Special Issue

Adaptive fuzzy control of nutrients concentration in fed-batch culture of mammalian cells

Petros Lenas^{1,3}, Tamotsu Kitade¹, Hideki Watanabe², Hiroyuki Honda¹ & Takeshi Kobayashi^{1,*}

¹ Department of Biotechnology, Graduate School of Engineering, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-01, Japan; ² Eisai Co. Ltd., Tsukuba Research Laboratories, 1–3, Tokodai 5-chome, Tsukuba, Ibaraki 300–26, Japan; ³ present address: Institute for Molecular Science of Medicine, Aichi Medical University, Yazako, Nagakute, Aichi 480–11, Japan; * (corresponding author)

Received 30 November 1996; accepted 22 May 1997

Key words: adaptive control, ammonia, antithrombin III, BHK cells, fed-batch culture, fuzzy control, HPLC

Abstract

An adaptive fuzzy controller was developed to control the glucose and glutamine concentrations in the reactor constant at the desired level. The parameter values of the controller change during the cultivation according to the culture phase which was detected by the lactate concentration. Cultivations with different glucose and glutamine set point concentrations of a recombinant BHK anchorage-dependent cell line were performed in a fed-batch reactor on-line connected with an HPLC system. Glucose and glutamine concentrations were satisfactorily controlled at each set point during all cultivation periods.

Ammonia had a determining effect on productivity since it inhibited cell growth and protein specific production. Ammonia production increased with an increase of glutamine or a decrease of glucose set point concentrations, indicating the importance of glucose to glutamine ratio for the optimization of productivity in mammalian cell cultures.

Introduction

The accumulation of inhibitory waste products, lactate and ammonia, is one of the most important factors that limit the recombinant protein productivity in batch and fed-batch cultivations of mammalian cells. One of the methods to avoid their rapid buildup at toxic levels is the controlled supply of nutrients glucose and glutamine in order to maintain their concentrations at low levels. Although it is well known that maintaining the glucose concentration low results in a decrease of the lactate concentration, an increase of glutamine consumption and ammonia production can be observed under these conditions (Zielke *et al.*, 1978; Hu *et al.*, 1987; Glacken, 1988; Gaertner and Dhurjati, 1993). An optimal glucose to glutamine ratio that depends on the metabolic characteristics of the specific cell line and its relative sensitivity to lactate and ammonia seems to

be a more promising control strategy (Luan *et al.*, 1987; Wang *et al.*, 1995).

In our previous study (Kurokawa *et al.*, 1994), the performance characteristics of different control strategies (adaptive, fuzzy and PI) for the control of glucose and glutamine concentrations in the reactor have been analyzed. Since each one of the above strategies had some advantageous characteristics, we tried to retain them in a combined strategy with more general applicability designing a fuzzy PI-like controller with continuous adaptation of the parameters through another fuzzy strategy. The simplicity of the fuzzy controllers and the easiness in the incorporation of partial knowledge from experimental experience combined with an adaptation scheme to account for dynamical changes of the process (Konstantinov and Yoshida 1990; Shiba *et al.*, 1994) and to minimize the tuning procedure lead to an effective control strategy for the cultivation of mammalian cells.

Materials and methods

Cell line and culture media

A BHK21 cell line producing a recombinant variant of antithrombin III was used in this study. The basal medium was glucose-, glutamine- and serum-free Dulbecco's modified Eagles medium (DMEM) supplemented with 10 mg l^{-1} insulin 5 mg l^{-1} transferrin, $30 \mu\text{M}$ ethanolamine, 40 nM sodium selenite and 100 mg l^{-1} LDL. Different amounts of glucose and glutamine were added to the above medium for the different experiments. The cells were cultured in plastic dishes until the inoculation amount was reached. Static attachment with intermittent shaking to Cytodex-3 microcarriers (Pharmacia Co., Uppsala, Sweden) was performed in dishes in the medium supplemented with 5% FCS. After an attachment period of 4 hours, the microcarriers were transferred in the reactor in the serum free-medium.

Culture system

Figure 1a shows the flow diagram of the culturing system we previously developed (detailed description of Kurokawa *et al.*, 1994). Cells attached to microcarriers were inoculated in a spinner flask (culture volume 750 ml , cell concentration $2.5 \times 10^{-5} \text{ ml}^{-1}$ and microcarriers concentration 3.5 g l^{-1}). The agitation rate was 35 rpm and the culture temperature was maintained at 37°C by a water jacket. Oxygen was supplied by surface aeration. DO concentration was controlled at 70% of air saturation by a control system previously reported (Ogawa *et al.*, 1992). The value of pH was controlled at 7.5 ± 0.1 with addition of 1M NaHCO_3 solution and the use of an on-off controller. Culture broth was drawn out from the reactor every 2 hours automatically by an autosampler (LC-ROBO, Able Co., Tokyo) combined with a timer. The autosampler injected the sample into the on-line HPLC measuring system. Analyzed data of glucose, glutamine and lactate were sent to a computer for glucose-glutamine control. The glucose and glutamine feeding pumps worked every 10 min for a time period decided by the computer.

High concentration media of glucose 55 g l^{-1} and glutamine 5 g l^{-1} were used for feeding to avoid dilution effects. Another feeding medium with glucose and glutamine concentrations equal to the set points was used to compensate for the culture broth loss from the frequent sampling when needed, maintaining the culture volume constant.

Figure 1. (a) Flow diagram of the culturing system. 1. reactor, 2. glucose feeding medium, 3. glutamine feeding medium, 4. volume control feeding medium, 5. pumps, 6. autosampler, 7. timer, 8. DO electrode, 9. pH electrode, 10. stainless-steel filter, 11. mobile phase solution, 12. HPLC pump, 13. HPLC column, 14. RI detector, 15. integrator, 16. RS-232C cable, 17. pH on-off controller, 18. DO meter-gas flow controller, 19. computer for DO control, 20. computer for gluc.-glut. control, 21. base, 22. HPLC waste. (— material flow, --- information flow). (b) Structure of the adaptive fuzzy controller.

Off-line analyses

The viable cell concentration was determined by hemocytometer with the dye exclusion method using 0.3% trypan blue solution. Glucose and lactate concentration measurements of HPLC were confirmed by a glucose-lactate analyzer (Model 27, Yellow Springs Instruments, Yellow Springs, OH). Ammonia concentration was enzymatically measured by an ammonia assay kit (170-A, Sigma Chemical Co., St. Louis, MO). Amino acids were determined by a HPLC amino acids analyzing system (JASCO Co., Tokyo) using *o*-phthalaldehyde (OPA) post-column derivatization method. Periodic check of amino acids during the cultivations showed that no one was in limiting amount. Antithrombin III was quantified by an enzyme-linked immunosorbent assay (ELISA).

Figure 2. (a) Static fuzzy controller rules. Controller inputs are the error (E) and change of error (ΔE) of glucose (or glutamine) concentration and output the change of the feeding time (ΔF). (b) Fuzzy rules for adaptive controller. Inputs are the lactate concentration (LAC) and the sum of four previous errors (SE) of glucose (GC) (or glutamine (GT)) and output the change of FTMAX(MIN) parameter values (ΔM) of the static fuzzy controller.

Control strategy analysis

The structure of the adaptive fuzzy controller used in the nutrients controlled experiments is shown in Figure 1b. A fuzzy adaption strategy was combined with a static PI-like fuzzy controller in an hierarchical structure.

Static fuzzy controller

The process state variables are the glucose (or glutamine) error (E) and the change of the error (ΔE). The control output variable is the change of the feeding time (ΔF) of the glucose (or glutamine) feeding pump. Since the variables are treated as fuzzy variables, they take linguistic instead of numerical values such as negative small (NS) etc. (Figure 2a). The membership functions which represent the correspondence of numerical to linguistic values are in all cases composed of symmetrical triangular functions. The transition function of the controller is expressed as a set of *if-then* rules that can be seen in tabular form in Figure 2a. Any cell defined by the intersection of a row and a column represents one rule. For example the right

corner cell represents the rule:

if E is PB and ΔE is NB then ΔF is ZO

These rules try to reverse, accelerate or retard the movement toward the set point depending on the relative magnitude and sign of E and ΔE . The controller performs the necessary computations called fuzzy reasoning that give ΔF (Mamdani and Assilian, 1975). The range of the ΔF membership function from zero to FTMAX determines how much is any positive (increase) change of the feeding time and the range from zero to -FTMIN how much is any negative (decrease) change.

The influence of the parameters FTMAX and FTMIN on the controller performance was checked in simulations using the glucose (or glutamine) balance equation in the reactor every two hours. An average consumption rate time profile calculated from preliminary experiments was used in the mass balance (Figure 3b). For simplicity FTMAX was set equal to FTMIN. An optimal value of FTMAX(MIN) around 20 gave satisfactory performance (Figure 3a). For this

Figure 3. (a, b and c) Static fuzzy controller performance in simulations. (a) Glucose concentration during the cultivation (set point 2.0 g l^{-1}). (b) Glucose feeding. (c) FTMAX(MIN) parameter values and membership functions of ΔF during the cultivation. (d, e and f) Adaptive fuzzy controller performance in experiments. (d) Glucose concentration during the cultivation (set point 2 g l^{-1}). (e) Glutamine concentration during the cultivation (set point 0.6 g l^{-1}). (f) FTMAX(MIN) parameter values of glucose ΔF membership function. (Controller parameters values: $A=0.16$ (gluc.), $B=0.08$ (gluc.), $L1=0.7$, $L2=4.2$, $S=0.3$ (gluc.), $AD=2.5$, $A=0.03$ (glut.), $B=0.015$ (glut.), $S=0.06$ (glut.)).

value, the feeding determined by the controller coincided with the consumption rate. Below this value, the system exhibited undershoot in the early phase of the cultivation and overshoot in the late phase (Figure 3a for FTMAX(MIN)=2.5) because the feeding changes which were restricted by the low parameter value could not follow the consumption changes. When the FTMAX(MIN) values were above the optimal, the system exhibited oscillatory behavior caused by the overestimation of the feeding time changes by the controller which led to oscillatory feeding (Figure 3b for FTMAX(MIN)=25). We encountered the same problem during the turning of FTMAX parameter value of a fuzzy controller for the control of glucose and ethanol concentration in a baker's yeast fermentation where the optimum value was experimentally determined (Park *et al.*, 1993). The above examples support the idea of

an adaptation scheme for the parameter values since the optimal ones are not apriori known and vary with the culturing conditions.

Adaptive fuzzy controller

At the early stage of the cultivation, the consumption is very low because of the low cell concentration and the lag phase exhibited by the cells after they are transferred in the reactor. Therefore, a low value of FTMAX must be adopted initially to avoid any erroneous increase of the feeding. This value must be increasing gradually during the cultivation following the increase of nutrients consumption caused by the increasing cell concentration. In the later phase when cell death predominates, FTMAX must fall to lower values and simultaneously FTMIN must increase to

cause the rapid decrease of the feeding time which at this stage will have reached high values.

The above considerations are included in the rules of the adaptation schema (Figure 2b), for which inputs are the lactate concentration (LAC) and the sum of the four previous errors (SE) and output the change of the FTMAX(MIN) parameter value (ΔM). The lactate concentration is used as an indicator of culture phase. Because the exact values of lactate when cell death starts are different for different experiments and not apriori known, the sum of the four previous errors is used as a controller performance index in combination with the lactate concentration. From these two inputs, the fuzzy adaptation strategy decides the change of the parameter values. If the change is positive (negative), FTMAX is increased (decreased) and FTMIN is decreased (increased) by the same magnitude. The adaptation of FTMAX(MIN) parameters was performed every four sampling time intervals (eight hours). The combination of the static fuzzy controller with the above adaptation strategy in an hierarchical structure gives an adaptive fuzzy controller (Figure 1b).

The adaptive controller exhibited satisfactory performance in simulations performed with the consumption function and the lactate function of Figure 3b calculated from preliminary experiments. The under(over)shoot of the early(late) phase observed with the static fuzzy controller was avoided with the adaptation of the parameters as can be seen in Figure 3a. FTMAX after passing from a maximum value started to decrease at the later stage of the cultivation (Figure 3c). Opposite trend was observed for FTMIN. Different membership functions for ΔF were adopted during the cultivation according to the parameters values (Figure 3c). Oscillations of the nutrients concentrations were not observed since the values of the FTMAX(MIN) parameters were initially low and increased gradually. Experimental results of glucose and glutamine controlled at 2.0 and 0.6 g l^{-1} , respectively, are shown in Figures 3d, e and f. The parameter values followed generally the same trends observed in the simulations (Figure 3f). Since the exact correspondence of lactate concentration values to the optimal parameter values were not known, some oscillations of FTMAX(MIN) were observed. However the nutrients concentrations could be controlled close to the set points.

Figure 4. Results from controlled cultivations. (a) Viable cell concentration. (b) Specific growth rate. (c) Ammonia concentration. (d) Lactate concentration. (e) Recombinant protein (Antithrombin III) concentration. (f) Specific recombinant protein production rate. (Glucose-glutamine set point concentrations in g l^{-1} : $\blacktriangle$ 2–0.2, $\bullet$ 0.5–0.6, $\blacksquare$ 0.5–0.2.)

Results from controlled cultivations and discussion

Results of three cultivations with different glucose-glutamine set point concentrations (2.0–0.2, 0.5–0.2, 0.5–0.6 g l^{-1}) are presented in Figure 4. The instantaneous specific growth rate (calculated using variable and derivative values from polynomial functions fitted by the least square method) was declining with time for all cultivations after the lag phase (Figure 4b) because of the decreasing surface area of the microcarriers (Frame and Hu, 1988). The maximum cell concentration depended on the ammonia concentration. In the 2.0–0.2 experiment in which the concentration of ammonia was lower (Figure 4c), the maximum cell concentration was 70% higher than that of the 0.6 g l^{-1} glutamine experiment (Figure 4a). When the ammonia reached the concentration of 2.5 mM, cell growth inhibition was observed in the experiment with 0.6 g l^{-1} glutamine set point. Cell death started at higher ammonia concentration (around 3.5 mM). In the two experiments of 0.2 g l^{-1} glutamine set point, no inhibitory phase was evident. Instead of that, cell growth ceased and cell concentration declined rapidly when ammonia

accumulated at 2.5 mM. Lactate seems not to play a determining role in cell viability since cell death started at very different lactate concentrations from 20 mM in the 0.5–0.2 experiment to 40 mM in the 2.0–0.2 experiment (Figure 4d). The rapid decline of viable cell concentration in the experiments of 2.0–0.2 and 0.5–0.2 gl^{-1} gluc.-glut. set points that reached higher cell concentration could be attributed to the exhaustion of a protective factor or the production of some toxic byproducts other than lactate and ammonia that made the cells more sensitive to ammonia.

The decrease of glutamine or the increase of glucose set point had as a consequence less ammonia concentration as can be concluded comparing the 0.5–0.2 and 0.5–0.6 or 0.5–0.2 and 2.0–0.2 gl^{-1} gluc.-glut. experiments (Figure 4c). The less ammonia accumulation through controlled cultivation at low glutamine concentration has been well demonstrated in bioreactors (Glacken *et al.*, 1986; Ljunggren and Häggström, 1994). Evidence for the negative effect of glucose in the glutamine utilization in bioreactors has been reported for hybridoma cells (Ljunggren and Häggström, 1994). In a study with hybridoma cells cultured in flasks (Gaertner and Dhurjati, 1993), high concentration of glucose and lactate caused decrease in ammonia production rate. Since lactate had not so detrimental effect as ammonia, high glucose concentration above 8 mM (1.5 gl^{-1}) and glutamine concentration of 1 mM (0.146 gl^{-1}) were suggested in the study as the nutrients concentrations that optimize the antibody productivity. The recommended nutrient concentrations are similar to that we found, implying that some metabolic characteristics can be generally observed in different cell lines. However for cells that are sensitive to both, lactate and ammonia, low glucose and glutamine concentrations are the optimal (Kurokawa *et al.*, 1993).

The less ammonia production at the late stage of 2.0–0.2 cultivation compared with that of 0.5–0.2 could be attributed to the effect of ammonia which has been reported to increase the glucose consumption for BHK (Wentz and Schügerl, 1992) and hybridoma cells (Miller *et al.*, 1988). It seems that ammonia is the critical factor that triggers the alteration of the cell metabolism which could be facilitated by the high glucose to glutamine ratio.

The highest final protein concentration was achieved in the cultivation of 2.0–0.2 gl^{-1} gluc.-glut. set points (Figure 4e) which had also the higher maximum cell concentration. In the 2.0–0.2 cultivation, the final protein concentration was 65% higher than that of the 0.5–0.6 culture which had the lowest final pro-

tein concentration. The protein concentration for the cultivation of 0.5–0.2 was almost the same as that of 0.6 gl^{-1} glutamine set point experiment although the cell number was 45% higher. This is a consequence of the specific protein productivity drop at the lower glutamine level (Figure 4f), which is in agreement with other studies (Dalili *et al.*, 1990; Shaughnessy and Kargi, 1990). The specific productivity was declining during the cultivation for all experiments possibly following the specific growth rate decrease for positively growth associated production. The decline of initial high metabolic and protein production rates, which reached relatively constant at 5–10 times lower level, has been previously reported for cells grown on microcarriers (Wagner *et al.*, 1990). The highest drop of the productivity (10 times lower than initial one) was observed for the experiment of the 0.6 gl^{-1} glutamine, indicating ammonia inhibition effect which has been reported by several researchers (reviewed by Schneider *et al.*, 1996).

Conclusions

Because of the difficulties in the mathematical modeling of bioprocesses, fuzzy logic approaches can be used effectively incorporating the imprecise and partial knowledge acquired from experiments for control purposes. In the present work using the minimum information for the behavior of the system, an adaptive fuzzy controller that efficiently controls the glucose and glutamine concentrations in the culture broth was designed. At least two phases can be distinguished in the nutrients-controlled cultivations. During the early phase, the factor that determined the cell growth was the decreasing surface area of the microcarriers. This resulted in declining growth rate and specific protein productivity (under the assumption of positively growth associated production). In the later phase, ammonia seems to play the critical role lowering the cell viability and specific protein productivity. Ammonia production was increased by an increase of glutamine or decrease of glucose set point concentrations. A combination of low glutamine with high glucose for cells with higher sensitivity to ammonia than lactate is more efficient in maximizing the cell concentration and protein productivity.

Acknowledgment

The fellowship from European Union (P. L.) is gratefully acknowledged.

References

- Dalili M, Sayles GD and Ollis DF (1990) Glutamine-limited batch hybridoma growth and antibody production: Experiment and model. *Biotechnol. Bioeng.* 36: 74–82.
- Frame KK and Hu WS (1988) A model for density-dependent growth of anchorage-dependent mammalian cells. *Biotechnol. Bioeng.* 32: 1061–1066.
- Gaertner J and Dhurjati P (1993) Fractional factorial study of hybridoma behavior. 2. Kinetics of nutrient uptake and waste production. *Biotechnol. Prog.* 9: 309–316.
- Glacken MW (1988) Catabolic control of mammalian cell culture. *Bio/technology* 6: 1041–1050.
- Glacken MW, Fleischaker RJ and Sinskey AJ (1986) Reduction of waste product excretion via nutrient control: Possible strategies for maximizing product and cell yields on serum in cultures of mammalian cells. *Biotechnol. Bioeng.* XXVIII: 1376–1389.
- Hu WS, Dodge TC, Frame KK and Himes VB (1987) Effect of glucose on the cultivation of mammalian cells. *Develop. Biol. Standard.* 66: 279–290.
- Konstantinov KB and Yoshida T (1990) An expert approach for control of fermentation processes as variable structure plants. *J. Ferment. Bioeng.* 70: 48–57.
- Kurokawa H, Ogawa T, Kamihiro M, Park YS, Iijima S and Kobayashi T (1993) Kinetic study of hybridoma metabolism and antibody production in continuous culture using serum-free medium. *J. Ferment. Bioeng.* 76: 128–133.
- Kurokawa H, Park YS, Iijima S and Kobayashi T (1994) Growth characteristics in fed-batch culture of hybridoma cells with control of glucose and glutamine concentrations. *Biotechnol. Bioeng.* 44: 95–103.
- Ljunggren J and Haggström L (1994) Catabolic control of hybridoma cells by glucose and glutamine limited fed batch cultures. *Biotechnol. Bioeng.* 44: 808–818.
- Luan YT, Mutharasan R and Magee WE (1987) Effect of various glucose/glutamine ratios on hybridoma growth, viability and monoclonal antibody formation. *Biotechnol. Letters* 9: 535–538.
- Mamdani EH and Assilian S (1975) An experiment in linguistic synthesis with a fuzzy logic controller. *Int. J. Man-Mach. Stud.* 7: 1–13.
- Miller WM, Wilke CR and Blanch HW (1988) Transient responses of hybridoma cells to lactate and ammonia pulse and step changes in continuous culture. *Bioprocess Eng.* 3: 113–122.
- Ogawa T, Kamihiro M, Yoshida H, Iijima S and Kobayashi T (1992) Effect of dissolved oxygen concentration on monoclonal antibody production in hybridoma cell cultures. *J. Ferment. Bioeng.* 74: 372–378.
- Park YS, Shi ZP, Shiba S, Chantal C, Iijima S and Kobayashi T (1993) Application of fuzzy reasoning to control of glucose and ethanol concentration in baker's yeast culture. *Appl. Microbiol. Biotechnol.* 38: 649–655.
- Schneider M, Marison IW and von Stockar U (1996) The importance of ammonia in mammalian cell culture. *J. of Biotechnol.* 46: 161–185.
- Shaughnessy TS and Kargi F (1990) Growth and product inhibition kinetics of T-cell hybridomas producing lymphokines in batch and continuous culture. *Enzyme Microb. Technol.* 12: 669–675.
- Shiba S, Nishida Y, Park YS, Iijima S and Kobayashi T (1994) Improvement of cloned α -amylase gene expression in fed-batch culture of recombinant *Saccharomyces cerevisiae* by regulating both glucose and ethanol concentrations using a fuzzy controller. *Biotechnol. Bioeng.* 44: 1055–1063.
- Wagner A, Marc A, Engasser JM, Villiermaux S and Einsele A (1990) Continuous production of p-aminobenzoic acid in fed-batch and perfusion cultures. *Biotechnol. Bioeng.* 36: 623–629.
- Wang J, Honda H, Lenas P, Watanabe H and Kobayashi T (1995) Effective tPA production by BHK cells in nutrients-controlled culture using an on-line HPLC measuring system. *J. Ferment. Bioeng.* 80: 107–110.
- Wentz D, and Schügerl K (1992) Influence of lactate, ammonia and osmotic stress on adherent and suspension BHK cells. *Enzyme Microb. Technol.* 14: 68–75.
- Zielke HR, Ozand PT, Tildon JT, Sevdalian DA and Cornblath M (1978) Reciprocal regulation of glucose and glutamine utilization by cultured human diploid fibroblasts. *J. Cell. Physiol.* 95: 41–48.

Address for correspondence: Takeshi Kobayashi, Department of Biotechnology, Graduate School of Engineering, Nagoya University, Furocho, Chikusa-ku, Nagoya 464-01, Japan.