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Effects of Different Osmotic Pressure of the Cultivation Media on Hybridoma Cell Growth and Monoclonal Antibodies Production Kinetics in Batch Culture

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Abstract: The effects of osmotic pressure on the cell growth and Monoclonal antibody (MAb) production by hybridoma cells in static and agitated cultures were investigated. The preliminary studies in static cultures showed that the maximal total cell number reaching about 11.1×10^5 cells mL^{-1} was obtained in cultures of 375 mOsmol kg^{-1}, while the maximal MAb production of 61 mg L^{-1} was obtained in cultures of 400 mOsmol kg^{-1}. On transferring the cultivation process to agitated cultures in spinner flasks at high shear of 150 rpm, the maximal cell number was reduced to 8.4×10^5 cells mL^{-1}. This was obtained in medium of osmotic pressure of 325 mOsmol kg^{-1}. Further increase in medium osmolarity resulted in significant decrease in total cell number. On the other hand, the cultivation in spinner flask gave higher yield of MAb than static culture. The maximal concentration of MAb of 135 mg L^{-1} was obtained in medium of 375 mOsmol kg^{-1}. Based on these data, an experiment was designed to increase the volumetric MAb production by shifting the medium osmolarity from 325 mOsmol kg^{-1} to 375 mOsmol kg^{-1} during the late exponential growth phase of cells. This resulted to an increase in MAb volumetric production reaching 165 mg L^{-1}. This value is about 68% higher than those obtained in culture of constant osmolarity of 325 mOsmol kg^{-1}. This value is also about 22% higher than the maximal volumetric MAb production obtained in culture of 375 mOsmol kg^{-1} adjusted from the beginning of cultivation time.

Key words: Antibody production, osmotic stress, hybridoma cells

INTRODUCTION

Nowadays, Monoclonal antibodies (MAbs) have many potential applications in different fields such as diagnostic assays, biosensors, catalysis, downstream processing, therapeutics and may other areas of biotechnological interest (Funaro *et al*., 2000; Micheel, 2003; Kokai-Kun and Mond, 2004; Stern and Herrmann, 2005; Boccardo *et al*., 2006). By the mid of 2005, there were 18 therapeutic monoclonal antibodies products on the US market. Worldwide, there were an estimated 500 MAb products in development by more than 200 companies for the treatment of virtually every debilitating disease. Therefore, the worldwide market for therapeutic and diagnostic antibodies reached about $15 billion in 2005 and will rise at an average annual growth rate of 11.5% to nearly 26 billion in 2010 (Dynamic antibody industry, 2005). This resulted in an increasing demand for the improvement of MAbs *in vitro* production techniques. The *in vitro* production processes for MAbs are ranged from small scale micro well plates upto bioreactors of more than 10 m^3 volume. Therefore, the influence of medium composition and cultivation conditions on MAbs was matter of interest for many scientists and industries as well Among different abiotic factors influencing *in vitro* cell cultivation, medium osmolarity is one of the most critical factors in many studies (Chua *et al*., 1994; Oh *et al*., 1995; deZengotita *et al*., 1998; 2002.

Under different abiotic stress conditions, cell growth and metabolic activities are highly influenced in all types of living organisms. Medium osmolality is usually one of those factors affecting different types of biological systems in different ways. In general, mammalian cells are more sensitive to osmotic stress than other types of lower eurkaryotes and prokaryotes because of the absence of cell wall and the less osmotic stress regulation mechanism and the inability for rest cell production (Kültz and

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Chakravarty, 2001). However, even in the same organ of higher eukaryotes the degree of osmoregulation mechanism is highly variable and dependent on the type of cells (Lee *et al*., 2002). Most cell culture media are designed to have an osmolarity of 280 to 330 mOsmol kg^{-1} through balancing the concentration of nutrients and osmolytes (Freshney, 1994). However, the results of previous studies concerning the effect of osmotic stress on hybridoma cells can be summarized in four main points. First, the stimulatory or inhibitory effect of osmotic stress on monoclonal antibody production is cell line specific (Reddy and Miller, 1994; Lee and Park, 1995). Second, for most hybridomas, hyperosmotic pressure induced by either sugar or ionized salt suppresses cell growth but it increases the specific antibody secretion rate (Oh *et al*., 1993; Øyaas *et al*., 1994; Oh *et al*., 1995). Third, the enhanced specific antibody secretion rate resulting from hyperosmotic pressure is not transient and is maintained in successive passages (Park and Lee, 1995). Fourth, hypo-osmotic pressure also suppresses cell growth, but it does not enhance specific antibody production rate (Ryu and Lee, 1997). However, the major component in maintaining osmotic pressure in cell culture media is sodium chloride. Other inorganic ions (such as potassium, magnesium and calcium) and glucose also contribute significantly. On the other hand, larger molecules like proteins play a lesser role and changes in their concentrations do not affect osmolarity significantly. In the present work we represent results demonstrating the significant influence of osmotic stress as well as osmotic stress shift on the kinetics of hybridoma cell growth/death as well as MAb production in batch cultures.

MATERIALS AND METHODS

Cell line and medium: The hybridoma cell line HD-24 was used in this study. This cell line produces IgG2b monoclonal antibodies. Cells were maintained at 37°C in medium consisting of DMEM (Gibco, USA) supplemented with 10% (v/v) FBS (Sigma, USA). Cells were maintained in 75 ml T-flask and were removed from the mid exponential phase of the culture for use in experimental work.

Cell cultivation: For 6-well plate experiment, cells were cultivated at the initial concentration of 1×10^5 cells mL^{-1} in 6 well plates using DMEM medium of different osmotic pressures from 325 to 500 (mOsm kg^{-1}). Medium osmolarity was adjusted by additions of 10% NaCl solution. Cells were cultivated at 37°C and 5% CO_2. In case of spinner flask cultures, A 1 L spinner flask containing 200 mL of medium was used, agitated at 150 rpm by magnetic stirrer and maintained in an incubator at 37°C in an atmosphere of 5% CO_2 and 95% relative humidity. The culture was inoculated with 1×10^5 cells mL^{-1}.

Analytical: In case of 6-well plate cultures, 0.2 mL samples were taken at different time intervals. In case of spinner flasks, samples were obtained from the side arm of the spinner flasks. The spinner flasks were shaken well before the sample was taken in order to ensure high homogeneity in the cultivation vessel before sampling. The samples were taken within 5 sec from the moment the shaking of the spinner flasks was stopped to ensure that a representative sample of the homogeneous mixture was taken. Cell concentration and percentage viability were determined by counting live and dead cells (approximately 1000 cells per sample in total) in a haemocytometer using an inverted microscope after staining with trypan blue. Medium osmolarity was determined by the measurement of the depression of the freezing point using an osmometer (Automatic semi-micro osmometer, model A0300, Knauer, Berlin, Germany). Glucose and lactate concentrations were measured using a glucose/lactate analyzer (Biochemistry analyzer 2700, Yellow Springs Instruments, OH, USA). Monoclonal antibody was determined by sandwich Enzyme-Linked Immunosorbent Assay (ELISA) using Goat anti-Rat IgG (H+L) (KPL, Maryland, USA) coated plates as first antibody and Goat anti-Rat IgG phospahatase labelled (KPL, Maryland, USA) as second antibody. The developed colour was measured at 405nm using an ELISA reader (Spectra Max 250 and software Softmax Pro) after treating the plates with pNPP substrate solution.

RESULTS AND DISCUSSION

Batch cultivation of hybridoma cells under different medium osmolarity in 6-well plate: Cells were inoculated at a cell concentration of around 1×10^5 cells mL^{-1} in 6-well plate in media of different osmotic pressure ranged from 325 up to 500 mOsmol kg^{-1}. Media were prepared by addition of a 0.1 g mL^{-1} sodium chloride solution to DMEM medium supplemented with 10% FBS. The cultures were sampled at different times intervals of 96 h, 144 and 240 h for determination of viable and non-viable cells, glucose and lactate concentrations. Experiments were done in triplicate and the data in Table 1 demonstrates the results of this experiment. As shown, the number of living cells increased with time up to 240 h in all cultures of medium osmolarity ranged from 320 to 375 mOsmol kg^{-1}. In all other cultures of 400 and 425 mOsmol kg^{-1}, the number of living cells decreased after 144 h and the final cell number was in all cases more than

Table 1: Effect of medium osmolarity on cell growth, viability and MAb production after 240 h cultivation in 6-well plate in DMEM medium

	Medium osmolarity (mOsmol kg^{-1})							
Parameters	325	350	375	400	425	450	475	500
Living cells ($\times10^5$ cells mL^{-1})	8.1	8.3	7.9	6.3	5.0	3.2	2.1	1.2
Dead cells ($\times10^5$ cells mL^{-1})	1.2	2.5	3.2	4.0	4.8	5.4	5.1	4.9
Total cells ($\times10^5$ cells mL^{-1})	9.3	10.8	11.1	10.3	9.8	8.6	7.2	6.1
MAb (mg L^{-1})	44	53	59	61	50	32	16	13

those obtained after 96 h. However, the number of living cells in culture of medium osmolarity of 450 mOsmol kg^{-1} or higher increased with time up to 144 h and then decreased significantly and reached very low values even less those observed after 96 h. On the other hand, the number of dead cells increased gradually with time in all cultures under study. The maximal cell concentration of 11.1×10^5 cells mL^{-1} was obtained in medium of 375 mOsmol kg^{-1} and decreased gradually with the increase in medium osmolarity. However, the changed in osmotic pressure of medium showed also significant effect on MAb production. The volumetric MAb production increased gradually with time up to 240 h in medium osmolarity between 325 and 425 mOsmol kg^{-1}. In other medium of osmolarity of 450 mOsmol kg^{-1} or higher, the maximal MAb concentration was obtained after 144 h and kept more or less constant for the rest of cultivation time. The maximal volumetric MAb production of about 61 mg L^{-1} was obtained after 240 h cultivation using medium of 400 mOsmol kg^{-1}. However, these preliminary experiments on multi-well plate, shows the significant effect of medium osmolarity on the cell growth and MAb production as observed by other authors using different types of hybridoma cells (Ozturk and Palsson, 1991; Lee and Park, 1995; Wu *et al*., 2004). It have been also observed by Bibila *et al.* (1994) that the increase of medium osmolarity from 300 up to 400 mOsmol kg^{-1}, by addition of sodium chloride, resulted in significant increase in MAb production. Other study of Franco *et al.* (1999) shows also significant increase in MAb production by about 30% by increasing medium osmolarity up to 400 mOsmol kg^{-1}. The increased MAb production of hybridoma cells under osmotic stress was due to an increase in gene expression which result in high protein transcription rate and general metabolic flux change as observed recently by many authors (Lin *et al*., 1999a; Sun *et al*., 2003; Wu *et al*., 2004; Shen and Sharfstein, 2005). Thus, based on our previous results, the influence of osmotic pressure on the kinetics of cell growth, metabolism and MAb production were further studied in spinner flasks. The suspension culture mode is considered to be more suitable for studying the effect of osmotic pressure on the performance of hybridoma cell from the viewpoint of bioengineering and process scalability.

Cultivation of hybridoma cells in spinner flasks at different osmotic pressures: Based on the results of the influence of osmotic pressure on cell growth kinetics and MAb production in static culture (6-well plates), cultivations were conducted in spinner flask. The agitated flask, spinner flask studies, is usually an important step for scaling up of MAb production process which is transition step between small scale and bioreactor level. Therefore, several cultivations in media of different osmolarity ranged from 325 up to 425 mOsmol kg^{-1} were carried out under high shear (150 rpm). The growth curves of these cultivations are represented in Fig. 1-5. Beside these curves, the kinetic data for cell growth, glucose, consumption, lactate production and MAb production are summarized in Table 2 for comparison. In general, the osmotic pressure had significant influence on cell growth. Cells grew exponentially in all cultures during the first 84 h of cultivations and the maximal total cell concentration of 8.4×10^5 cells mL^{-1} was obtained in culture medium of 325 mOsmol kg^{-1}. Further increase in medium osmolarity resulted in slightly decrease in total

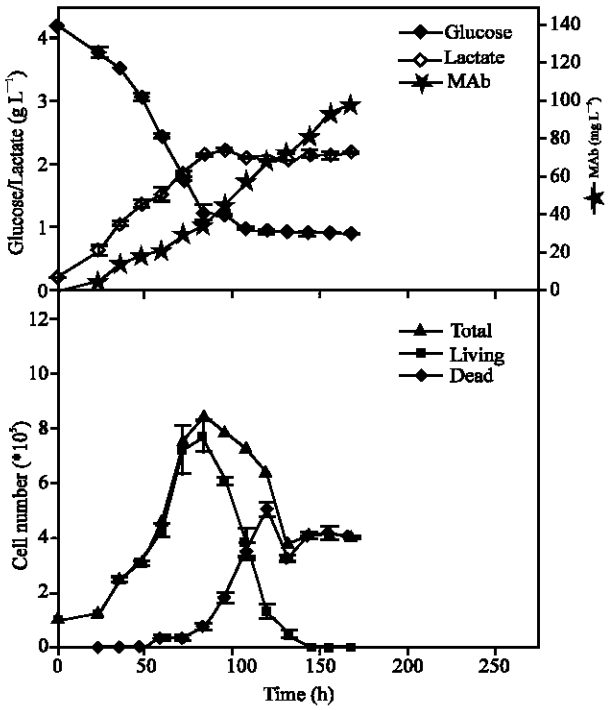

Fig. 1: Hybridoma cell growth and monoclonal antibodies production during cell cultivation under 325 mOsmol kg^{-1} osmotic stress culture

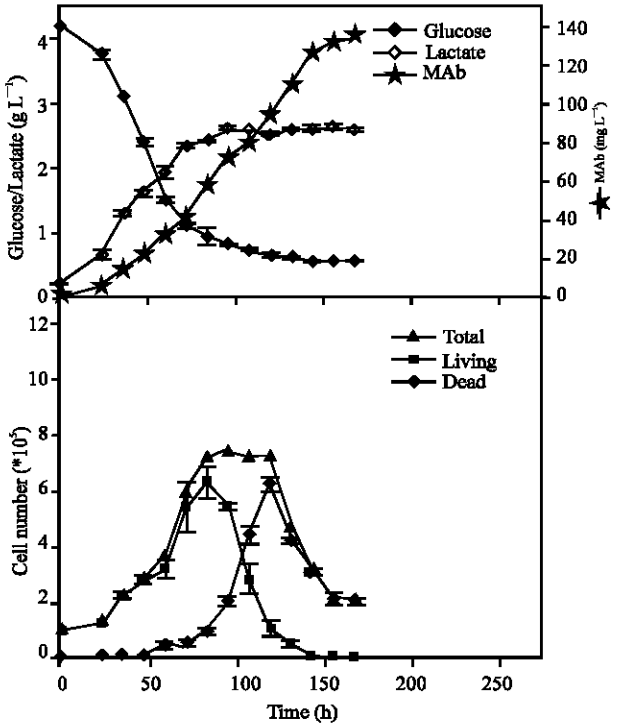

Fig. 2: Hybridoma cell growth and monoclonal antibodies production during cell cultivation under 375 mOsmol kg^{-1} osmotic stress culture

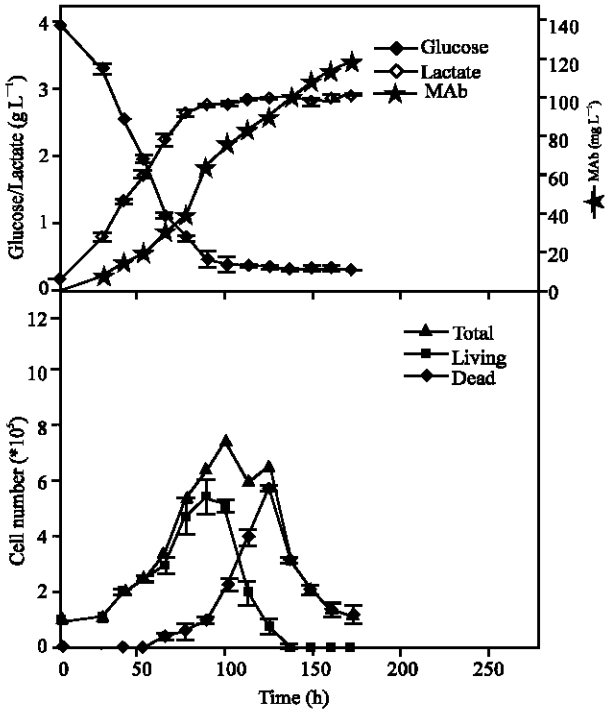

Fig. 3: Hybridoma cell growth and monoclonal antibodies production during cell cultivation under 400 mOsmol kg^{-1} osmotic stress culture

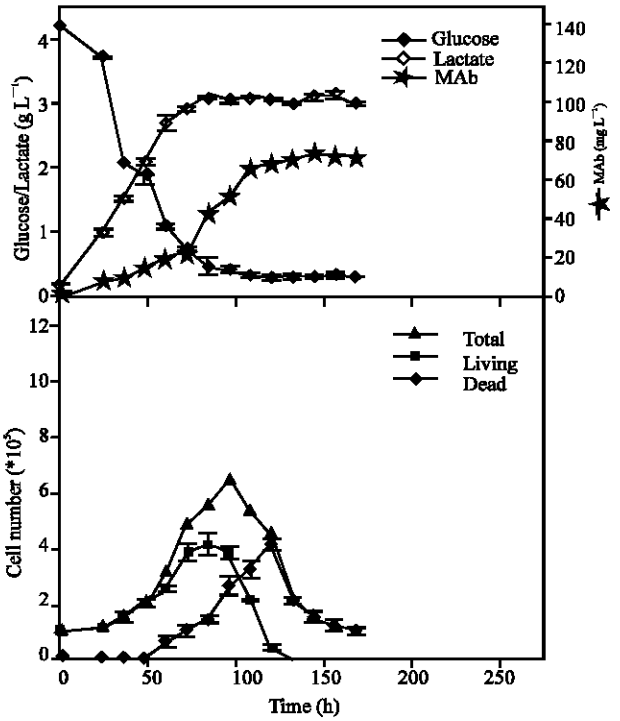

Fig. 4: Hybridoma cell growth and monoclonal antibodies production during cell cultivation under 425 mOsmol kg^{-1} osmotic stress culture

cell number concomitant with more significant decrease in cell viability. On the other hand, the volumetric rate of glucose consumption ($-Q_{glu}$) as well as the volumetric rate of lactate production (Q_{lac}) in all culture was found to be directly proportional to the medium osmolarity. Moreover, the final residual glucose in cultures was higher at low osmotic pressure. However, in all osmotic pressures under study glucose was not completely consumed until the end of cultivation time. On the other hand, the final volumetric lactate produced was directly proportional with the increase in medium osmolarity. Beside all these differences in cell growth and cellular metabolism, MAb production was also highly influenced by the medium osmolarity by different manner. By increasing the initial medium osmolarity from 325 mOsmol kg^{-1} up to 375 mOsmol kg^{-1}, the maximal volumetric MAb production increased by about 38%. Further increase in cell osmolarity resulted in significant decrease in both final concentration of MAb produced as well as the MAb production rate. Its also worthy to note that, for all applied medium of osmolarity between 325 mOsmol kg^{-1} and 400 mOsmol kg^{-1} the MAb concentration increased gradually with time reaching its maximal concentration at the end of cultivation time (168 h). In case of 425 mOsmol kg^{-1}, the MAb production terminated after 132 h and reached its maximal value of 70 mg L^{-1}.

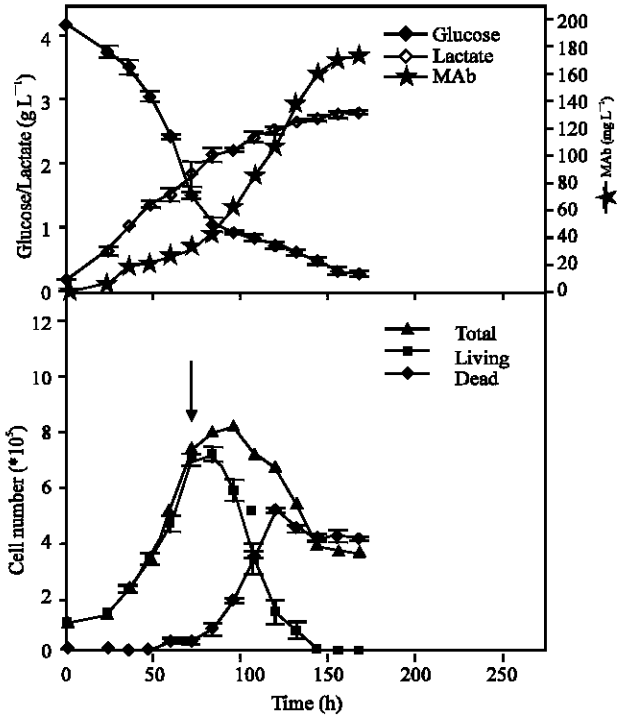

Fig. 5: Hybridoma cell growth and monoclonal antibodies production during cell cultivation in spinner flask with an osmotic shift from 325 mOsmol kg^{-1} to 375 mOsmol kg^{-1}. Arrow shows the time of osmotic shift in culture medium

Table 2: Effect of medium osmolarity on the kinetics of cell growth, substrate consumption and MAb during the cultivation of hybridoma cells in spinner flask

	Medium osmolarity (mOsmol kg^{-1})			
Parameters	325	375	400	425
$X_{max(v)}$ ($\times 10^5$ cells mL^{-1})	7.66	6.3	5.5	4.1
$X_{max(t)}$ ($\times 10^5$ cells mL^{-1})	8.4	7.4	7.5	6.4
$t(X_{vmax})$ (h)	84	84	84	84
$t(X_{tmax})$ (h)	84	96	96	96
$-Q_{glu}$ (mg L^{-1} h^{-1})	35.4	39.0	43.8	44.4
Q_{lac} (mg L^{-1} h^{-1})	23.3	26.4	32.7	35.2
P_{MAb} (mg L^{-1})	98	135	121	70
$t(P_{max})$ (h)	168	168	168	132
Q_{MAb} (mg L^{-1} h^{-1})	0.583	0.882	0.764	0.575

The antibody production depends obviously on cell viability and any competition with maintenance requirements for the limiting nutrients. The increased antibody production in hyperosmotic culture by hybridoma cells was studied recently by Sun *et al.* (2004) in protein translation and transcription level. They observed that the changed in immunoglobulin transcription played a minor role in the increase in antibody (Ab) production in response to osmotic stress. On the other hand, hyper osmotic stress altered the cell cycle distribution, increasing the fraction of the cells in S-phase. The Ab secretion rates increased approximately 50% in response to osmotic stress, with a commensurate increase in the antibody assembly rate. They observed also that the main two factors dominate the increase in Ab production in response to hyperosmotic stress are the increase in total protein translation concomitant with the increase in cell size. The correlation between the decrease in cell growth and MAb production was found as result of cell cycle control. The increased MAb synthesis in the G_1 phase of the cell cycle has been proposed to explain the enhanced productivity of slow or growth arrested cell population based on cell cycle control (Hayter *et al.*, 1992; Al-Rubeai *et al.*, 1992). This supports also our result of higher MAb produced at reduced cell growth as function of increase in medium osmolarity.

On the other hand, the glucose consumption rates and lactate production rates are generally good indicator for cell health. It have been reported that the characteristic feature of the early phase of the response of mammalian cells to stresses such as shock, hypoxia, metabolic poisons or exposure to osmotic stress is an increase in the rat of cellular glucose uptake (Baldwin *et al.*, 1997; Barros *et al.*, 2001). The increase in nutrient transport through cell membrane as function of osmotic stress is dependent on cell line. The increased glucose uptake is an adaptive response, allowing the cells to maintain their normal ATP levels when energy demand increases or oxidative phosphorylation is impaired and thus promotes the cell survival. In general, stress induced increases in glucose uptake originated from an increase in the rate of glucose transport across the cell membranes, which is rate limiting step for glucose metabolism in most mammalian cell types (Barros *et al.*, 1995). Moreover, in some cases, the glucose transport rate is not influence the cell metabolism and cell productivity, it was also reported that apoptosis is highly regulated by the rate of glucose transport (Kan *et al.*, 1994). In our study, the increase in the rate of glucose consumption was reflected directly on lactate production rate in all culture under study. The proportional strait line relation between these two rates was also found in other mammalian cells such as stem cell (El-Demellawy *et al.*, 2005). On the other hand, there was no direct relation between MAb production rate and glucose consumption rate in cultures of medium osmolarity more than 375 mOsmol kg^{-1}.

Effect of osmotic shift on kinetics of hybridoma cell growth and MAb production: In general, as shown in the previous experiments, cell growth was depressed at high osmotic pressure, while the MAb production rate (Q_{MAb}) was increased by the increase of medium osmotic pressure up to 400 mOsmol kg^{-1}. The maximal Mab

production rate and total volumetric production was obtained on using medium with osmolarity of 375 mOsmol kg^{-1}. Based on these data, a cultivation strategy was designed to cultivation cells at low osmolarity of 325 mOsmol kg^{-1}, the optimal value of cell growth, during the first 72 h of cultivation to increase the cell number and thereafter the medium osmotic pressure was shifted up to 375 mOsmol kg^{-1} which is optimal for MAb production. As shown in Fig. 5, cells grew exponentially during the first phase reaching about 7×10^5 cells mL^{-1} after 72 h with more than 90% viability. After this phase, the osmotic pressure was increased to 375 mOsmol kg^{-1} by the addition of 10% sodium chloride solution. The increase in medium osmolarity resulted in significant increase in both glucose consumption and lactate production, compared to controlled culture without osmotic pressure shift (Fig. 1). On the other hand, the volumetric MAb production increased significantly and reached about 165 mg L^{-1}. This value is about 68% higher than the production obtained in culture of constant osmolarity of 325 mOsmol kg^{-1}. This value is also about 22% higher than the maximal volumetric MAb production obtained in culture of 375 mOsmol kg^{-1}, adjusted from the beginning of cultivation time.

However, these results are in agreements with many authors studied the influence of osmotic pressure shift on the production of MAb by hybridoma cells. Lin *et al.* (1999b) studied the influence of gradual increase of medium osmotic pressure from 300 mOsmol kg^{-1} to 400 mOsmol kg^{-1} using different profile during batch cultivation. They found that a maximal increase in MAb production of about 55.9 mg L^{-1} was obtained on one step increase of osmotic pressure of medium from 300 up to 360 and further increased up to 400 after just only 6 h. This strategy gave an increase of about 7.5% in MAb production compared to the optimal constant osmotic pressure for MAb production. From different profile studied, they conclude that the rapid increase in osmotic pressure shift is the most suitable pattern for MAb production. Other study of Takagi *et al.* (2001) who investigated the effect of changes in osmotic pressure on the production of tissue plasminogen activator (tPA) by Chinese Hamster Ovary (CHO) cells in suspension culture showed also the significant influence of osmotic pressure on tPA production. Shifting the osmotic pressure cyclically between 300 and 500 mOsmol kg^{-1} during the production phase improved the tPA production 1.13-fold compared with the amount produced at a constant osmotic pressure of 300 mOsmol kg^{-1}.

Thus, using two levels of osmolarity during cultivation, (low level to promote cell growth in high concentration) and shift it to higher value during the late exponential growth phase (which promote MAb production), is good alternative cultivation strategy to increase the MAb production industrially with cheap technique compared to the conventional improvement of MAb production by change of osmotic pressure at the beginning of cultivation time.

CONCLUSIONS

It can be conclude that, increasing the osmotic pressure of culture medium by simple addition of cheap salts to culture media is suggested as being an economical solution to increase MAb production in hybridoma cell cultures. Moreover, using medium of low osmotic pressure (which is optimal for cell growth) and shift it to higher value (which is preferable for MAb production) at the end of exponential growth phase, increased the volumetric production rate significantly and could be used to increase the yield of MAb significantly. This gave also better MAb yield compared to cultures of constant osmotic pressure during the cultivation process.

ABBREVIATIONS

$X_{max(v)}$: Maximal viable cell density;
$X_{max(t)}$: Maximal total cell density;
$t(X_{vmax})$: Culture time at maximum viable cell density;
μ : Specific growth rate;
$-Q_{glu}$: Volumetric glucose consumption rate;
Q_{lac} : Volumetric lactose production rate;
P_{MAb} : Maximal volumetric MAb produced;
$t(P_{max})$: Culture time at maximal volumetric MAb production;
Q_{MAb} : Volumetric MAb production rate.

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