

A High-Yielding, Generic Fed-Batch Process for Recombinant Antibody Production of GS-Engineered Cell Lines

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An animal-component-free and chemically defined fed-batch process for GS-engineered cell lines producing recombinant antibodies has been developed. The fed-batch process relied on supplying sufficient nutrients to match their consumption, simultaneously minimizing the accumulation of by-products (lactate and osmolality). The proportionalities of nutritional consumption were determined by direct analysis. The robust, metabolically responsive feeding strategy was based on the offline measurement of glucose. The fed-batch process was shown to perform equivalently in GS-CHO and GS-NS0 cultures. Compared with batch cultures, the fed-batch technology generated the greater increase in cell yields (5-fold) and final antibody concentrations (4–8-fold). The majority of the increase in final antibody concentration was a function of the increased cell density and the prolonged culture time. This generic and high-yielding fed-batch process would shorten development time, and ensure process stability, thereby facilitating the manufacture of therapeutic antibodies by GS-engineered cell lines.

Keywords: High-yielding, generic fed-batch process, GS-engineered cell lines, glucose, recombinant antibody

The demand for monoclonal antibodies (Mab) for therapeutic and diagnostic applications is rising constantly. This puts up a need to improve the efficiency of processes used for Mab production by mammalian cells. The fed-batch culture process has emerged as the predominant mode for producing Mab at a manufacturing scale, because it allows product accumulation to a higher concentration, and obtains a higher yield of product in medium than other operation modes [2]. In the past decade, most fed-batch processes of

hybridoma, NS0, and CHO cells were terminated by the accumulation of toxic waste products, such as ammonia and lactate. Several strategies can be followed to minimize the by-product accumulation in fed-batch cultures [6, 18–21]. Limited glucose or/and glutamine concentration in the fed-batch culture was the most effective way to enhance the performance.

The glutamine synthetase (GS) expression system, developed by scientists at Celltech and Lonza Biologics, has been widely used in NS0 and CHO cells [1]. Moreover, NS0 and CHO cells have been proven to be the most effective host cells for the expression of antibodies [3]. The introduction of the GS system has dramatic effects on the growth, metabolism, and antibody production compared with those of non-GS ones [4, 13, 14]. According to the published data, GS-engineered cell lines could synthesize glutamine from glutamate and ammonia, resulting in a reduction of ammonia production. In addition, cell growth and antibody production would be inhibited under the nutrient-limited culture condition. Owing to the specialty of GS-engineered cell lines, the development of process control has focused on how to fulfill nutritional requirements of cell lines continuously [7, 9, 17, 22, 23]. However, overfeeding would increase the concentration of medium components to inhibitory levels. Moreover, overfeeding of nutrients would lead to a high lactate production rate. The level of lactate accumulated eventually requires the addition of sodium bicarbonate to maintain pH. As a result, redundant nutrients supplement combined with the addition of sodium bicarbonate would increase medium osmolality.

As discussed above, the development of the fed-batch process that has general applicability to GS-engineered cell lines was aimed at sufficient nutritional supplements and minimization of lactate and medium components accumulation, which in turn restrict osmolality accumulation. As far as we know, there was little information about the development of an efficient and productive fed-batch

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platform for GS-engineered cells. The fed-batch process that we describe in this work had three elements: (1) the animal-component-free, chemically defined basal and feed medium; (2) the robust, metabolically responsive feeding strategy; and (3) the general application of this high-yielding fed-batch process in GS-engineered cell lines.

MATERIALS AND METHODS

Cell Lines and Cell Maintenance

The host cell lines GS-NS0 and GS-CHO, which constitutively produce Mab against CD25 and the dimeric fusion protein (the human tumor necrosis factor receptor linked to the Fc portion of human IgG1, named TNFR-Fc), respectively, were employed for this study. Cells were maintained in shake flasks and incubated at 37°C on a rotary shaker agitated at 50 rpm.

Basal Medium

The basal medium for NS0 (BM-NS0) was a fully defined animal-component-free medium containing only two proteins (transferrin and insulin). BM-NS0 was a 1:1 mixture of DMEM and Ham's F-12, supplemented with amino acids, insulin, transferrin, ethanolamine, β -mercaptoethanol, β -cyclodextrin, cholesterol, vitamins, and Pluronic F-68. The basal medium for CHO (BM-CHO) was identical to BM-NS0 except that β -cyclodextrin, and cholesterol were removed. The osmolality of the basal medium (BM-NS0, BM-CHO) was 340 mOsm/kg.

Feed Medium

The feed medium, both for GS-NS0 and GS-CHO cells, was developed in a manner similar to that described by Wei-Shou Hu [18]. The consumption rates of medium components were estimated by the direct analysis of glucose, amino acids, sodium pyruvate, phosphorus, and trace metals. The well-formulated feed medium was designed to add nutrients at appropriate stoichiometric rates equal to their consumption rates. Glucose was chosen as the reference nutrient. All nutritional compositions were stoichiometrically balanced. Glucose concentration in the feed medium ($\text{glucC}_{\text{feed}}$) was 200 mmol/l, and the osmolality of the feed medium was 430 mOsm/kg.

Fed-Batch Bioreactor Protocol

The feeding protocol was based on maintaining a constant glucose level during the culture. Supernatant samples were withdrawn periodically (an approximate sampling interval was 12 h). Glucose analysis was carried out immediately after sampling. The amount of glucose (Δgluc) required to sustain growth and production until the next point was estimated by Eq. (1). The volume of feed medium (V_{feed}) was calculated according to Eq. (2). Feed medium was pumped into the bioreactor continuously.

$$\Delta\text{gluc} = q_{\text{gluc}}^{\text{Ave}} \int_n^{n+1} X_v dt \quad (1)$$

$$V_{\text{feed}} = \frac{\Delta\text{gluc}}{\text{glucC}_{\text{feed}}} \quad (2)$$

where the average specific glucose consumption rate ($q_{\text{gluc}}^{\text{Ave}}$) and integral of viable cell concentration ($\int_n^{n+1} X_v dt$, named IVC) were calculated using regression on the cumulative consumption method (IVC method).

Bioreactor Equipment

A 2-l B. Braun reactor was used with a starting volume of 1.5 l. The MFCS software (B. Braun) was used for process control. The controller set points were as follows: pH, 7.00; DO, 40% air saturation; temperature, 36.8°C; and agitation, 80 rpm.

Analytical Methods

Viable cell density (VCD) and viability were determined by hemocytometer counts after trypan-blue staining. Glucose, sodium pyruvate, lactate, and ammonia concentrations were measured enzymatically (NanJing Jian Chen). The assay for amino acids in the culture broth utilized a Accq-Tag assay kit (Waters). The concentration of phosphorus was performed as previously described [11]. Trace metals were quantified using ICP-OES (Thermo). Osmolality was measured using the auto freezing-point osmometer. The antibody concentration was determined by ELISA.

RESULTS

Batch Culture

Bioreactor batch cultivation of GS-CHO in our in-house-developed basal medium was carried out to investigate the nutritional requirements of the GS-engineering cell (Fig. 1). VCD increased steadily up to 1.90×10^6 cells/ml at 106 h with a viability of 95%, which sharply dropped to 34% by the end of culture. The apparent specific growth rate (μ) was maintained constantly in the first 72 h, and then declined linearly for the remaining time of the culture. A final antibody concentration reached 47 mg/l (Fig. 1A).

Glucose, lactate, and pyruvate were measured during the batch culture (Fig. 1B). The glucose consumption rate was quite similar during the exponential growth phase. The glucose was completely exhausted prior to cessation of proliferation. At the same moment, lactate and pyruvate, which exhibited as products during the exponential growth phase, transitioned to consumption when the culture entered the stationary phase. It suggested that lactate and pyruvate were used as carbohydrate fuel sources for the TCA cycle in the absence of glucose, and the metabolic shift could not compensate for the energy requirement of mammalian cells. The results also indicated that pyruvate would not be contained in the feed medium when sufficient glucose was added.

The relationship of glutamine, glutamic acid, and ammonia with the culture time is shown in Fig. 1C. It was observed that the trends of glutamic acid and ammonia were analogous, whereas glutamine acted in the opposite way. Glutamine was utilized at the beginning, and maintained at a low constant concentration (around 0.4 mmol/l) afterwards. At the same time, the concentrations of glutamic acid and ammonia were decreased. It implied that glutamic acid and ammonia would be combined by the action of glutamine synthetase to produce glutamine, and the accumulation of ammonia (about 0.6 mmol/l) would be ignored during the

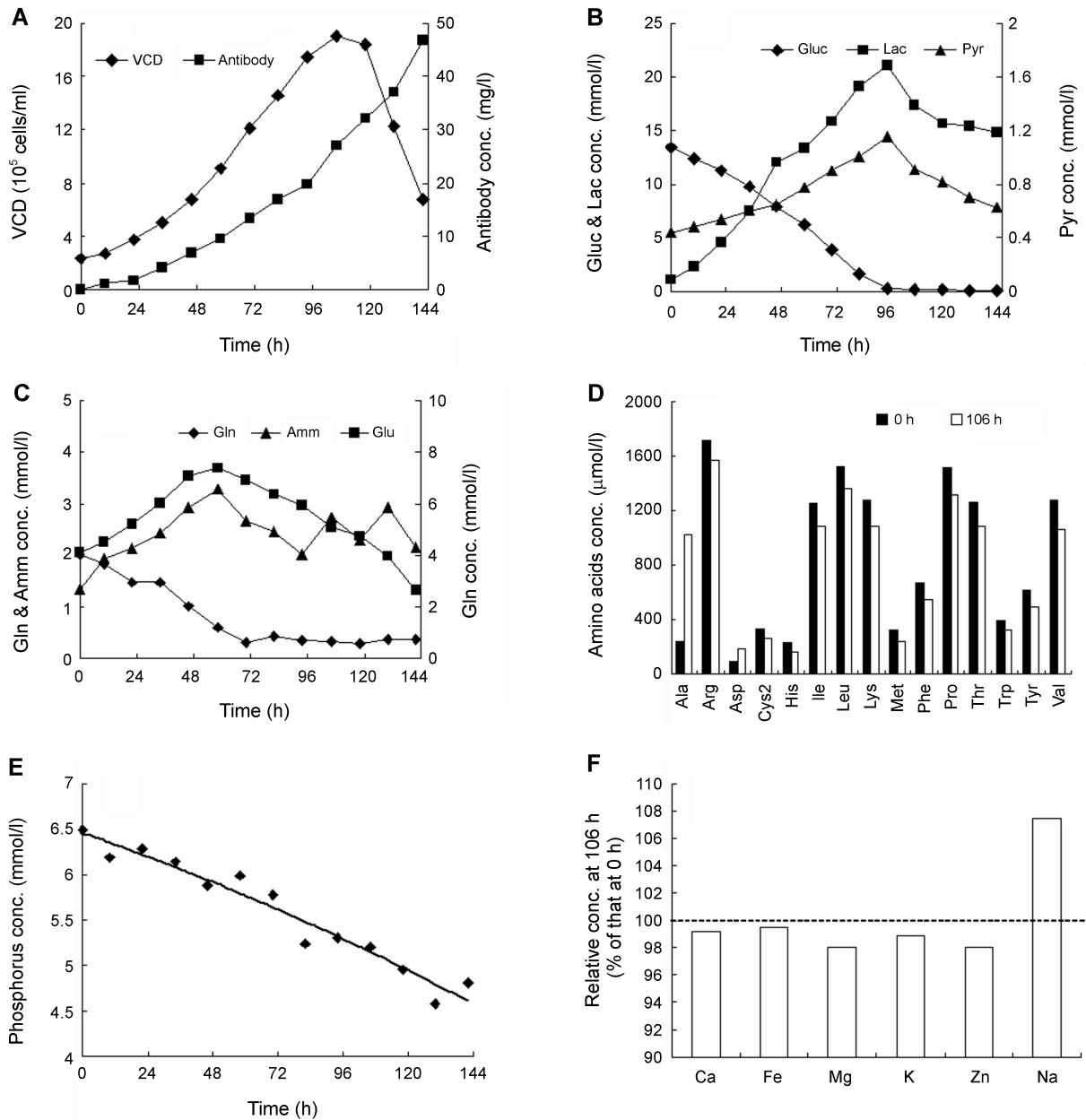


Fig. 1. Batch culture of GS-CHO cells.

The seeding cells were inoculated in a 2-l bioreactor at a concentration of 2.36×10^5 cells/ml using BM-CHO basal medium. **A**, Cell growth and Mab production; **B**, Metabolism of glucose, lactate, and pyruvate; **C**, Profiles of glutamine, glutamic acid, and ammonia concentrations; **D**, Concentrations of amino acids at 0 h and 106 h; **E**, Profile of phosphorus concentration; **F**, Ratio of the concentrations of trace metals at 106 h to those at 0 h.

GS-engineering cell culture. These results demonstrated that the enzymatic reaction not only provided another pathway for glutamine supplementation, but also solved problems brought about by the accumulation of ammonia.

Other amino acids in the culture broth were also measured using the Accq-Tag assay kit (Fig. 1D). Analyses of residual amino acids in the medium at the stalling point of growth (106 h) revealed their different degrees of utilization by GS-CHO cells. Arginine, leucine, isoleucine, lysine, proline, threonine, and valine were consumed at

high rates. Moreover, certain amino acids (leucine and phenylalanine) were required at high concentration, because these used the same amino acid transporter (transporter L) on the cell membrane. Therefore, amino acid supplementation should be aimed at keeping the same levels of all amino acids as those in the initial medium; that is, to replenish amino acids to match their consumption. It was also found that alanine and aspartic acid accumulated during culture and thus were not given any further considerations in the design of the feeding medium.

Phosphate was also quantified during the batch culture, as shown in Fig. 1E. The requirement was approximately constant at 0.26 mmol/(10⁹ cell-day). Among all of the trace metals analyzed, it was found that calcium, iron, magnesium, potassium, and zinc were consumed at a very low rate and presented in sufficient amounts (Fig. 1F). The amount of sodium was increased slightly owing to the addition of sodium bicarbonate to maintain pH. The addition of sodium bicarbonate would destroy the balance of sodium/potassium at the high-density culture process, which may contribute to termination of the culture. Thus, upregulation of pH should be accomplished by adding a mixture of sodium bicarbonate and potassium carbonate. The proportion of sodium and potassium was chosen according to the proportion of sodium and potassium in BM-CHO.

Feeding Protocol

As discussed above, an optimal feeding process of GS-engineered cell lines would keep nutrients within their desired concentration ranges that have no negative effect on cell growth and antibody production while minimizing the accumulation of by-products, such as lactate and osmolality.

It was hypothesized that linking the feeding protocol to glucose could lead to overfeeding or underfeeding of the culture. To test this hypothesis, one batch culture with an initial glucose concentration of 45 mmol/l and two fed-batch cultures with relatively steady glucose level control (2 and 10 mmol/l) were carried out. Fig. 2A shows that the apparent specific growth rate reached a maximum level (about 0.7 day⁻¹) and stayed constant when sufficient glucose was supplied. However, cell growth was limited by the concentration of glucose below 5 mmol/l. Antibody production gave similar results. The relationship of IVC and total antibody production is shown in Fig. 2B. The slope of the line was equal to the average specific antibody production rate, which was found to be 5.67 and 1.84 mg/(10⁹ cells-day) in the 10 mmol/l and 2 mmol/l of glucose-controlled fed-batch cultures, respectively. These behaviors strongly suggested that different glucose levels in the broth resulted in different glucose uptake rates, which in turn affected cell growth and antibody production significantly. However, glucose overfeeding would lead to the accumulation of lactate quickly. As shown in Fig. 2C and 2D, increased glucose level resulted in significant lactate production and high overall lactate/glucose yield coefficients. The accumulation of lactate eventually required the addition of base and led to excessive osmolality buildup. Thus, reducing lactate production was an effective way to slow and defer the aggravation of culture environment caused by the osmolality increasing. Generally, the overall lactate/glucose yield coefficient of 1 or less was considered acceptable for long-term culture.

Stoichiometric ratios, the rate proportionalities of consumption of nutrients relative to glucose, were also

investigated when glucose was controlled in the range of 5 to 15 mmol/l. The relationship between cumulative consumption of nutrients and that of glucose is shown in Fig. 2E, 2F, 2G, and 2H. The slopes of these lines are the stoichiometric ratios of components with respect to glucose. The data, shown as cumulative consumption for a nutrient vs. glucose, can be reasonably well fit by a single straight line. These clearly illustrated that the specific consumption rates of various nutrients were relatively constant over the course of the culture with the glucose concentration between 5 and 15 mmol/l. It would be advantageous to use a single feed medium throughout the culture, simultaneously avoiding excessive accumulation or depletion of a component.

In conclusion, these results showed that it was possible to: (1) underfeed a culture (glucose concentration <5 mmol/l), resulting in reduced cell growth and antibody production, because of insufficient energy supplementation; (2) overfeed a culture (glucose concentration >15 mmol/l), resulting in rapid lactate production and increased osmolality; and (3) have a relatively wide range of glucose target values (5–15 mmol/l), resulting in extending culture longevity, improving antibody production, and minimizing process failure.

Fed-Batch Process

Three fed-batch runs for GS-CHO cells were performed in the 2-l B. Braun bioreactors to establish process consistency. The well-formulated feed medium was designed according to the specific consumption rates of medium components, which were calculated by the direct measurement of glucose, amino acid, phosphorus, and trace metals. The robust, metabolically responsive feeding strategy was based on the offline measurement of glucose in order to keep it in the range of 5 to 15 mmol/l. This process exhibited a high degree of reproducibility. The maximal viable cell density reached 9.40×10⁶ cells/ml, and 207 mg/l of the accumulated TNFR-Fc concentration was achieved within the 9.5 days of fed-batch cultures (Fig. 3A and 3C). The final antibody concentration was a 4.4-fold improvement relative to that of 47 mg/l in batch culture. The majority of increase in final antibody concentration was a function of increase in the integral of viable cell concentration with culture time. The waste products such as lactic acid and ammonia accumulated in the fed-batch cultures to a maximum of 42 mmol/l and 4.9 mmol/l, respectively (Fig. 3E). Reduction in the specific production rate of lactate was prominent in fed-batch compared with that in batch cultures (Table 1). Fig. 3G, 3H, 3I, and 3J showed that the feeding strategy was successful in terms of keeping the concentration of nutrients (except sodium, potassium, alanine, and aspartic acid) constant at the initial level during the exponential growth, even though a slight overfeeding occurred towards the end of the culture. These results indicated that the nutritional environment was well controlled under this

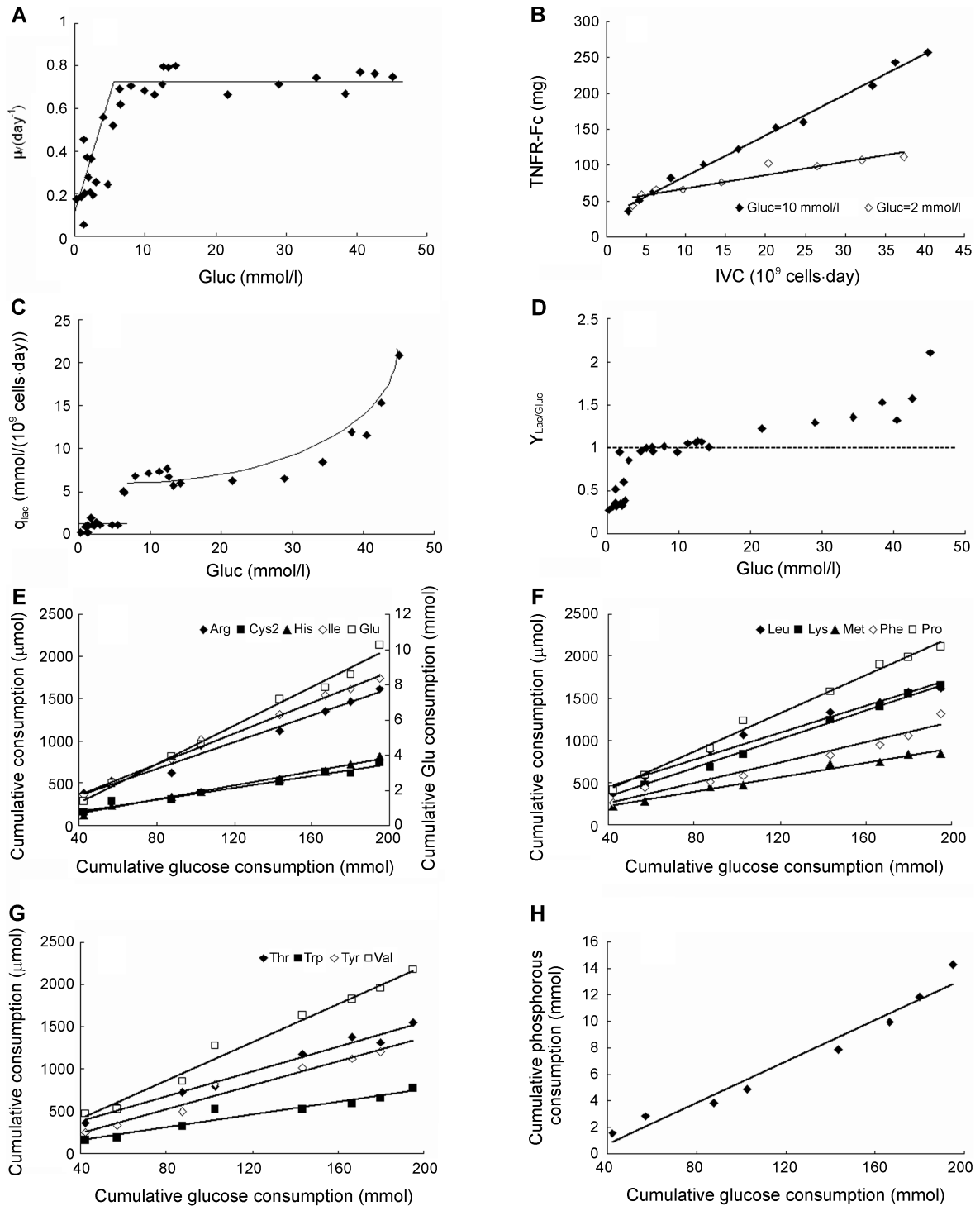


Fig. 2. Data obtained from a batch and two fed-batch cultures of the GS-CHO cells. The initial glucose concentration was 45 mmol/l in batch culture. During fed-batch culture processes, the glucose concentrations were targeted at 2 and 10 mmol/l, respectively. **A, B.** Effect of glucose concentrations on cell growth and antibody production; **C, D.** The relationship of glucose consumption and lactate production. **E, F, G, and H.** The cumulative consumption of nutrients relative to that of glucose.

feeding protocol. As a result of good control in lactate production and nutrient supplements, the final level of osmolality was only 420 Osm/kg (Fig. 3F).

General Applicability of the Fed-Batch Process

The fed-batch process applied equally well to the GS-NS0 cell lines. The same fed-batch protocol was used, except

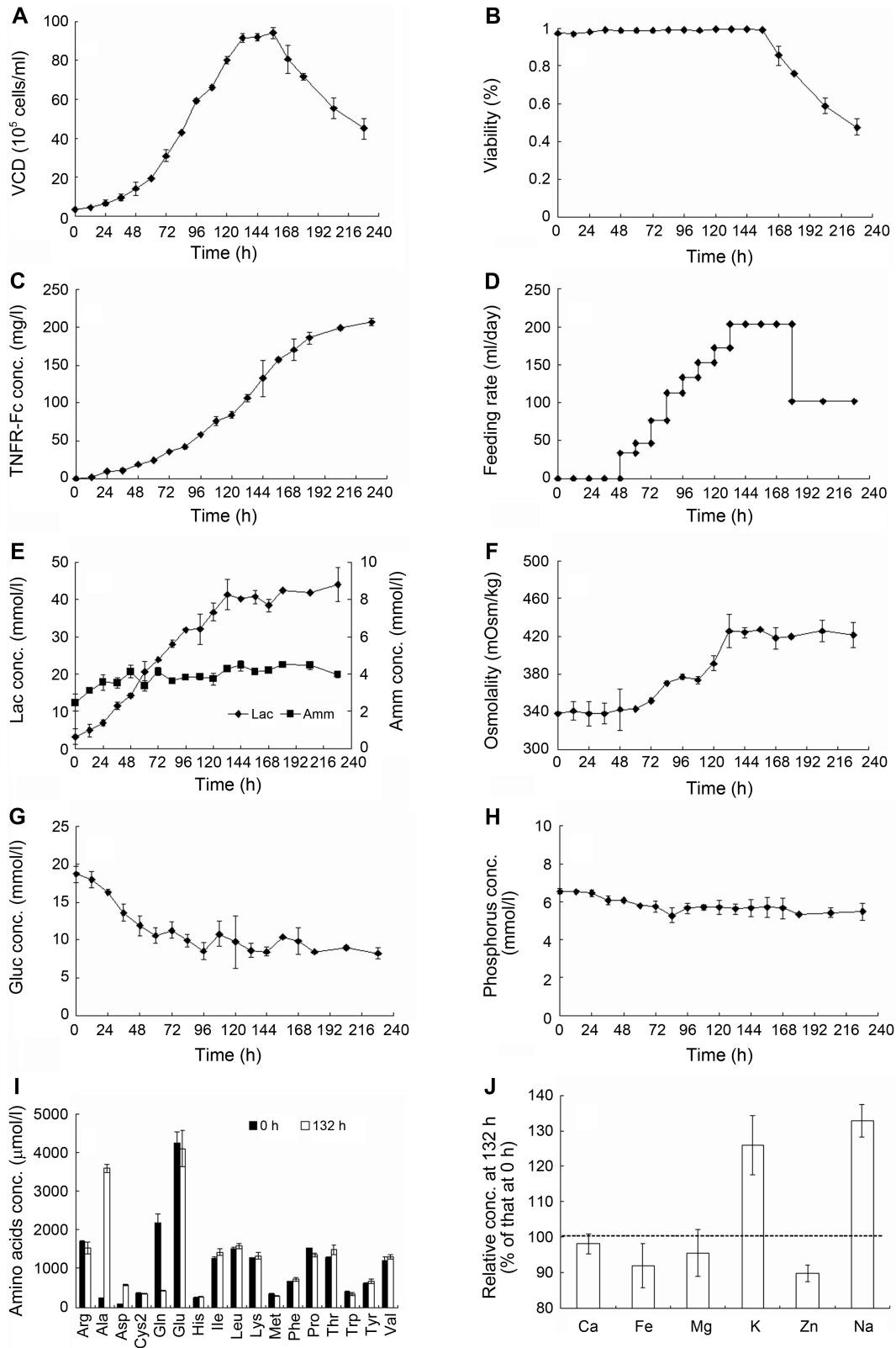


Fig. 3. Processes of fed-batch cultures of GS-CHO cells.

Data represent three runs in 2-l bioreactors using BM-CHO basal medium and stoichiometric balanced feed medium. The feeding protocol specified that the addition of nutrients matched the nutritional requirements of GS-CHO cells. **A.** Viable cell density; **B.** Viability; **C.** TNFR-Fc concentration; **D.** Feeding rate; **E.** Lactate and ammonia concentrations; **F.** Osmolality; **G.** Glucose concentration; **H.** Phosphorus concentration; **I.** Concentrations of amino acids at 0 h and 132 h; **J.** ratio of the concentrations of trace metals at 132 h to those at 0 h.

Table 1. Comparison of the data from batch and fed-batch cultures of the GS-CHO and GS-NS0 cells.

	Batch		Fed-batch	
	GS-CHO	GS-NS0	GS-CHO	GS-NS0
Run time (day)	6	4	9.5	9
Peak viable cell density (10^6 cells/ml)	1.9	1.7	9.4	8.9
Integral of viable cell concentration (10^9 cell day/l)	7.0	5.4	40.1	44.3
Apparent specific growth rate (day^{-1})	0.58	0.64	0.74	0.71
Final antibody concentration (mg/l)	47	133.5	207	1,005
Specific antibody production rate ($\text{mg}/(10^9 \text{ cell}\cdot\text{day})$)	6.03	18.32	5.18	22.14
Volumetric productivity ($\text{mg}/\text{l}\cdot\text{day}$)	7.83	33.38	21.79	111.67
Peak lactate concentration (mmol/l)	21	23	42	41
Specific lactate production rate ($\text{mmol}/(10^9 \text{ cell}\cdot\text{day})$)	6.11	6.22	3.99	2.41
Specific glucose consumption rate ($\text{mmol}/(10^9 \text{ cell}\cdot\text{day})$)	4.79	5.09	4.04	2.05
Lactate produced/glucose consumed (mmol/mmol)	1.28	1.22	0.98	0.85
Peak ammonia concentration (mmol/l)	3.2	1.7	4.5	1.9

The fed-batch data of GS-NS0 cells were generated using the same feeding protocol as GS-CHO cells.

that the BM-CHO basal medium was replaced with BM-NS0 basal medium. The data from GS-NS0 cell lines were presented along with those from GS-CHO cells in batch and fed-batch cultures (Table 1). In the final fed-batch culture, 8.9×10^6 viable cells/ml and 1,005 mg antibody/l were obtained, as compared with 1.7×10^6 viable cells/ml and 133 mg antibody/l in batch culture. The increase of viable cell density combined with a prolonged culture time resulted in a 5.2-fold increase of cell yield and 7.6-fold increase of product yield, respectively. The consistent and substantial improvement in cell yield and final antibody concentration exposed the general applicability of this fed-batch process in GS-engineered cell lines.

DISCUSSION

This paper described the design and development of an animal-component-free and chemically defined fed-batch process for GS-engineered cell lines producing clinical antibodies. The successful applications of this fed-batch protocol in GS-CHO and GS-NS0 cell cultures were reported. It generated the magnitude of the increase in cell yields and the final antibody concentrations. The reproducibility of this fed-batch strategy was also verified. This standardized fed-batch process may have potential application in cultures of other GS-engineered cell lines producing various therapeutic proteins.

Usually, the final product concentration in cultures equals the specific production rate times the integral of viable cell concentration. In our fed-batch process, the majority of increase in the final antibody concentration was a function of an increase in the integral of viable cell concentration as opposed to the constant specific antibody production rate (Table 1). Further process optimization for improving the final product concentration should rely on

increasing the specific antibody production rate. Solutions to the aforementioned problem have been attempted using various methods. The nutrient feed compositions and feeding rate could significantly affect the specific antibody production rate in fed-batch cultures [5]. The effects of environmental stresses were utilized and reported to improve antibody synthesis [8]. The addition of protein inducers or chemical enhancers (DMSO, sodium butyrate) was also used to stimulate the Mab secretion [10, 12].

In addition, the possible reasons for cessation of the cell proliferation in fed-batch are as yet unknown. Excessive accumulation or depletion of nutrients was not observed throughout the culture. The levels of lactate, ammonia, and osmolality were probably not the factors causing limitation or inhibition. Some particular substances (such as vitamins, hormones, and antioxidants) have also been fed in the parallel fed-batch cultures, but had little effect on cell growth and antibody accumulation (data not shown). It is possible that the accumulation of autocrine factors could inhibit cell growth [15, 16]. In that case, identifying such factors or eliminating negative effects will be the efficient way to further increase the integral of viable cell concentration.

Abbreviations

Amm	ammonium
Ala	alanine
Arg	arginine
Asp	aspartic acid
Cys2	cysteine-2HCl
Gln	glutamine
Glu	glutamic acid
Gluc	glucose
His	histidine
Ile	isoleucine

Lac	lactate
Leu	leucine
Lys	lysine
Met	methionine
Phe	phenylalanine
Pro	proline
Thr	threonine
Trp	tryptophan
Tyr	tyrosine
Val	valine

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