the cells.

Conclusions

We have shown that there is a good correlation between loss of culture viability and LDH release. In flask culture, there is also good agreement between LDH release and decline in glucose utilisation. The data also suggest that an increase in LDH release occurs before either the decline in glucose utilisation or viable cell numbers, allowing earlier detection of sub-optimal growth conditions.

In complex, long-term growth systems, where it is not possible to assess culture viability directly, LDH release provides a more accurate indicator of changes in culture viability than glucose utilisation because it is not affected by changes in cell metabolism. Measurement of LDH has enabled us to optimise the process conditions for maintaining immobilised cultures.

Comparison of the LDH release data for culture F2 (Fig. 2) with the data for culture I2 reveals differences between the two culture systems. Therefore these results show that the growth system used to culture hybridoma cells has a marked effect on the change of culture viability with time. The data in Fig. 3 show that the medium change regime has a profound affect upon culture viability, and also on the metabolism of the culture.

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