Pilot production of u-PA with porous microcarrier cell culture

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Abstract

A recombinant DNA CHO cell line secreting urokinase-type plasminogen activator (u-PA) was cultivated with Cytopore cellulose porous microcarriers in a 30l Biostat UC stirred tank reactor. After 26 days of culture, using a spinfilter to retain cells in bioreactor, the cell density could reach 1.33×10^7 ml⁻¹. The maximal u-PA activity in supernatant was 7335 IU·ml⁻¹, and 204l supernatant containing 7.1 g u-PA was harvested. After 100 days of culture with 0.1% fetal bovine serum medium, a modified cell retention system which can be washed-out backward, substituted the spinfilter to prevent filter clogging. The maximal cell density was over 10^7 ml⁻¹, the maximal u-PA activity in supernatant reached 6250 IU·ml⁻¹, and 1604l supernatant containing about 51 g u-PA was harvested. Compared to perfusion culture, batch medium-replaced culture could raise the utilizing efficiency of the medium, increase cell specific productivity and improve the quality of the product which was not steady in a 37 °C environment. Cells can move from seed porous microcarriers occupied by cells to vacant microcarriers spontaneously, without trypsinization, and continue to grow until all microcarriers contained cells. It shows that Cytopore porous microcarriers are very useful and convenient to scale up cultivation step by step.

Abbreviations: DO, dissolved oxygen; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; scu-PA, single-chain urokinase-type plasminogen activator; tcu-PA, two-chain urokinase-type plasminogen activator; u-PA, urokinase-type plasminogen activator; μ_{app} , apparent cell specific growth rate.

Introduction

Cytopore is one of the excellent porous microcarriers made of cellulose for animal cell culture, which possesses almost all characteristics of an ideal cell culture support, as described by Griffiths (Griffiths, 1990). It can be used for both anchorage dependent and suspension cells, is capable of long-term continuous operation with low-serum/serum-free media, has good mechanical stability, and considerable volumetric scale-up potential (Shirokaze, 1994). In this study, we report that a rCHO cell line which expresses prourokinase was cultivated on Cytopore porous microcarriers in a 30l Biostat UC stirred tank reactor with a spinfilter to retain cells. The cell density could

reach 1.33×10^7 ml⁻¹, the maximal u-PA activity was over 7000 IU·ml⁻¹. But the spinfilter was completely fouled by cells and microcarriers after 21 days of culture. So we developed a new cell retention system to overcome this problem, and continuously cultivated rCHO cells for over 100 days without serious clogging.

Materials and methods

Cell line and media

CL-11G is a new genetically-engineered CHO cell line secreting prourokinase. The growth medium for CL-11G cells was DMEM:F12 (1:1), supplemented with

Figure 1. Schematic diagram of batch medium-replaced culture.

the low-serum medium supplement BIGBEF-3 (Xiao, 1996), as well as with $20\text{KIU} \cdot \text{ml}^{-1}$ aprotinin to inhibit scu-PA degradation. The growth medium with 1% FBS was adopted when cells cultivated in the reactor equipped with spinfilter, while 0.1% FBS medium was utilized when cells cultivated in the reactor equipped with a modified cell retention system.

Microcarrier and bioreactor

The porous microcarriers, Cytopore (Pharmacia Co., Sweden), were hydrated in $0.1\text{ mol} \cdot \text{l}^{-1}$ PBS without Ca^{2+} and Mg^{2+} , prewashed and autoclaved for 30 min at 121°C according to manufacturer's recommendation. After sterilizing, they were washed twice with growth medium, and kept at 4°C until use.

A 30l (working volume = 20l) Biostat UC stirred tank reactor (B. Braun, German) was equipped with bubble-free silicone tubing oxygenator for gas supply and with a spinfilter (pore size = $20\text{ }\mu\text{m}$) to retain cells in the reactor for continuous perfusion culture. During 100 days of cell culture, a modified cell retention system which can be washed-out backward was substituted for spinfilter to prevent filter clogging (Xiao, 1994a; Hu, 1999).

Cell culture and growth rate

The Biostat UC reactor was supplemented with some fresh medium and vacant porous microcarriers before inoculation. The agitate rate was 70 rpm. Temperature was maintained at $37 \pm 0.2^\circ\text{C}$ with a circulating water bath through the reactor jacket. The pH was kept at 7.1 ± 0.1 by automatic addition of $1\text{ mol} \cdot \text{l}^{-1}$ NaOH solution. DO was maintained at $40 \pm 5\%$ of air sat-

uration. The concentration of porous microcarrier was $2.5\text{ g} \cdot \text{l}^{-1}$. The seed microcarriers with CL-11G cells obtained from 5l CelliGen reactor or 1000 ml spinner flasks, were transferred directly to the 30l reactor via tube without trypsinization. The frequency and extent of medium replenishment depended on the residual glucose concentration. The apparent specific growth rate (μ_{app}) is determined by linear regression of the exponential increase of cell concentration C, by plotting the natural log of C vs. time t.

Perfusion culture mode was adopted when cells grew in the reactor with spinfilter, and chemostat mode was used after spinfilter was fouled. Batch medium-replaced culture and perfusion culture were adopted when cells were cultured in the reactor with a modified cell retention system. In batch medium-replaced culture, when the medium replacement rate was about $1.0 \sim 1.2$ working volume per day ($20\text{ l} \cdot \text{d}^{-1} \sim 24\text{ l} \cdot \text{d}^{-1}$), about 0.5 working volume of supernatant was replaced with fresh medium by filtering every 12 hours in batch mode (Figure 1), and all the microcarriers were retained in the reactor all the time.

Assays

Cells were enumerated by counting nuclei, stained with 0.1% crystal violet (w/v) in $0.1\text{ mol} \cdot \text{l}^{-1}$ citric acid solution. The cell adherent ratio of microcarriers was determined by dyeing with $5\text{ mg} \cdot \text{ml}^{-1}$ MTT solution. Viable cell counts were performed with exclusion trypan blue method after trypsinization. Glucose concentration was measured using a clinical glucose analyzer. The u-PA activity in supernatant was measured by a modified frebrin-agarose-plate assay

Figure 2. Cells moving from seed bead to vacant bead. (a) After 10 days of culture, cells had moved into the vacant microcarriers; (b) At day 18, all the microcarriers were full of cells. Cells were dyed in black with MTT.

(Xiao, 1994b). For distinguishing the proportion of single chain and two-chain u-PA in supernatant, the samples were analyzed both in non-reducing and reducing condition with western blotting analysis (Han, 1997).

Results

Cells move from seed microcarriers to vacant microcarriers

Seed microcarriers with CHO cells obtained from a 5l CelliGen reactor or 1000 ml spinner flask was transferred to the 30l Biostat UC reactor directly via tube without trypsinization. Cells could move from seed microcarriers to vacant microcarriers spontaneously (Kamiya, 1995; Xiao, 1999). As reported by Kamiya, the cell transferring speed depends on the inoculated cell density and the scale-up ratio, the higher the cell

(A) Growth curve and u-PA activity curve

(B) Bioreactor productivity and cell specific productivity

Figure 3. Cell culture in the 30l reactor equipped with spinfilter.

density in seed microcarriers and the lower scale-up ratio, the faster the cells move from seed microcarriers to vacant microcarriers. When the initial cell density was $3.35 \times 10^5 \text{ ml}^{-1}$ and the scale-up ratio was 1:4, it took 6 days to reach 100% adherent ratio. While the inoculated cell density was $2.0 \times 10^5 \text{ ml}^{-1}$ and the scale-up ratio was 1:20, it need 10 days to move. Figure 2 shows the procedure of cell moving when CHO cells were cultivated in the reactor with spinfilter.

Cell culture in bioreactor equipped with spinfilter

Growth experiment in a 30l Biostat UC reactor with spinfilter is shown in Figure 3. The inoculated cells obtained from 5l CelliGen reactor were $3.35 \times 10^5 \text{ ml}^{-1}$. During 20 days of perfusion culture, the perfusion rate increased from $51 \cdot \text{d}^{-1}$ to $201 \cdot \text{d}^{-1}$ gradually to keep the residual glucose concentration at $2.0 \text{ g} \cdot \text{l}^{-1} \sim 3.0 \text{ g} \cdot \text{l}^{-1}$, and the u-PA activity increased along with cell dens-

ity. The cell specific productivity (q_{u-PA}) was around $420\text{IU}\cdot(10^6\text{cells}\cdot\text{d})^{-1} \sim 540\text{IU}\cdot(10^6\text{cells}\cdot\text{d})^{-1}$, and the μ_{app} was 0.182d^{-1} . The maximal cell density reached $1.33 \times 10^7\text{ml}^{-1}$ after 18 days of culture, the u-PA activity in the supernatant was $7335\text{IU}\cdot\text{ml}^{-1}$ at the same time, and the max. bioreactor productivity ($q_{reactor}$) was about $1.47 \times 10^8\text{IU u-PA}\cdot\text{d}^{-1}$ ($\sim 1.41\text{g u-PA}\cdot\text{d}^{-1}$ calculated according to u-PA specific activity = $104,000\text{IU}\cdot\text{mg}^{-1}$). But cells and microcarriers clogged the spinfilter completely. At day 20, chemostat mode was adopted, the dilution rate $D = 0.3\text{d}^{-1}$, the cell density dropped to $4.0 \times 10^6\text{ml}^{-1}$ due to a large quantity of cells lost, and u-PA activity decreased to $3000\text{IU}\cdot\text{ml}^{-1}$ or so. In 26 days of cell culture, 204l supernatant which contained 7.1 g products was harvested. It seemed that spinfilter is not the best method to retain cells in reactor for long-term cell culture.

Cell culture in bioreactor equipped with a modified cell retention system

Cell growth and replenishment of medium

CL-11G cells had been successfully cultivated on Cytopore cellulose porous microcarriers in a 30l Biostat UC stirred tank reactor for 100 days. The culture process could be divided into five stages (Figure 4). During stage I, the μ_{app} was 0.112d^{-1} , lower than that of 0.182d^{-1} at the initial cell density of $3.5 \times 10^5\text{cells/ml}$ when 1% FBS culture medium was used. The expanded lag phase and relatively lower specific growth rate were due to (1) the inoculated cell density was low ($2 \times 10^5\text{cells/ml}$); (2) 0.1% FBS growth medium was not very suitable at the beginning of cell subculture, especially at low inoculated cell density. The culture process of stage II started from the viable cell density of $9.8 \times 10^5\text{cells/ml}$, the insulin concentration in the growth medium increased from 40 U/l to 120 U/l. The cells grew up rapidly and the cell density reached $1.01 \times 10^7\text{cells/ml}$ after another 15 days culture. The μ_{app} at stage II was 0.153d^{-1} , higher than that of stage I. It indicated that higher inoculated cell density and higher insulin concentration were beneficial to shorten lag phase and speed up cell proliferation.

During stage III, cell growth was at stationary phase, and perfusion culture mode was adopted. The cell density kept around $9.0 \times 10^6\text{cells/ml}$, 20l ~ 23l medium were replaced per day to keep glucose concentration around 2.0 g/l. After 15 days of perfusion culture, the filter was almost clogged, and the perfusion rate was only $12\text{l}\cdot\text{d}^{-1}$, the residual glucose con-

centration decreased down to $1.2\text{g}\cdot\text{l}^{-1}$. The culture medium couldn't supply enough nutrients to support cell growth, and a large number of cells died due to undernourishment. Batch medium-replaced culture was adopted to prevent filter clogging. During the early period of stage IV, the μ_{app} was 0.172d^{-1} at the initial cell density of $4.0 \times 10^6\text{cells/ml}$, showing that μ_{app} increased with inoculation density (Table 1). Comparing to the μ_{app} of 0.182d^{-1} in 1% FBS culture medium, low-serum growth medium seemed to have a little effect on cell growth at relatively high inoculation density. The cell viability was about 70% when the sample of the 87th day was enumerated with exclusion trypan blue method after trypsinization. It seemed to indicate that the cells in the center of microcarriers might encounter mass transfer limitation, especially under the circumstance, which the cells in the porous microcarriers were very dense after long term culture.

After 90 days culture, the reactor couldn't control pH at a proper state, and the pH even dropped below 6.5, so the CHO cells began to die gradually, and at the 98th day, the cells which were detached from microcarriers with trypsin shrank, only some of them suspended in the medium were viable. Cell death occurred due to sharp change in pH.

Bioreactor productivity and product quality

The u-PA activity curve was shown in Figure 4(A). The u-PA activity reached its peak of 6250IU/ml in stage II, and reduced to $4500 \sim 5500\text{IU}\cdot\text{ml}^{-1}$ during perfusion culture process. During stage IV, cell density and product expression were in relatively steady state, the u-PA activity fluctuated about $2800\text{IU}\cdot\text{ml}^{-1} \sim 3600\text{IU}\cdot\text{ml}^{-1}$. In decline phase, the u-PA activity decreased from $2500\text{IU}\cdot\text{ml}^{-1}$ to $500\text{IU}\cdot\text{ml}^{-1}$ as a result of cell death.

The bioreactor productivity and cell specific productivity was given in Figure 4(C) and Table 1. The maximum bioreactor productivity could reach $1.375 \times 10^8\text{IU}\cdot\text{d}^{-1}$ ($\sim 1.32\text{g u-PA}\cdot\text{d}^{-1}$), and the u-PA yield of 100 days culture added up to 51 g more or less. Cell specific productivity in stage I and II was around $600 \sim 730\text{IU}\cdot(10^6\text{cells}\cdot\text{d})^{-1}$, a little higher than that in stage III ($640 \sim 540\text{IU}\cdot(10^6\text{cells}\cdot\text{d})^{-1}$). It appears that comparing to perfusion culture, batch medium-replaced culture has some advantages in utilizing culture medium and cell expression. This may be attributed, that the environment after replenishing medium was more suitable for cell growth like fresh medium, while some fresh medium would flow out of the reactor without utilizing, and the environment

(A) Growth curve and u-PA activity of CL-11G cells cultivated on Cytopore porous microcarriers with 0.1% FBS medium
 stage I: batch medium-replaced culture at initial cell density of 2.0×10^5 cells/ml (day 0 – day 22);
 stage II: batch medium-replaced culture at initial cell density of 9.8×10^5 cells/ml (day 24 – day 39);
 stage III: perfusion culture at stationary phase (day 40 – day 54);
 stage IV: batch medium-replaced culture (day 55 – day 90);
 stage V: cell decline phase, the pH in the reactor was not properly controlled (day 91 – day 100);

(B) Medium replacement rate and residual glucose concentration

(C) Bioreactor productivity and cell specific productivity

Figure 4. CL-11G cells cultivated in a 30l Biostat UC reactor with a modified cell retention system.

Table 1. rCHO cells cultivated on porous microcarriers for 100 days

	stage I	stage II	stage III	stage IV	stage V
cell density ($\times 10^5 \text{ ml}^{-1}$)	2.0~23.8	9.8~101.2	100~90	40~93	~45
μ_{app} (d^{-1})	0.112	0.153	—	0.172	—
medium replacement ($1 \cdot \text{d}^{-1}$)	7~12	7~22	20~23	20~22	22
i-PA activity ($\text{IU} \cdot \text{ml}^{-1}$)	1000~2980	2000~6500	4000~5500	2500~3700	2000~500
scu-PA ratio	>90%	~85%	80%~30%	70%~80%	~70%
cell specific productivity ($\text{IU} \cdot (10^6 \text{ cell} \cdot \text{d})^{-1}$)	620~730	640~730	540~660	400~440	—
bioreactor productivity ($\text{g u-PA} \cdot \text{d}^{-1}$)	0.05~0.23	0.118~1.32	~1.0	0.54~0.75	0.1~0.42
total productivity (g u-PA)	2.73	10.82	12.42	22.12	2.8

Figure 5. Western blot analysis of supernatant. Lane 1,2,3,4: non-reducing condition; lane 5,6,7,8: reducing condition; lane 1,5: sample of stage I, >90% was scu-PA; lane 2,6: sample of day 54 (perfusion culture), lots of cells died, and the scu-PA ratio dropped to 30%~40%; lane 3,7: adopting batch medium-replaced culture, the ratio of scu-PA got back to ~80%; lane 4,8: sample of day 80.

was not always kept at a very perfect state in perfusion culture. In stage IV, cell specific productivity maintained in the range of $400 \sim 440 \text{ IU} \cdot (10^6 \text{ cells} \cdot \text{d})^{-1}$, the relatively low value was due to cell degeneration after long term culture and not taking dead cells into account when calculating cell specific productivity.

Scu-PA (i.e. prourokinase) is a potential thrombolytic drug with fewer side effect than tcu-PA (i.e. urokinase) (Sasahara, 1995), so the quality of u-PA product depends on the ratio of scu-PA. Scu-PA is easily activated by plasmin, trypsin and plasma kallikrein due to the cleavage of a single internal peptide bond, resulting in the conversion of single chain prourokinase into two-chain urokinase. Thrombin also can convert prourokinase to a two-chain form that is not activatable by plasmin, plasma kallikrein and factor XIIa (Ichinose, 1986). The main problem in prourokinase production by cell culture is how to keep unsteady prourokinase in single chain form. In stage I and II, 85%~90% of u-PA was maintained in single chain form in the harvested supernatant. During perfusion culture, scu-PA ratio decreased from about 80% to 60% gradually along with culture process, and even dropped to 30%~40% when large number of cells died due to filter clogging. In stage IV and V, adopting batch medium-replaced culture, the ratio of scu-PA

got back to 70%~80% (Figure 5). This suggested that cell death in large quantities would release some protease to convert scu-PA into two chain form, and batch medium-replaced culture could harvest supernatant with relatively high ratio of scu-PA due to the short residence time of the product after secretion.

Discussion

Spinfilter was efficient to retain cells in reactor to increase cell density and productivity, but it was easy to be clogged after about one month of running. A modified cell retention system was developed to prevent clogging in long-term and large-scale continuous cell culture. After 100 days culture, the filter worked excellent and 1604L supernatant containing 51 g u-PA was harvested.

Batch medium-replaced culture has advantages of batch culture and perfusion culture when medium replacement rate was about 1~2 working volume per day. Just like batch culture, it could utilize medium more efficient and obtain supernatant with relatively high concentration product. On the other hand, it reduced accumulation of toxic metabolites and operation expenses to raise utilizing efficiency of equipment. Moreover, when the product was unsteady in 37°C

environment such as prourokinase, batch medium-replaced culture will be an ideal choice for product quality assurance.

Cytopore porous microcarrier was very useful for long-term cell culture with low-serum medium, and subculture method was simple and safe. There is no fear for contamination during inoculation process because cells can move from seed microcarriers to vacant ones automatically without trypsinization, this was convenient for preparing seed cells and enlarging culture scale. It can reduce serum concentration in culture medium that can't result in cells detaching from microcarriers. Low-serum culture medium seemed to have no significant effect on cell growth and product secreting. The μ_{app} in 0.1% FBS culture medium was a little lower than that in 1% FBS culture medium, while the cell specific productivity in 0.1% FBS medium was $600\sim 700 \text{ IU}\cdot(10^6\text{cells}\cdot\text{d})^{-1}$, a little higher than $450\sim 500 \text{ IU}\cdot(10^6\text{cells}\cdot\text{d})^{-1}$ in 1% FBS growth medium (Hu, 1998).

Cells cultivated in porous microcarriers may encounter mass transfer limitation after long term culture, this would result in decrease of cell viability and cell productivity. Furthermore, cell apoptosis would happen in long term cell culture due to nutrients, pH, shear forces, accumulation of waste products and availability of oxygen (Cotter, 1995). Recently, we developed a new and simple process to deal with these problems when cell grew in the 30l bioreactor. During the culture process, 1/4~1/5 of microcarriers in the reactor were replaced with vacant microcarriers once every 12 days~13 days, the cell density could keep at $1.3\sim 2.6 \times 10^7 \text{ ml}^{-1}$ with serum-free growth medium, and u-PA activity in the supernatant was around $6000\sim 12000 \text{ IU}\cdot\text{ml}^{-1}$, cell viability was more than 90% all the time. In 91 days of cell culture, we have got 1800l supernatant contained 114 g u-PA (the ratio of scu-PA is >85%). Together with 310l supernatant from cell culture in a 7.5l bioreactor, about 80 g eligible u-PA (scu-PA ratio >80%) was obtained for clinical trial after purification (Hu, 2000).

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