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Enhanced monoclonal antibody production by gradual increase of osmotic pressure

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Abstract

The time length required for the adaptation of AFP-27 hybridoma cells to high osmotic pressure and the effect of a gradual increase of osmotic pressure on monoclonal antibody production were investigated. When the cells were subjected to an increase of osmotic pressure from 300 mOsmol kg^{-1} to 366 mOsmol kg^{-1}, the intracellular content of osmoprotective free amino acids reached a maximum level 6 h after the osmotic pressure was increased to 366 mOsmol kg^{-1}. The same time period of 6 h incubation at 366 mOsmol kg^{-1} was required to obtain a high growth rate of AFP-27 cells at 440 mOsmol kg^{-1} when the cells were subjected to a two-step increase of osmotic pressure from 300 mOsmol kg^{-1} to 366 mOsmol kg^{-1} and then to 440 mOsmol kg^{-1}. The time length for the physiological adaptation of the cells to 366 mOsmol kg^{-1} was consequently estimated to be 6 h. Osmotic pressure during batch cultivation was gradually increased from 300 mOsmol kg^{-1} to 400 mOsmol kg^{-1} with an adaptation time of at least 6 h. The specific growth rates following a gradual increase of osmotic pressure were higher than those at a constant osmotic pressure of 400 mOsmol kg^{-1}, while the specific monoclonal antibody production rate increased with the increase in the mean osmotic pressure. As a result, the cells grown under a gradual increase of osmotic pressure produced higher amounts of monoclonal antibodies than did those grown under constant osmotic pressure.

Introduction

Recently, the effectiveness of high osmotic pressure on the increase of specific monoclonal antibody production rate (q_{MAb}) has been confirmed (Reitzer et al., 1979; Berg et al., 1990; Reddy et al., 1992; Banik and Health., 1995; Lee and Park, 1995; Oh et al., 1993; Øyaas et al., 1994b). However, in many cases, the enhanced q_{MAb} did not lead to a higher final monoclonal antibody (MAb) titer because of depressed cell growth at high osmolarity (Ozturk and Palsson, 1991; Lee and Park, 1995). In general, MAbs can be produced at a high rate even after the cessation of cell growth during the batch cultivation of hybridoma cells. Consequently, MAb productivity may be increased by an increase of osmotic pressure in late growth phase during cultivation, so that the cells grow well at the physiologically appropriate osmotic pressure in the early culture phase and q_{MAb} is increased and maintained by the increase of osmotic pressure in the later phase.

In the cultivation of hybridoma cells, the rate of increase of osmotic pressure may be limited in accordance with the physiological state of cells. When cells are subjected to an increase of osmotic pressure, at the first stage, the rapid passive efflux of water from the cells results in cell shrinkage. These events may terminate cell growth unless the cells have some means to counterbalance the osmotic gradient. The mechanism of growth inhibition by osmotic stress was assumed to be that high concentrations of potassium inhibit the activity of many enzymes (Yancey et al., 1982).

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Simultaneously, an energy-dependent osmoregulation system might function to accumulate organic solutes which counteract the toxic effects on the macromolecules inside the cells (Yancey et al., 1982). The accumulation of osmoprotective solutes inside the cells can counterbalance the perturbing solutes outside the cells to restore cell volume in later stage and to prevent any external perturbing solutes from entering into the cells. Ozturk and Palsson (1991) ever reported the cell size decreased at the beginning when the cells were first introduced to high osmolarities, but increased in the later stages of cultivation. We also observed the swelled cell volume of the viable cells some time later after the cells were introduced to high osmolarities in this experiment (data not shown). Besides, the osmoprotective solutes also have stabilizing effects on the structure and function of the macromolecules inside the cells (Yancey et al., 1982). There are three kinds of osmoprotective organic osmolytes: (1) polyhydric alcohols, (2) free amino acids and aminoe acid derivatives and (3) urea and methylamines (Yancey et al., 1982). By addition of different kinds of free amino acids to the stressed hybridoma cell cultures, several kinds of them could enhance cell growth rate and exhibit the osmoprotective effects on hybridoma cells (Øyaas et al., 1994a). It is speculated that this active osmoregulation involving amino acid transportation takes a significant amount of time for completion considered as the time length required for the cells to adapt to an increase of osmotic pressure (Ta).

In this study, the extent of Ta was estimated in the batch culture of hybridoma cells subjected to an increase of osmotic pressure and the effect of a grudual increase of osmotic pressure on MAb productivity was investigated.

Materials and methods

Cell line and medium

Mouse-mouse AFP-27 hybridima cells producing IgG against human α-fetoprotein are employed (Frame and Hu, 1990; Takagi et al., 1995). DMEM/F-12 medium (Gibco Co. Ltd., NY, USA) supplemented with 1.35 g l^{-1} glucose, 219 mg l^{-1} glutamine, 0.4 g l^{-1} pluronic F-68, 10 μg l^{-1} insulin, 2 g l^{-1} BSA, 20 mg l^{-1} transferrin, 10 μl l^{-1} ethanolamine, 2.44 g l^{-1} $NaHCO_3$, 10,000 U l^{-1} penicillin and 10 mg l^{-1} streptomycin (Sigma, USA) was used as the cell growth medium. Changes in osmolarity was made by the addition of 1M NaCl solution.

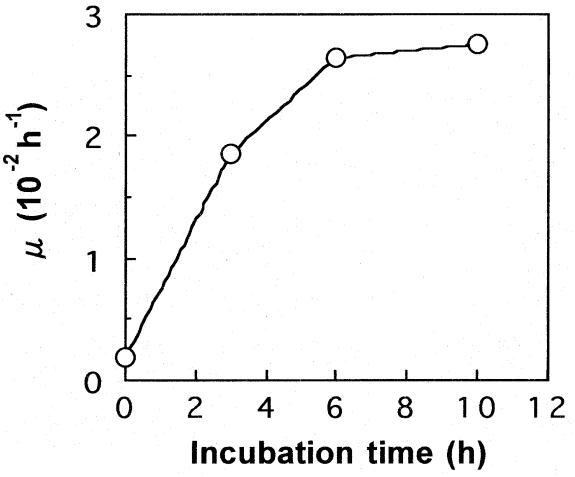

Figure 1. Effect of cell incubation time in a 366 mOsmol kg^{-1} medium on the specific growth rate in a 440 mOsmol kg^{-1} medium. The average specific growth rate from the beginning of the culture to 30 h in the 440 mOsmol kg^{-1} medium was calculated.

Cultivation

A T-flask for suspension cell culture (25 cm^2, Sumilon Co. Ltd., Japan) containing 10 ml of medium was inoculated with 1×10^6 cells and incubated at 37 °C for 65 hr in a conventional CO_2 incubator with a 5% CO_2 atmosphere. In experiment of growth adaptation, 4 sampling times were made from 0 to 30 h of the culture with the sample volume of about 1 ml. In the experiment measuring MAb production with gradual increase of osmotic pressure, samples were made about every 12 h with the sample volume of about 0.5 ml. The experiments were duplicated.

Analyses

Viable cells were counted using the trypan blue dye exclusion method. Glucose and lactic acid concentrations were measured by the glucose oxidase-peroxidase and lactate oxidase-peroxidase methods, respectively, using an auto-analyzer (Biochemistry Analyzer 2700; YSI Inc., Ohio, USA). Glutamine and glutamic acid concentrations in culture broth were measured using an enzymetic analyzer (model BF-4 biosensor, Able Co. Ltd., Japan). Ammonia was measured using an enzymatic assay kit (Boehringer Mannheim, Germany). MAb concentration was measured by an enzyme-linked immunosorbent assay (ELISA) method. Medium osmolarity was measured using an osmometer (model OM-80, VOGEL, Germany).

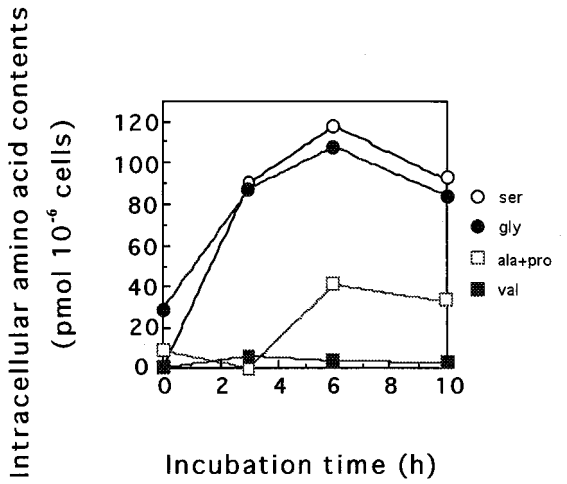

Figure 2. Changes in the intracellular osmoprotective free amino acid contents during incubation. The intracellular amino acid contents were measured after incubating cells in 366 mOsmol kg^{-1} medium for 0, 3, 6, 10 h. The values are the mean of three measurements with the standard deviation less than ± 5%.

Intracellular amino acid content

Amino acid concentration of the intracellular samples was determined using the Pico-Tag system developed by Waters (Millipore Co., USA). To identify and quantify amino acid peaks on the chromatogram, the H-type amino acid standard (2.50 μmol ml^{-1}, Wako Co., Japan) was used. The results obtained were analyzed using the software '805 data Workstation' provided by Waters.

Sample preparation was carried out using the method described by Øyaas et al. (1994a) and Oh et al. (1995). A total of $5 \times 10^6 \sim 5 \times 10^7$ cells were harvested at 3, 6 and 10 h of the culture in a 366 mOsmol kg^{-1} medium, and washed twice with 10 ml of cold phosphate-buffered saline (PBS) with an osmolarity of 366 mOsmol kg^{-1} (same osmolarity as that of the culture medium). As the control, sample preparation was also carried out using cells precultured in a 300 mOsmol kg^{-1} medium and washed with 10 ml of cold PBS with an osmolarity of 300 mOsmol kg^{-1}. After the second wash, total cell number was again determined. The standard deviation of the mean of replicate cell counts was typically less than ± 5% of the mean. Samples were centrifugated and intracellular compounds were extracted by resuspending the cell pellets in 0.5 ml of deionized water. The sample tubes were placed in boiling water for 10 min and cooled at room temperature for 10 min. Samples were then kept at 4 °C for 1 h and the precipitated proteins were removed by centrifugation at 8,000 g for 20 min at 4 °C. The supernatants were then deproteinated by ultrafiltration using a filter (0.4 μm, Millipore, Japan), and subsequently stored at –20 °C until measurement.

Results and discussions

Time length required for adaptation to high osmotic pressure

The time length required for cell adaptation to an increase of osmotic pressure, Ta, was studied with the aim of designing an adequate profile of osmotic pressure change to obtain higher MAb productivity. The cells previously cultured in medium of 300 mOsmol kg^{-1} were incubated in a 366 mOsmol kg^{-1} medium for different time periods, i.e. 3, 6 and 10 h. After incubation in the 366 mOsmol kg^{-1} medium, the cells were transferred into the 400 mOsmol kg^{-1} medium. A control was made without the incubation in the 366 mOsmol kg^{-1} medium. The average specific growth rate (μ) in the 440 mOsmol kg^{-1} medium increased markedly with the increase in incubation time in the 366 mOsmol kg^{-1} medium up to 6 h (Figure 1). However, there was no further increase of specific growth rate, μ, for incubation time longer than 6 h. This suggests that Ta was 6 h.

It has been reported that the intracellular amino acid contents of animal cells increased at high osmotic pressure (Chua et al., 1994; Øyaas et al., 1994a; Oh et al., 1995). The contents of the following intracellular free amino acid: aspartic acid, glutamic acid, glycine, arginine, threonine, alanine and proline, tyrosine, valine, methionine and phenylalanine were determined. Among them proline and glycine were reported to have strong, alanine, serine and valine to have weak osmoprotective effects on hybridoma cells under potassium stressed condition (Øyaas et al., 1994a). In order to estimate the time length required for the accumulation of intracellular osmoprotective amino acids in response to the increase of osmotic pressure, cells previously cultured in the 300 mOsmol kg^{-1} medium were incubated in the 366 mOsmol kg^{-1} medium for 0, 3, 6 or 10 h and the intracellular amino acid contents was determined.

A 3 h incubation in high osmolarity decreased the intracellular contents of alanine and proline, while the

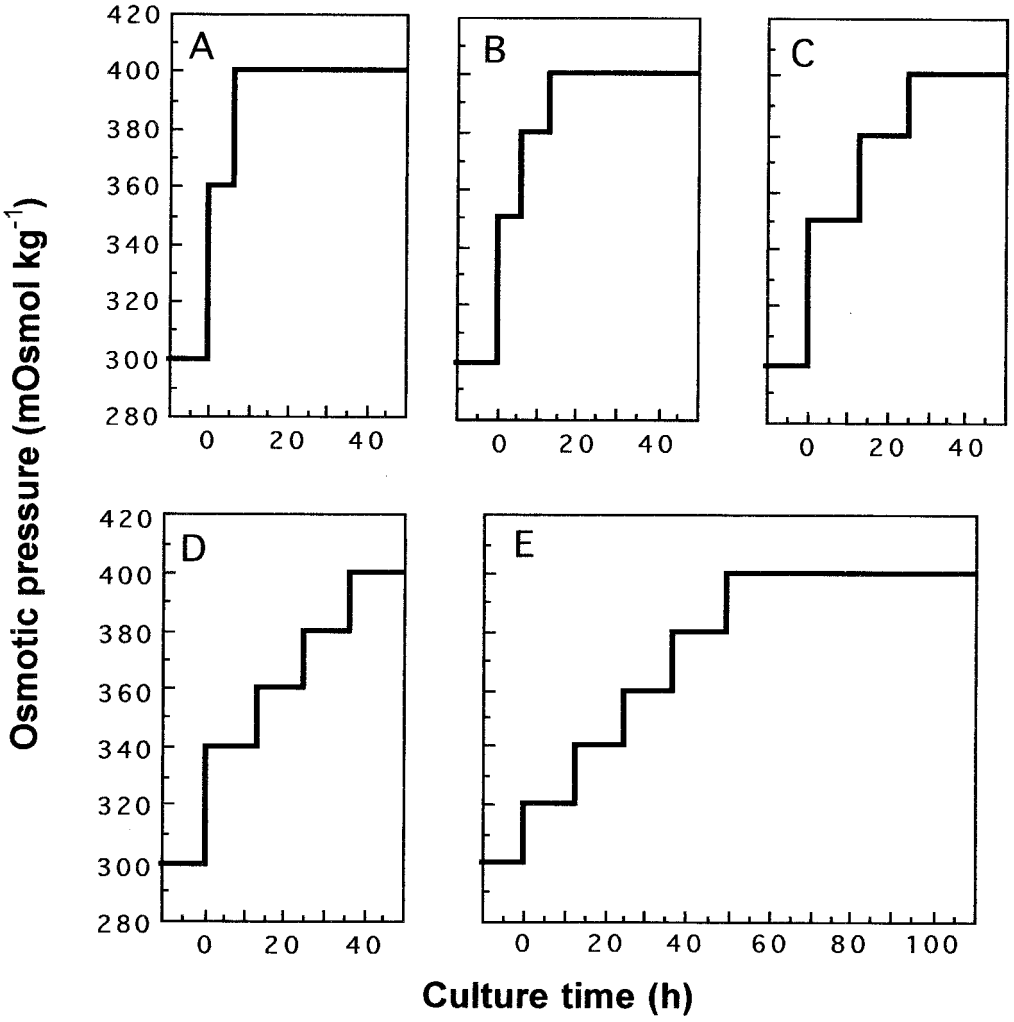

Figure 3. Profiles of osmotic pressure change. The osmotic pressure of the medium was changed stepwise by addition of various amounts of a 1 M NaCl solution at the designated times.

intracellular contents of glycine and serine increased markedly. After 6 h incubation, proline and alanine intracellular contents increased to reach the maximum levels, and the high levels of glycine and serine were maintained. Further incubation for 4 h did not result in a further increase in the intracellular contents of the above osmoprotective amino acids. The valine intracellular contents of the above osmoprotective amino acids. The valine intracellular content increased only a little and remained at a low level throughout the entire incubation period (Figure 2). For the unmeasured free amino acids, none of them had strong osmoprotective effect on hybridoma cells (Øyaas et al., 1994a). Except for free amino acids, amino acid derivatives (e.g., β–alanine), methylamines (e.g., glycine betaine, sarcosine), polyhydric alcohols (e.g., myo-inositol) were also reported to have osmoprotective effects on hybridoma cells (Øyaas et al., 1994a). But, these compounds were not included in the medium in the experiment. According to Øyaas et al. (1994a), the osmoprotactants of the hybridoma cells were taken up from the medium, rather than synthesized by the cells. Consequently, the intracellular contents of the measured osmoprotective free amino aacids could represent the osmoprotective effect in this experiment and they reached the maximum levels 6 h after the increase of osmotic pressure. The phenomena of the dynamic

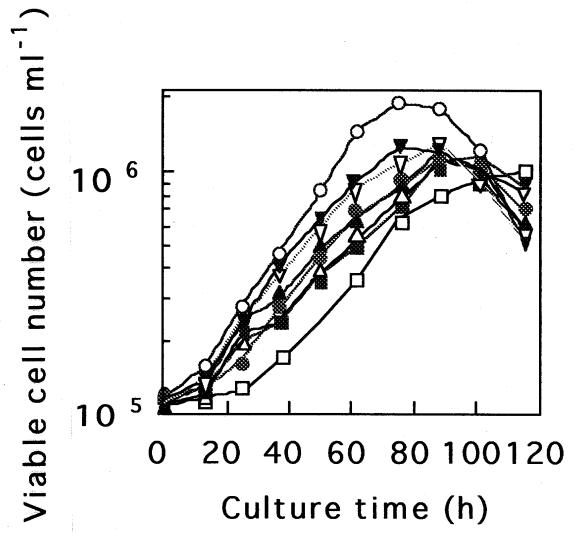

Figure 4. The cell growth of cultures subjected to various osmotic pressure changes. Osmotic pressure was kept constant at 300 (○), 388 (●) or 400 mOsmol kg^{-1} (□) and increased according to the profiles A (■), B (△), C (▲), D (▽) and E (▼) as shown in Fig. 3.

change in the intracellular osmoprotective amino acid contents explains why Ta was extractly 6 h.

Effects of a gradual increase of osmitic pressure on sell growth and MAb production

In general, cell growth was depressed at high osmotic pressure, while the specific MAb production rate, q_{MAb}, was increased. In order to increase the cell growth rate to obtain a high final MAb titer, we investigated cell growth and MAb production during a gradual increase of osmotic pressure. The time interval between the stepwise increase of osmotic pressure was the same, i.e. 6 h or longer of Ta to allow for the adaptation of cells to the increase of osmotic pressure at each step. The profile of osmotic pressure increase is shown in Figure 3. The main rate of increase was highest in profile A and lowest in profile E. Three control cultures were made under constant osmotic pressure of 300, 388 and 400 mOsmol kg^{-1}. 388 mOsmol kg^{-1} is the optimal value for MAb production under constant osmotic pressure (Lin et al., 1998). Cell growth is shown in Figure 4. The gradual increase of osmotic pressure used for profiles A and B, although the final osmotic pressure, 400 mOsmol kg^{-1}, was higher than that of the control culture under constant osmotic pressure of 388 mOsmol kg^{-1}, the maximum cell concentrations, Xm, and the specific growth rates, μ, were almost same with that of the 388 mOsmol kg^{-1} control. In case of profiles C, D, and E, μ were 3.5%, 13.8% and 25.0%, respectively, higher than that of the constant control culture of 388 mOsmol kg^{-1}. The culture time required to reach Xm in case of the lowest osmotic pressure increase profile E was the same with that of the control culture grown under the physiological osmolarity, 300 mOsmol kg^{-1}, and was at least 12 h shorter than that of the other cases. Even in the case of the most rapid osmotic pressure increase rate profile A, the cell growth rate was almost the same with that of the control culture grown under constant osmotic pressure of 388 mOsmol kg^{-1} and was 3.6% higher than that of the control culture grown under the constant osmotic pressure of 400 mOsmol kg^{-1}. This indicates that adaptation of cells to a gradual increase of osmotic pressure occurs.

Table 1. MAb production under gradual increase of osmotic pressure

Mode	Osmotic pressure	MAb (mg/L)
Constant	300 mOsm/kg	37.58
	388 mOsm/kg	51.98
	400 mOsm/kg	46.50
Increasing	Profile A	55.90
	Profile B	54.93
	Profile C	50.77
	Profile D	49.61
	Profile E	44.80

The value is the mean of three measurements with the standard deviation less than 5%.

In our previous study (Lin et al., 1998), among various constant osmotic pressures with batch cultivation, the osmotic pressure of 388 mOsmol kg^{-1} yielded the highest MAb productivity. However, the MAb productivities in cultures subjected to a gradual increase of osmotic pressure, as indicated in profiles A and B, were 7.5% and 5.7% respectively higher than that of the control culture grown under the optimal constant osmotic pressure for MAb production of 388 mOsmol kg^{-1}. The highest MAb productivity was obtained in the culture grown under the most rapid osmotic pressure increase profile A (Table 1).

The q_{MAb} increased under high osmotic pressure. But the mechanism still needs to be investigated. Using NaCl as the osmolty, the medium Na^+/K^+ ratio is changed. This can affect the transmembrane transport of some substrates (Christensen et al., 1973; dePont et al., 1977). The optimal Na^+/K^+ ratio for animal cells in general and for hybridoma cell in particu-

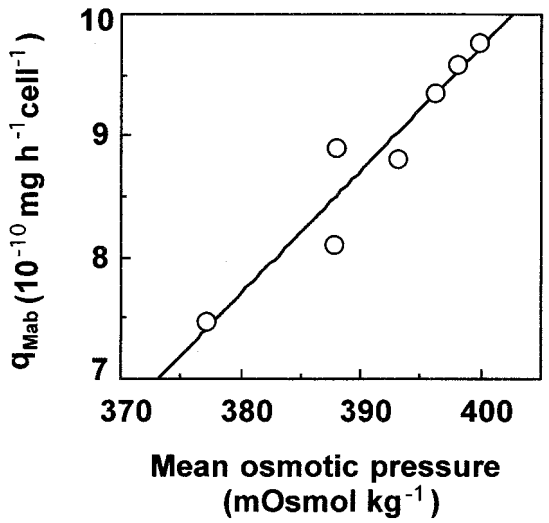

Figure 5. Effect of mean osmotic pressure on the specific MAb production rate. Mean osmotic pressure was calculated by the weighted average method with the time length for each osmotic pressure as the weighted variable, and the total time length for calculation was from the beginning of the culture until the viable cell number reachted the maximum level.

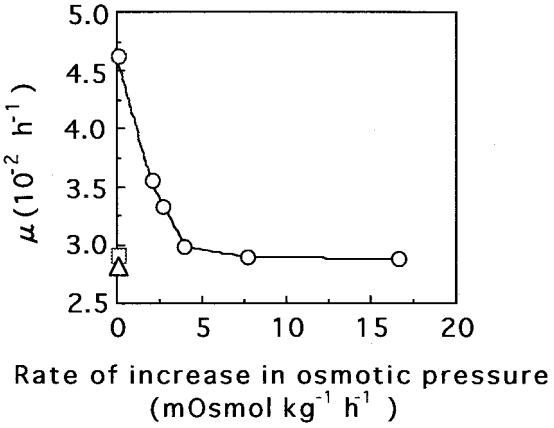

Figure 6. Effect of the rate of increase in osmotic pressure on the specific growth rate. Osmotic pressure was gradually increased (○) or kept constant at 300 (●), 388 (□), and 400 mOsmol kg^{-1} (△). The rate of increase was calculated using the final osmotic pressure divided by the time taken to reach this pessure.

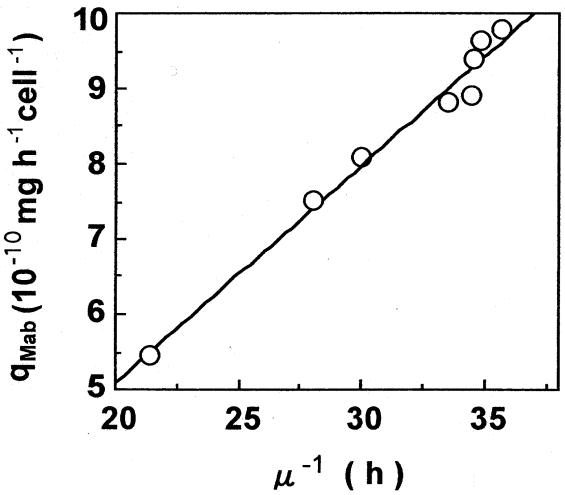

Figure 7. Relationship between the specific MAb production rate (qMAb) and the reciprocal of specific growth rate (μ^{-1}).

lar is around 20 : 1 and 25–35 : 1, respectively (Eage, 1995; Wolfe, 1988). The Na^+/K^+ ratio of the 300 mOsmol kg^{-1} medium used in this experiment is 36.4 : 1, which is near the optimal range for hybridoma cells. The Na^+/K^+ ratio of the 388 and 400 mOsmol kg^{-1} medium are 51.8 : 1 and 54.1 : 1, respectively, which are higher than the optimal range. Therefore, optimal Na^+/K^+ ratio was not the reason for the increased q_{MAb} under high osmotic pressure in the experiment. The fact that q_{MAb} is increased under high osmotic pressure stressed by either sucrose or KCl indicates that high q_{MAb} is caused by high osmotic pressure not dependent on the kinds of the osmolytes used (Chua et al., 1994). In this experiment, Figure 5 shows the dependence of q_{MAb} on osmotic pressure. In our previous study under constant high osmotic pressure, the specific consumption rates of glucose (ν_G) and glutamine (ν_{GLN}) and the yield of ammonia from glutamine ($Y_{A/GLN}$) increased with the increase of osmotic pressure (Lin et al., 1998). The increases in ν_G, ν_{GLN} and $Y_{A/GLN}$ indicate the increase in specific ATP production rate which is considered as one reason for the increased q_{MAb} uner high osmotic pressure. Besides, the decreased μ, which can be controlled by the increasing rate of osmotic pressure (Figure 6), may also be a reason for the increased q_{MAb} (Figure 7).

In the previous study, a linear relationship between q_{MAb} and medium osmolarity was also obtained under constant osmotic pressure within the whole experimental osmotic pressure span from 300 to 424 mOsmol kg^{-1} (data not shown). The highest q_{MAb} under the osmotic pressure of 424 mOsmol kg^{-1} was 2 times higher than that under physiological osmotic pressure (Lin et al., 1998). With the strategy of gradual increase of osmotic pressure, high q_{MAb} can be maintained while high cell concentration can be obtained even though the final osmotic pressure was increased high. As a result, an even higher MAb productivity is expected at a higher osmotic pressure than 400

mOsmol kg^{-1}. The reason why we arbitrarily chose the final osmotic pressure of 400 mOsmol kg^{-1} in this experiment is simply because the MAb productivity under this osmotic pressure is the second highest under constant condition of osmotic pressure (Lin et al., 1998).

Conclusion

In conclusion, cells have an osmoregulation system which allows adaption to increase in osmotic pressure. It takes about 6 h to accumulate osmoprotectants inside cells to give an effective osmoprotective effect when the cells are subjected to the increase of osmotic pressure. Using the strategy of gradual increase of osmotic pressure with the increasing speed not higher than $\Delta P_I/Ta$ (ΔP_I, a step increase of osmotic pressure which can strongly inhibit cell growth), growth inhibition can be relieved. Meanwhile, high q_{MAb} can be obtained under high osmotic pressure. As a result, a higher MAb productivity can be got with gradual increase of osmotic pressure than that with constant osmotic pressure.

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