Analysis of CHO-K1 cell growth in a fixed bed bioreactor using magnetic resonance spectroscopy and imaging

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Abstract

Non-invasive magnetic resonance imaging and spectroscopy techniques have been used to monitor the growth and distribution of Chinese hamster ovary K1 cells growing in a fixed bed bioreactor composed of macroporous carriers. Diffusion-weighted ^1H magnetic resonance spectroscopy was used to monitor the volume fraction of the bioreactor occupied by the cells and diffusion-weighted ^1H magnetic resonance imaging was used to map cell distribution. The imaging measurements demonstrated that cell growth in the bioreactor was heterogeneous, with the highest cell densities being found at the surface of the carriers. The increase in the volume fraction occupied by the cells during cell growth showed a close correlation with bioreactor ATP content measured using ^{31}P magnetic resonance spectroscopy. These magnetic resonance measurements, in conjunction with measurements of bioreactor glucose consumption, allowed estimation of the specific glucose consumption rate. This declined during the culture, in parallel with medium glucose concentration.

Abbreviations: ADC – Apparent diffusion coefficient; CHO-K1 – Chinese hamster ovary K1 cells; DMEM – Dulbecco's modified Eagle's medium; HFBR – Hollow fiber bioreactor; MDP – Methylene diphosphonate; MRI – Magnetic resonance imaging; MRS – Magnetic resonance spectroscopy; PBS – Phosphate buffered saline; STEAM – Stimulated echo acquisition mode

Introduction

Intensive bioreactors, such as the hollow-fiber and fixed bed, have attracted considerable interest as systems for the commercial production of proteins from mammalian cells (Banik et al., 1995; Chresand et al., 1988; Knazek et al., 1972; Knight, 1989). The high cell densities that can be maintained by these systems result in higher volumetric protein productivity and can also lead to increased specific protein production rates (Knight, 1989). Intensive reactors are also being used in the field of tissue engineering (Hubbell et al., 1995; Jauregui et al., 1996; Langer et al., 1993), where they can be used to produce large quantities of cells for subsequent transplant, or where the cell-filled reactor itself can serve as a surrogate organ, such as bioartificial liver (Jauregui et al., 1996). However, despite their

recognised potential, a lack of knowledge concerning the influence of reactor design on cell growth, physiology and protein productivity has been perceived as inhibiting their wider commercial exploitation (Banik et al., 1995; Knight, 1989).

The principle problem with intensive reactors is that concentration gradients of nutrients and waste products can be formed (Chresand et al., 1988; Drury et al., 1988; Piret et al., 1990a; Piret et al., 1990b). These can have an adverse effect on cell growth and may also affect the quality of a secreted protein product. For example, culture pH and glucose concentration have both been shown to affect the glycosylation patterns of secreted proteins (Hayter et al., 1992; Borys et al., 1993). These concentration gradients may have an even greater negative effect on the function of a bioartificial tissue, such as bioartificial liver, where

the metabolic activity of the cells is intrinsically linked to their function.

In order to develop these systems there is a need for techniques which can provide information on aspects of the reactor environment, such as pO_2 , pH and media flow rates and their influence on cell growth and physiology. The intrinsically inaccessible nature of these systems has made the application of conventional monitoring techniques more difficult. Invasive electrode techniques, for example for measuring reactor pH or pO_2 , may perturb reactor function (Weismann et al., 1994) whereas indirect techniques may rely on assumptions which are not always justified. A commonly used method to determine cell concentration in hollow-fiber reactors is to make estimates based on nutrient uptake rates (Mancuso et al., 1990). However this method assumes constant specific uptake rates during the culture. The rates of glucose and oxygen consumption and lactate production, which have been used to estimate cell concentrations, can vary strongly with changes in growth conditions (Lin et al., 1993; Ljunggren et al., 1994; Neermann et al., 1996).

Magnetic resonance spectroscopy (MRS) and imaging (MRI) are non-invasive techniques which have already been used to monitor cellular metabolism (Gillies et al., 1989), cell density (Mancuso et al., 1990; Gillies et al., 1991; Barry et al., 1993), cell and dissolved oxygen distribution (Callies et al., 1994; Williams et al., 1997) and medium flow rates in HFBRs (Hammer et al., 1990; Donoghue et al., 1992), (Reviewed in Fernandez, 1996; Brindle 1998). Mancuso et al. (1990) used ^{23}Na and ^{31}P MR measurements of reactor sodium and ATP content respectively to estimate cell concentration in an HFBR and compared these measurements with measurements of oxygen consumption rate. Gillies et al. (1991) used similar methods to estimate cell density in an HFBR but concluded that the changes in ATP levels might represent fluctuations in cellular ATP concentrations, rather than changes in cell density, and that the ^{23}Na MR methods were not reliable enough. Subsequently this same group reported a ^{31}P MR method for measuring cell volume in an HFBR based on measurement of the intra- and extracellular signal intensities of a probe molecule, dimethyl methylphosphonate (Barry et al., 1993). In this study we have measured the cell content of a fixed-bed bioreactor using diffusion-weighted 1H MR spectroscopy measurements of cell volume and compared these with ^{31}P MR measurements of ATP content and measurements of bioreactor glucose consumption. There was good agreement be-

tween the cell volume measurements and estimates of reactor ATP concentration, showing that the cellular ATP concentrations did not vary significantly during the culture. However, comparison of these data with measurements of glucose consumption showed that glucose uptake rate cannot be used to estimate cell content in this reactor system under the conditions used. Diffusion-weighted 1H MRI was used to monitor cell distribution. This showed that cell growth was heterogeneous and that cell densities were highest at the edge of the macroporous carriers. The study further demonstrates the utility of these non-invasive MR methods for assessing the performance of intensive bioreactor systems.

Materials and methods

Materials

Enzymes, cell culture media, foetal calf serum and glucose assay kits were obtained from Sigma Chemical Company (Poole, Dorset, U.K.).

Cell culture

Chinese hamster ovary cells (CHO-K1) were cultured in DMEM containing 4.5 g L^{-1} glucose supplemented with 10% foetal calf serum, 2 mM glutamine, 100 units mL^{-1} penicillin and $100\text{ }\mu\text{g mL}^{-1}$ streptomycin. The bioreactor system consisted of a stirred tank fermenter (FT Applikon, Tewkesbury, U.K.), containing 1.25 L of medium, which was pumped via water-jacketed tubing at $37\text{ }^\circ\text{C}$, to the bioreactor positioned in the NMR magnet. The bioreactor consisted of a capped polysulphone tube (20 mm internal diameter) forming a 28 mL volume fixed-bed, composed of CELLSNOWTM FN-S05T carriers (Biomaterials Co., Japan). The bioreactor was filled with phosphate buffered saline (PBS) and autoclaved. PBS was exchanged for media and control spectra, images and media samples were acquired from the cell-free system. To inoculate the bioreactor, 8×10^7 cells were harvested from dishes and resuspended in 300 mL medium. This was pumped through the reactor until the majority of the cells had adhered. The seeding density was thus approximately $2.6 \times 10^5\text{ cells mL}^{-1}$. The fixed bed was then perfused with medium from the fermenter at a flow rate between 5 and 45 mL min^{-1} . A flow rate of 45 mL min^{-1} is equivalent to a vertical speed of 2.4 mm sec^{-1} , or 1.6 volume changes per minute through this bioreactor.

Medium in the fermenter was replaced by a continuous bleed and feed system, with a maximum rate of 300 mL per day. The temperature was maintained at 37 °C. The medium oxygenation and pH were maintained by passing a mixture of 95% O₂/5% CO₂ through nine metres of silicone tubing wound on a former surrounding the impeller blade of the fermenter. The pO₂ of the medium was monitored using a polarographic oxygen electrode (Mettler Toledo, Leicester, U.K.). Flow rate through the fixed bed was increased when a reduction in intracellular pH (pH_i) was observed in the phosphorus spectra of the cells. Media glucose and lactate concentrations were measured using enzymatic assay kits from Sigma (glucose) and Boehringer Mannheim (lactate). Media glutamine concentrations were determined enzymatically, as described in (Lund, 1985). The feed and bleed rate was increased as the experiment proceeded, to maintain media glucose concentrations above 1 g L⁻¹. Media flow rates through the bioreactor and feed and bleed rates used in this experiment are indicated in Figure 1.

NMR methods

Experiments were performed using a Varian Unity Plus 400 MHz wide-bore spectrometer equipped with an unshielded gradient set. ¹H spectra and images were acquired using a Varian 25 mm ¹H imaging probe and ³¹P spectra using a Bruker 25 mm ¹H/³¹P probe. Conventional spin echo images (TE = 20 ms; TR = 3 s) were acquired from transverse slices of the bioreactor using 256 phase encode increments. The in-plane resolution was 0.1 × 0.1 mm and the slice thickness 2 mm. Diffusion-weighted images were acquired using a stimulated echo (STEAM) sequence (Callies et al., 1994), which included a pair of pulsed magnetic field gradients of 0.2 T m⁻¹ and 2.5 ms duration in a TE period of 40 ms. The mixing time (TM) was 0.3 s. This provided diffusion weighting of $b = 5.64 \times 10^9$ s m⁻². Sixty four phase encode increments, with 128 transients per increment, were acquired with a repetition time of 2 s. The slice thickness was 2 mm and the in-plane resolution was 0.1 × 0.4 mm.

Diffusion-weighted ¹H spectroscopy was performed using a STEAM sequence with a pair of pulsed magnetic field gradients of 2.5 ms duration in a TE period of 40 ms. The mixing time was 0.3 s. Fifteen gradient strengths between 0.02 and 0.3 T m⁻¹ were used, providing diffusion weighting of up to $b = 1.27 \times 10^{10}$ s m⁻². The relative proportions of in-

tracellular and extracellular signal were calculated as described in (van Zijl et al., 1991).

³¹P NMR spectra were acquired using a 40 μs, 90° pulse and a pulse repetition time of 1.4 s. The spectra, which were the sum of 2000 transients, were acquired into 8000 data points, with a spectral width of 10 kHz. Chemical shifts and signal intensities were referenced to the resonance of methylene diphosphonate (MDP), which was contained in a capillary tube within the fixed bed reactor. The chemical shift of MDP was measured as 19.32 ppm relative to the resonance of phosphocreatine.

Cell counting

At the end of the experiment the CELLSNOW carriers were removed from the bioreactor and washed in 100 mL PBS. These were then incubated for 3 sequential periods of 15 min in 100 mL of 0.25% trypsin in PBS to remove cells. Cell suspensions from these washes were centrifuged, the cells were then resuspended in PBS for counting. Cell concentration and viability were measured with a hemocytometer using the Erythrocin B dye exclusion method.

Results

The bioreactor was perfused with medium from a stirred tank fermenter containing 1.25 L of medium, which was replaced continuously using a bleed and feed system. Media flow rates through the bioreactor and the bleed and feed rates for the 17 days of the reactor run are shown in Figure 1.

A ³¹P MR spectrum obtained from the reactor on day 17 is shown in Figure 2. The spectrum shows prominent signals from the three phosphates in ATP, from inorganic phosphate (P_i), and from an MDP standard contained in a sealed capillary tube in the fixed bed. The chemical shift or frequency of the P_i peak, which was due predominantly to extracellular phosphate, can be used to estimate pH in the reactor (Gillies et al., 1991). The pH in the reactor on day 17 was 7.02. At various times during the reactor run the extracellular P_i peak showed an upfield shoulder, which was assigned to intracellular P_i (see Figure 2). The chemical shift of this resonance in the spectrum obtained on day 17 indicated an intracellular pH of 6.84. The appearance of this intracellular P_i peak from under the extracellular resonance indicated that there had been intracellular acidification, possibly as a result of lactate production due to inadequate oxygen

Figure 1. Media flow rate through the bioreactor (—) and media feed/bleed rate (· · ·).

delivery. For this reason media flow rates through the reactor were increased whenever this intracellular P_i peak was observed.

Figure 3 shows the increase with time of a water fraction within the reactor which has a low apparent diffusion coefficient (ADC). Also shown on this figure is the ATP content of the bioreactor determined using ^{31}P MR spectroscopy. The concentration of ATP was determined from the intensity of the β -phosphate resonance (see Figure 2). The water fraction with low ADC, which has been assigned previously to intracellular water (van Zijl et al., 1991), was determined using diffusion-weighted ^1H MR spectroscopy measurements on a region of the reactor which was similar to that interrogated in the ^{31}P MR experiment. The figure quoted for the percentage of intracellular water in the reactor is only approximate since its calculation depends on knowing the T_1 and T_2 MR relaxation times of intra- and extracellular water. These have not been determined and have been assumed to be

equal for intra- and extracellular water. However the increases in cell volume apparent from the water diffusion measurements showed good agreement with the increases in ATP content of the bioreactor. A plot of ATP content against reactor cell volume is shown in Figure 4. Linear regression analysis showed that there was a strong correlation between the two measurements ($r^2 = 0.97$). There was a small fraction of water in the bioreactor (0.5%) which had a low ADC prior to cell seeding. This was presumably water associated with the carrier matrix.

Figure 5 shows a conventional ^1H spin echo image and a diffusion-weighted image of water protons from the same transverse slice through the reactor. The position of the carriers can be clearly identified in the conventional image. The diffusion-weighted image shows water with a low ADC, i.e. intracellular water (van Zijl et al., 1991; Callies et al., 1994; Williams et al., 1997), giving a map of cell growth over the reactor. High signal intensity in this image corresponds

Figure 2. ^{31}P MR spectrum acquired 17 days after cell seeding. The spectrum was the sum of 2000 transients which were acquired into 8000 data points and Fourier transformed with 5 Hz exponential line broadening. Abbreviations: MDP – methylene diphosphonate; PME – phosphomonoesters; P_i – inorganic phosphate; PdE – phosphodiester; PCr – phosphocreatine; dPdE – diphosphodiester; α , β and γ phosphates of ATP.

to high cell density. The image shows that cell growth is heterogeneous and maximal at the periphery of the carriers.

The total amount of glucose consumed and lactate produced by the cells over the 17 day period of the culture is shown in Figure 6. These values were calculated from assays of the glucose and lactate concentrations in the feed and fermenter vessels and the medium feed and bleed rate. The lactate/glucose ratio was close to 1 for the first 6 days, after which it was reduced to approximately zero, as lactate production ceased. This decrease in the lactate/glucose ratio was preceded by a drop in the reactor glutamine concentration to undetectable levels by day 4 of the culture (data not shown). A similar decrease in the lactate/glucose ratio has been observed in glucose and glutamine limited fed batch

cultures of hybridoma cells (Ljunggren et al., 1994).

Estimation of the viable cell number throughout the experiment allowed calculation of the specific glucose uptake rate (Figure 7). The viable cell number was estimated from bioreactor ATP content, which we have already shown correlated with the cell volume measurements. The relationship between ATP content and cell number was determined by comparing the ATP signal intensity on day 17 with the total number of cells present. Trypsinisation of the carriers removed from the bioreactor on day 17 released a total of 8×10^8 cells, corresponding to a density of approximately 2.9×10^7 cells mL^{-1} over the 28 mL volume of the fixed bed. The specific glucose consumption rate estimated by this method declined in parallel with the medium glucose concen-

Figure 3. Cell volume, as a percentage of bioreactor volume (■), and bioreactor ATP concentration (▲, arbitrary units) during the 17 day bioreactor run.

tration. The glucose consumption rate at the end of the culture was approximately $0.5 \mu\text{mol}/\text{hour}/10^6$ cells, which is comparable with values of between of 0.6 – $0.2 \mu\text{mol}/\text{hour}/10^6$ cells determined for CHO-K1 cells growing in T-flasks (Neermann et al., 1996). However the values reported here may be overestimates due to incomplete extraction of the cells from the carriers at the end of the culture.

Discussion

Non-invasive magnetic resonance imaging and spectroscopy techniques have been used previously to investigate the growth and metabolism of mammalian cells in intensive bioreactor systems, in particular the hollow-fiber bioreactor (HFBR) (Gillies et al., 1991; Mancuso, 1990). We have shown previously that diffusion-weighted ^1H MRI can be used to monitor the distribution of CHO-K1 cells growing in an HFBR (Callies et al., 1994) and that ^{19}F MRI measurements

of dissolved oxygen distribution, using a perfluorocarbon probe molecule, can be used to investigate the relationship between the distribution of cells and dissolved oxygen (Williams et al., 1997). We have shown here that diffusion-weighted ^1H MR imaging and spectroscopy can be used to monitor the distribution and growth of CHO-K1 cells in a fixed-bed bioreactor composed of macroporous carriers. Cell growth was monitored by measuring the relative proportion of water in the bioreactor with a low ADC using diffusion-weighted ^1H MR spectroscopy. This water fraction with low ADC has been assigned previously to intracellular water (van Zijl et al., 1991; Callies et al., 1994). The validity of this method for assessing cell growth is supported by the good agreement between these measurements and the measurements of bioreactor ATP content using ^{31}P MRS. This also implies that there was negligible variation in the cellular ATP concentration during the culture. The good agreement between cell volume and ATP

Figure 4. Correlation between bioreactor ATP content and cell volume. The solid line was obtained by linear regression.

concentration also makes it unlikely that dead or dying cells contain a significant water fraction with low ADC. This conclusion was supported by experiments in which we induced large scale cell death, for example by reduction of medium flow rate or lowering of oxygen tension. In the cells that remained, following restoration of flow or oxygen delivery, the proportionality between reactor ATP content and cell volume was the same as that prior to the insult (data not shown).

On-line monitoring of cell mass in intensive bioreactors is an important requirement for optimisation of the bioprocess. This can be performed using direct methods, such as dielectric spectroscopy (Noll et al., 1996) and electrical conductivity measurements (Blute et al., 1988) or indirectly by measuring nutrient uptake rates. Estimates based on nutrient uptake rates, however, rely on the assumption that there is a constant specific uptake rate, which may not always be justified (Ljunggren et al., 1994; Neermann et al., 1996; Mancuso et al., 1990 and references cited therein). For example in a CHO cell line it has been shown

that the specific oxygen consumption rate can be reduced by 50% with no effect on cell growth rate (Lin et al., 1993) and in a number of cell lines glucose consumption rate has been shown to be influenced by glucose concentration (Ljunggren et al., 1994; Neermann et al., 1996, and references cited therein). The dissociation of nutrient uptake rate from cell mass is likely to be even greater in intensive reactor systems where there may be significant heterogeneity in nutrient concentrations (Chresand et al., 1988; Drury et al., 1988; Piret et al., 1990a; Piret et al., 1990b; Williams et al., 1997). By comparing bioreactor glucose consumption rate with cell mass, estimated from MR measurements of cell volume or ATP content, we have shown that the specific glucose consumption rate declines during the culture and that this correlates with the decline in media glucose concentration. Such a correlation has been observed previously in fed-batch cultures of hybridoma cells and in CHO-K1 cells cultured in T-flasks (Ljunggren et al., 1994, Neermann et al., 1996). In this previous study on

Figure 5. Conventional ^1H spin echo image acquired before cell seeding (A), and a diffusion weighted STEAM image (B) from a transverse slice through the reactor acquired after 16 days of cell growth. The media delivery tube and the small tube containing the MDP standard can be seen in the spin echo image.

CHO-K1 cells an approximately 70% decrease in specific glucose consumption rate was observed, which is comparable with the 75% decrease observed in this study. This result emphasises the problems associated with estimating cell mass from nutrient uptake rates and illustrates the utility of the MR methods described here for determining cell mass, in particular the diffusion-weighted ^1H MR measurements of cell volume. Cell mass has been estimated previously in a hollow-fiber bioreactor using ^{23}Na MR (Mancuso et al., 1990). As the intracellular sodium ion concentration is considerably lower than the extracellular, a decrease in the ^{23}Na MR signal was observed as cell number increased. The cell concentration estimated in this way showed good agreement with the cell mass estimated from ^{31}P MRS measurements of bioreactor ATP content and measurements of oxygen uptake rate. However this method relies on the measurement of a relatively small decrease in the ^{23}Na signal and therefore could be expected to be error prone at low cell densities. Gillies et al. (1991) estimated cell volume in an HFBR by measuring the intensities of the intra- and extracellular sodium signals following addition of a shift reagent and using the concentration of intracellular sodium determined in parallel bench experiments. The same group also determined intra-

cellular volume by using ^{31}P MR to measure the intra- and extracellular dimethyl methylphosphonate resonances following the addition of this probe molecule to the HFBR (Barry et al., 1993). The diffusion-weighted ^1H MRS technique described here has the advantage that it does not require the addition of exogenous probes and is expected to be more sensitive, particularly at low cell densities, as the technique monitors the increase in intensity of the intracellular water signal, that has been separated from the extracellular water signal. The small fraction of extracellular water in the reactor which has a low ADC and which is believed to be water associated with the carrier matrix, could compromise this measurement. However this is easily corrected for by measuring this volume fraction prior to cell seeding, provided that this fraction is not affected by subsequent cell attachment and growth. The relationship between bioreactor ATP content, the intracellular volume fraction of the bioreactor and the cell number was determined at the end of the culture by removing the carriers from the reactor and counting the number of cells present. The total number of cells was 8×10^8 and the density 2.9×10^7 cells per mL fixed bed volume. This is comparable to a density of 5×10^7 cells per mL found for hybridoma cells growing on similar carriers in a fixed bed reactor (Ong et

Figure 6. Total glucose consumed (■, mmoles) and total lactate produced (▲, mmoles) during the 17 day growth period.

al., 1994). Assuming a mean cell diameter of $12.8 \mu\text{m}$ (Seewoster et al., 1997), 8×10^8 cells corresponds to a total cell volume in the reactor of 0.87 mL or 3.1% of the fixed-bed volume. This, given our lack of knowledge regarding the size of the cells growing in the reactor, shows reasonable agreement with the total cell volume determined in the diffusion-weighted ^1H MR experiment. The volume estimated from cell number may also be an underestimate due to incomplete removal of the cells from the carriers for counting at the end of the experiment.

The measurements of cell distribution, using diffusion-weighted ^1H MRI, showed that cell density was greatest at the periphery of the carriers. This reflects presumably the limited diffusion of nutrients, in particular oxygen (see Williams et al., 1997) into the carriers. Estimates of the thickness of the cell layer at the edge of the carriers is compromised to a certain extent by the irregular arrangement of the carriers and the relatively low resolution of the image (2 mm slice

thickness). In similar experiments on an HFBR, where the fibres were oriented along the z axis (the image slice is perpendicular to this axis) we were able to estimate a cell layer thickness of approximately $200 \mu\text{m}$. This was very similar to that expected from the work of others (Williams et al., 1997). Image resolution in this experiment is limited by the sensitivity of signal detection. Images could be obtained from thinner slices, therefore, if the amount of signal detected were increased. This could be achieved by acquiring more transients per phase encode increment and by reducing the TE period, during which there is a T_2^* -dependent loss of signal intensity. By acquiring a series of these images during the 17 day culture we have been able to generate cine-images of cell growth in the reactor. This technique could thus give unique insights into the dynamics of cell growth in this and other types of intensive reactor.

MRI and MRS are useful techniques for investigating intensive bioreactor performance. Conversely,

Figure 7. Specific glucose consumption rate (■, $\mu\text{mole glucose hr}^{-1}$ per 10^6 cells) and glucose concentration in the medium (▲, mM).

intensive bioreactors are useful tools for MR studies of cell physiology and metabolism as they can provide the relatively high cell densities required for MRS studies *in vivo*. The fixed-bed bioreactor described here has significant advantages over other intensive bioreactors and cell immobilisation techniques which have been used previously in conjunction with MRS (Fernandez, 1996; Brindle, 1998). The reactor is simple to construct and can be sterilised by autoclaving. By comparison, HFBRs have a more complex structure and can be difficult to construct in the laboratory. The disadvantage of the reactor described here is that there are likely to be significant nutrient gradients across the carriers. However this is also a problem with HFBRs (see for example Williams et al., 1997). The reactor may be improved in this regard by the use of smaller carriers.

The spectrometer used in these studies has a relatively narrow bore (up to 40 mm for imaging experiments) and therefore cannot be used to study larger systems, such as those generally used for bioartificial liver devices. However high-field instruments are available which have much larger bore sizes and therefore the experiments described here are transferable to these larger bioreactor systems.

Conclusions

We have shown that diffusion-weighted ^1H MR can be used to monitor the growth of CHO-K1 cells in a fixed-bed bioreactor composed of macroporous carriers. The increase in total cell volume determined by this technique correlated with the increase in bioreactor ATP content determined by ^{31}P MRS. These

measurements, in conjunction with measurements of bioreactor glucose consumption, were used to estimate the specific glucose uptake rate. This declined throughout the culture in parallel with the glucose concentration in the medium. Diffusion-weighted ^1H MRI measurements showed that cell growth was maximal at the periphery of the macroporous carriers. This study has further demonstrated the utility of MR techniques for investigating the performance of intensive mammalian cell bioreactors.

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