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Optimization of monoclonal antibody production: combined effects of potassium acetate and perfusion in a stirred tank bioreactor

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Abstract

To increase the yield of monoclonal antibody in a hybridoma culture, it is important to optimize the combination of several factors including cell density, antibody productivity per cell, and the duration of the culture. Potassium acetate enhances the production of antibodies by cells but sometimes depresses cell density. The production of anti-(human B-type red blood cell surface antigen) antibody by Cp9B hybridoma was studied. In batch cultures, potassium acetate inhibited Cp9B cells growth and decreased the maximal cell density but the productivity of antibody per cell was increased. The balance of the two effects resulted in a slight decline of antibody production. In a stirred tank bioreactor, the inhibitory effect of potassium acetate on cell density was overcome by applying the perfusion technique with the attachment of a cell-recycling apparatus to the bioreactor. In such a reactor, potassium acetate at 1 g l^{-1} did not cause a decrease in the cell density, and the antibody concentration in the culture supernatant was increased from 28 μg ml^{-1} to 38 μg ml^{-1}. Potassium acetate also suppressed the consumption of glucose and the accumulation of lactate in batch cultures, but the glucose and lactate levels were kept stable by applying the perfusion technique in the stirred tank bioreactor.

Introduction

The continuous establishment of new hybridoma cell lines and the expanding applications of monoclonal antibodies have led to an increasing demand for large-scale production of antibodies. A number of advanced processes have been developed in the last decades. Nevertheless, the research and development of hybridoma cell technology remains to have great potential since all the techniques presently used give final antibody concentrations far inferior to those obtained from mouse ascetic fluid and furthermore, there is a constant demand for antibody products of better quality to satisfy specific needs. Some of the active research areas in this field include high density culture of hybridomas, serum-free and protein-free cultures for the production of monoclonal antibodies for *in vivo* applications, the improvement of cellular productivity, and the design of high performance equipment and processes.

Different cell growth kinetics and their respective relationship to monoclonal antibody production have been described. These include negative, positive, and non-growth associated production (Merten, 1988; Miller *et al*., 1988; Suzuki and Ollis, 1990; Gaertner and Dhurjati, 1993; Glacken *et al*., 1988). Studies on synchronous cultures showed the maximum antibody production took place during G1/S phase of the cell cycle (Al-Rubeai and Emery, 1990). Hence, a number of reagents which inhibit DNA synthesis, such as thymidine (Gentry *et al*., 1965; Al-Rubeai and Emery 1990; Al-Rubeai *et al*., 1992), hybroxyurea (Suzuki and Ollis, 1990) and sodium butyrate (Oh *et al*., 1993), or which inhibit synthesis of proteins other than antibodies, such as potassium acetate (Somenshein and Brawerman, 1976; Suzuki and Ollis, 1990; Xu *et al*., 1994), were used to slow the growth of cells and stimulate antibody synthesis. Hybridoma cultures could also be hyper-stimulated to produce higher immunoglobulin titres by suppressing cell growth and increasing cul-

ture longevity through adapting to media with higher osmolarity (Ozturk and Palsson 1991; Oh *et al*., 1993).

The present study further investigated the effects of potassium acetate on hybridoma cell growth and monoclonal antibody production in batch and perfusion cultures. The influences of potasssium acetate on glucose consumption and lactate accumulation were monitored. It is hoped that by manipulating these conditions a higher antibody yield can be achieved.

Materials and methods

Cell line

The hybridoma Cp9B was obtained in our laboratory by the fusion of SP2/0 myeloma cell to the spleen cell of Balb/c mice immunized with human B-type blood cells. The hybridoma Cp9B cells secreted the monoclonal antibody of IgG_3 subtype against the surface antigens of human B-type blood cells.

Cell culture

In batch cultures, the Cp9B cells were grown in 100 ml flasks each containing 10 ml of cells suspended in RPMI 1640 medium (Gibco) supplemented with 10% new born calf serum (GIBCO), 2 g l^{-1} D-Glucose and 2 mM L-glutamine at 37 °C in a 5% CO_2 incubator. After sub-cultured for two passages in the presence of various concentrations of potassium acetate, 2-ml suspensions with a cell density of 2.5×10^4 cells ml^{-1} were inoculated into wells of 24-well plates (Nunc). Contents of quadruplicate wells were sampled daily. The samples were mixed before viable cell count was performed by the trypan blue exclusion method. Samples were centrifuged at approximately 400 g for 5 minutes and supernatants were stored in a –20 °C freezer for later use in the determination of glucose and lactate.

In the perfusion mode, cells were cultured in 2.5 l CelliGen bioreactor (New Brunswick Scientific) with a working volume of 2 l. The perfusion rate was 1 l per day (i.e. 1/2 working volume per day). A cell recycling apparatus was constructed with a 3-way glass tube (Figure 1). Driven by a peristalic pump, the recirculation rate was twice of the drainage rate. The effectiveness of the system was verified by the estimation that the densities of dead and viable cells in the harvested supernatant was 27% and 7% of that in the bioreactor respectively, indicating that although a small number of cells were lost, the majority of the lost cells were dead cells.

Analytical methods

Monoclonal antibody titers were determined in 1:1 serially diluted supernatants by their coagulating activities against human B-type blood cells. Human B-type blood cells were washed with saline repeatedly and made into 3% suspension. 0.2 ml of a diluted culture supernatant was mixed with an equal volume of the blood cell suspension and centrifuged at approximately 200 g for 1 minute. Antibody titer was the maximal dilution at which coagulation appeared.

Monoclonal antibody concentration in harvested supernatants was determined using indirect enzyme-linked immunosorbent assay (ELISA) test kits for mouse IgG antibody (Boehringer Mannheim). Samples were diluted 100, 500 or 1000 times according to their antibody titers.

The concentrations of D-glucose and L-lactate in the culture supernatants were determined by the UV-method with the respective kits ('Test-Combinations', Boehringer Mannheim). Before the assays, supernatant samples were diluted 10 times with double distilled water and placed in a 80 °C water bath for 15 minutes to stop intrinsic enzymatic reactions. Ammonia was assayed using the method of Fawcett and Scott (1960).

Results and Discussion

Effect of potassium acetate in batch culture

It was found in preliminary experiments that potassium acetate at concentrations above 2 g l^{-1} (or approximately 20.38 mM), strongly inhibited Cp9B cell growth. In some previous studies (Suzuki and Ollis, 1990), 10–100 mM of patassium acetate was added to cell cultures. Hence, we first studied the effects of different concentrations pf potassium acetate on Cp9B cell growth and antibody production.

Cp9B cells grew rapidly after inoculation and the maximal specific growth rate (specific growth rate μ is defined as

$$\mu = \frac{dN}{dT} \cdot \frac{1}{N}$$

where *N* is the cell number) was reached at day 2 and the maximal cell density was attained 2–3 days later. After day 4, cell viability and cell densities declined gradually (Figure 2). This time course was independent

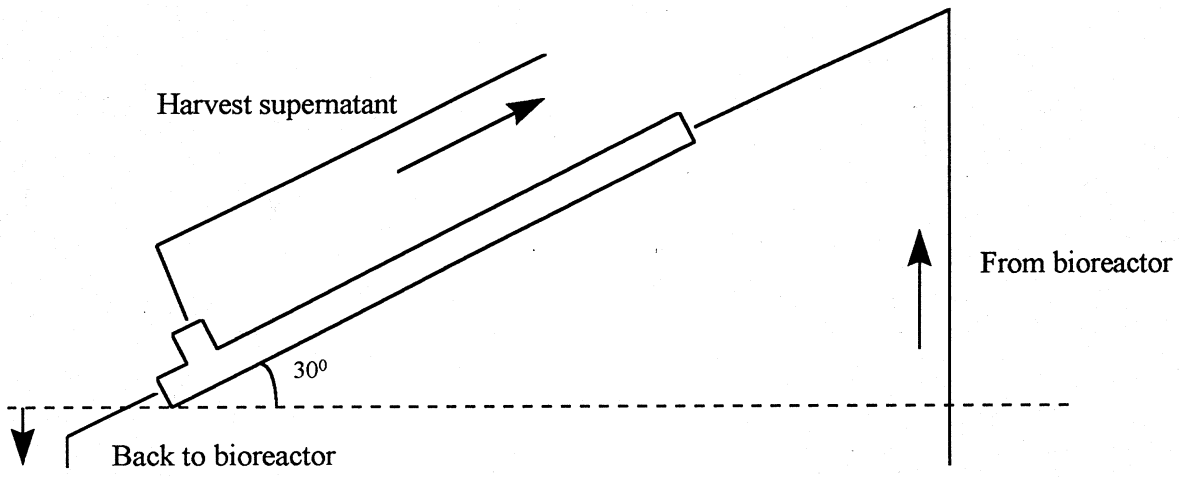

Figure 1. The cell recycling apparatus used in perfusion cultures was made of a 3-way glass flow settle tube.

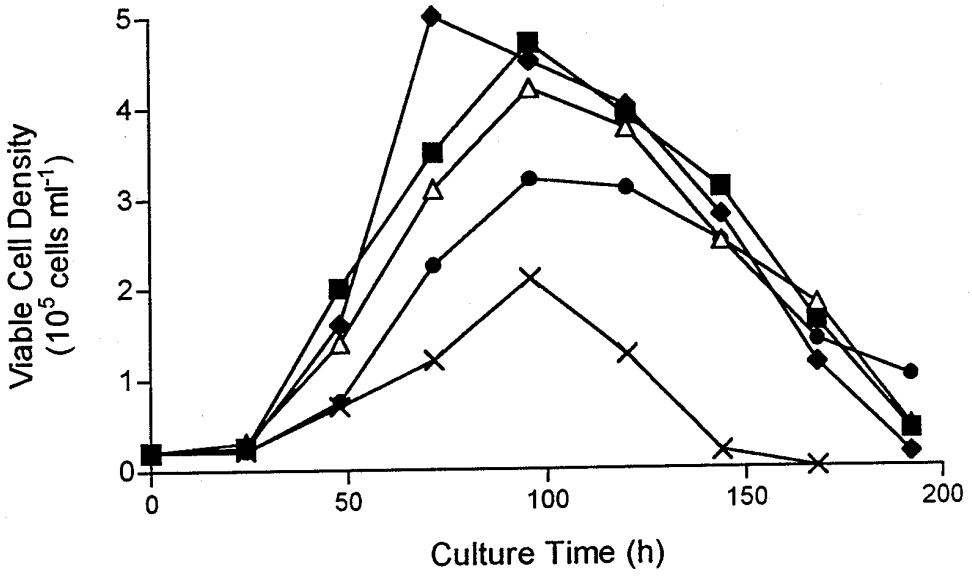

Figure 2. The growth profiles of Cp9B hybridoma cells in batch culture mode in the presence of various concentrations of potassium acetate. 0 (♦), 0.1 (■), 0,5 (△), 1 (●), 2 (×) gl $^{-1}$.

to changes in the concentration of potassium acetate. However the maximal specific growth rate and cell density decreased with the increase of potassium acetate at concentrations above 0.1 g l^{-1} (Figure 3). The maximal cell density at 2 g l^{-1} potassium acetate was less than half of that in the control.

Figure 4 shows the effects of different concentrations of potassium acetate on monoclonal antibody concentration and cell antibody productivity (or antibody productivity per cell) in batch hybridoma cultures. Cell antibody productivity is defined as

$$cell\ antibody\ productivity = \frac{(antibody\ concentration\ attained)}{(integral\ of\ cell\ density\ for\ the\ culture\ duration)}$$

Although high concentrations of potassium acetate slowed down cell growth, the total production of monoclonal antibody was less affected and the same super-

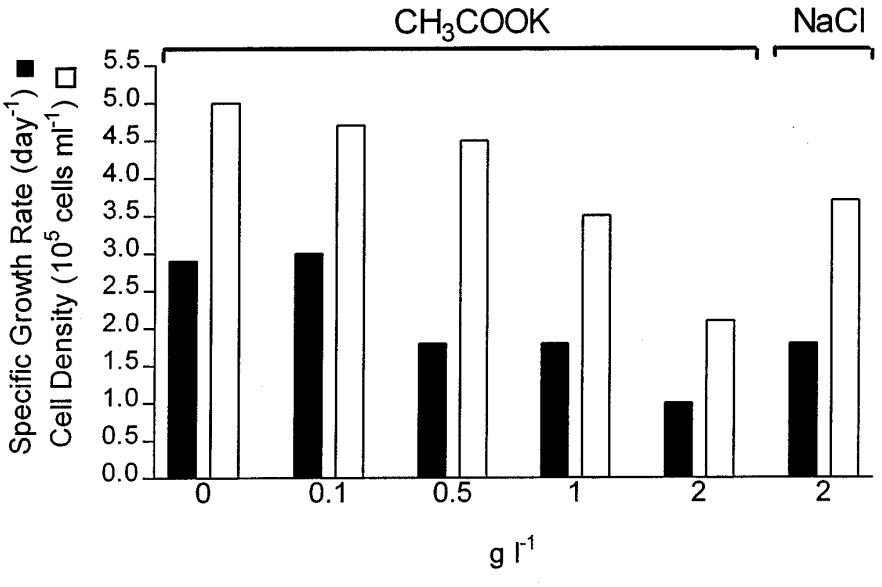

Figure 3. Effects of potassium acetate on the maximal specific growth rate (■) and cell density (□) achieved on day 7 of batch cultures of Cp9B hybridoma cells. Data shown were from one experiment typical of three separate experiments, data varied less than 10% amongst experiments.

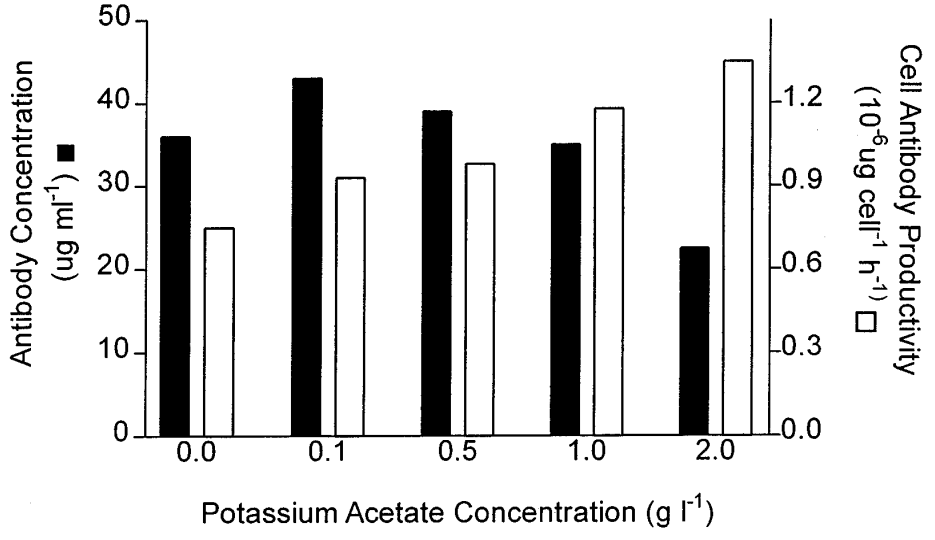

Figure 4. Effects of various concentrations of potassium acetate on the monoclonal antibody concentration (■) and calculated cell antibody productivity (□) in batch cultures of Cp9B hybridoma cells. Results were obtained from 7 days old cultures and cell antibody productivity was expressed as the antibody concentration at day 7 divided by the integral cell density for the 7 days culture period. Data shown were from one experiment typical of three separate experiments and they varied less than 10% amongst experiments.

natant antibody titer of 1:64 was maintained from 120th hours onwards. As a result cell antibody productivity increased with the concentration of potassium acetate. In other words, antibody production on a per cell basis was increased.

These results are in agreement with those obtained by Suzuki and Ollis (1990) and Xu *et al.* (1994) who demonstrated that potassium acetate stimulated the formation of antibody proteins while inhibited cell growth. Three possible mechanisms explaining

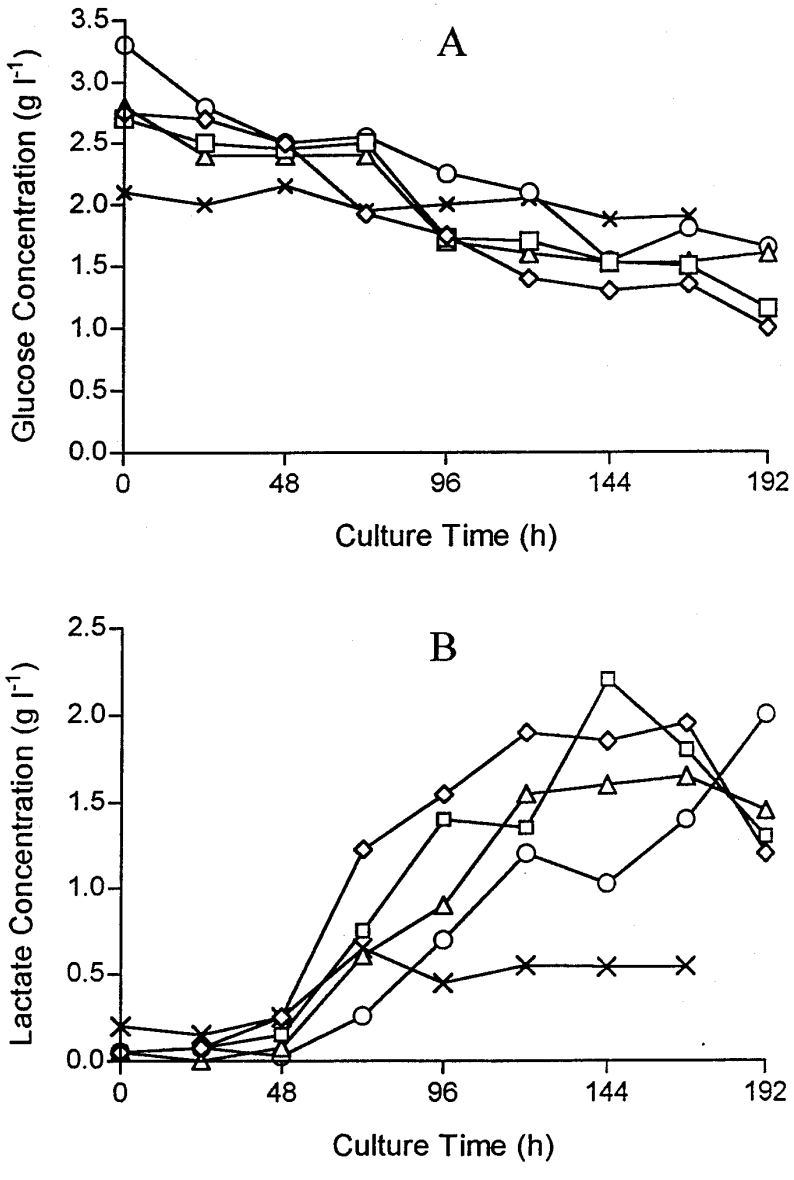

Figure 5. The glucose (panel A) and lactate (panel B) levels in batch cultures of Cp9B hybridoma cells in the presence of various concentrations of potassium acetate. 0 (◊), 0.1 (□), 0.5 (△), 1 (○), 2 (×) gl $^{-1}$.

this observation have been proposed. First, potassium acetate may inhibit the synthesis of proteins (Somenshein and Brawerman, 1976) which are essential for cell growth but not antibodies, and this may in turn favour immunolobulin synthesis by concentrating cellular energy and nutrients on the formation of antibody proteins. Or alternatively, slowed cell proliferation prolongs the duration of the cell cycle, especially the G1/S phases, during which antibody production and secretion are at maximal rates (Al-Rubeai and Emergy, 1990). A third possibility is that the increase of potassium ion may have some beneficial effects on the cells, such as the reduction of NH_4^+ uptake due to K^+ competition (Martinelle and Häggström, 1994) and it is also conceivable that changing the Na^+/K^+

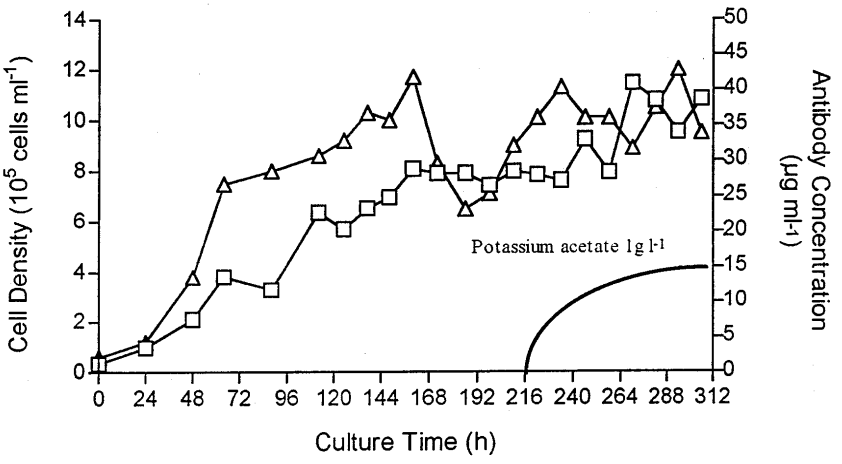

Figure 6. Cell density (△) and antibody concentration (□) in a stirred tank perfusion culture of Cp9B hybridoma cells. Potassium acetate was added to the infused medium at and after the 222nd hour.

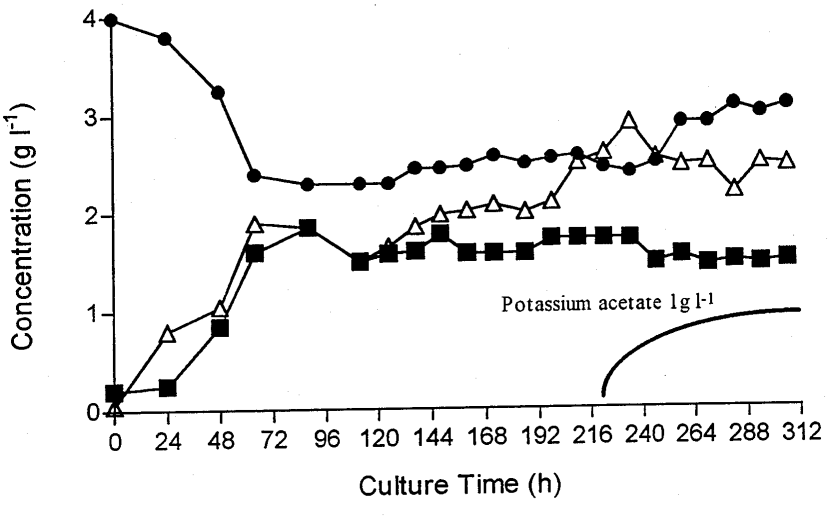

Figure 7. Levels of glucose (●), lactate (■) and ammonia (△) in a stirred tank perfusion culture of Cp9B hybridoma cells. Potassium acetate was added to the infused medium at and after the 222nd hour.

balance may have some complex effects on cellular metabolism.

It was not the purpose of this paper to fully addressed the question of effects of osmolarity changes on cell growth and antibody production. However data presented in Figure 3 would suggest that the cell growth inhibitory effect of 2 g l^{-1} (20.38 mM) of potassium acetate was higher than 2 g l^{-1} (34.22 mM) of NaCl. This would imply that osmolarity, within the range studied, was not the major factor as far as cell growth inhibition was concerned. Antibody production was not measured in the presence of 2 g l^{-1} of NaCl and this aspect needs further investigation.

Potassium acetate at 2 g l^{-1} suppressed the utilization of D-glucose and the accumulation of L-lactate (Figure 5). Due to different sizes of the innocula, varied initial glucose concentrations for different potassium acetate treatments were studied. Nevertheless since glucose was not depleted in any of the cultures, the effects of 2 g l^{-1} of potassium were apparant. The accumulation of ammonia, usually in the 2–3 mM range, was less affected by potassium acetate over a wide range of concentrations (data not presented),

which implied a lesser effect of potassium acetate on amino acid metabolism as compared to that on glucose metabolism.

Effect of potassium acetate in stirred tank perfusion culture

By the attachment of a cell recycling apparatus in the perfusion mode, it is possible to attain a higher cell density since viable cells are retained during medium recirculation and new nutrients are continuously supplied by infusion. A process in which a CelliGen stirred tank bioreactor operated in the perfusion mode was developed and antibody production was studied in the presence of 1 g l^{-1} potassium acetate (Figure 6).

The culture was started with an inoculum density of 6 $\times$ 10^4 cells ml^{-1} without the addition of potassium acetate. At the 65th hour, a cell density of 7.5 $\times$ 10^5 cells ml^{-1} was attained and perfusion with the same medium at a rate of 11 (approximately half of the bioreactor volume) per day was started. After the 222nd hour, perfusion of a medium containing 1.5 g l^{-1} potassium acetate was started and continued at the same rate as above for the subsequent 48 h. At the end of this period, the calculated concentration of potassium acetate in the bioreactor was 0.95 g l^{-1}. Thereafter, the concentration of potassium acetate in the perfusion medium was changed to 1 g l^{-1}.

As can be seen in Figure 6, the first 144 h was the active growth phase of the perfusion culture. After this period the culture entered into a stationary phase during which the cell density fluctuated at about 1 $\times$ 10^6 cells ml^{-1} and the antibody concentration was maintained at 28 μg ml^{-1}. This cell density was 2–3 times of that achieved in 24-well plate culture. The regular fluctuation of cell density in perfusion culture is an interesting and common observation (Vits and Hu, 1992) beyond the scope of this investigation. After the addition of potassium acetate, cell density was maintained or even slightly increased demonstrating the beneficial effect of the perfusion culture mode. More importantly the production of monoclonal antibody was improved. The antibody titre rose from 1:32 to 1:64 with the addition of potassium acetate and the overall yield of the antibody was improved from 28 μg ml^{-1} to 38 μg ml^{-1}.

Different from the batch culture mode, in the perfusion culture the addition of potassium acetate had little effect on glucose, lactate and ammonia levels despite the maintenance or even a small increase in the cell density (Figure 7).

In summary, the benefit of the addition of potassium acetate to hybridoma culture was potentiated by the perfusion culture technique which enabled the attainment of a high cell density in the presence of potassium acetate and in addition stabilized culture conditions. These ensured a higher yield of the monoclonal antibody over a prolonged culture duration.

Acknowledgement

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