EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 6
Issue: 5
590-595
Year: 2019

SUPERMACROPORUS CRYOGEL SYSTEM FOR CULTURING HYBRIDOMA CELLS

B. Premkumar[1]*, N. S. Jayaprakash[2] and M. A. Vijayalakshmi[2]

[1]Department of Biotechnology, Bhaskar Pharmacy College, Yenkapally, Moinabad (M), Ranaga Reddy (Dt), Hyderabad- 500 075, Telangana, India.
[2]Centre for Bio-separation Technology, VIT University, Vellore – 632 014, Tamil Nadu, India.

***Corresponding Author: B. Premkumar**
Department of Biotechnology, Bhaskar Pharmacy College, Yenkapally, Moinabad (M), Ranaga Reddy (Dt), Hyderabad- 500 075, Telangana, India.

Article Received on 21/03/2019 **Article Revised on 11/04/2019** **Article Accepted on 02/05/2019**

ABSTRACT
In this experimental study, a mini-bioreactor setup was developed with cryogel matrix (35.32ml bed volume) for hybridoma cell H9E10 secreting antibodies against β-lactoglobulin are immobilized and evaluated for mAb production. The hybridoma cells were inoculated inside the gelatin-cryogel column by passing the cell suspension (25ml; 2.2 x 10^7). The initial flow through of about 6ml was collected which contained about 6.6 x 10^5 cells. The column retained about 19ml of the cell suspension which contained a total cell number of about 2.134 x 10^7 cells. The cells were left to grow and to establish themselves in the cryogel matrix for 12 h. After 12 h of incubation, we observed that the flow through (25ml) contained 9.2 x 10^5cells, which indicated that most of the cells got immobilized. A very few cells were detected in the flow through of the column after 48 h and as the cells were bound on the cryogel support matrix, the media was continuously circulated through the mini-bioreactor at a flow rate of 0.6 ml/min. The cryogel reactor was continuously run for a total period of 26 days until the gel became loose and cells started releasing out of the gel matrix.

KEYWORDS: Supermacroporous Cryogel Matrix, Monoclonal Antibody.

INTRODUCTION
A fantastic technological breakthrough was achieved by Kohler and Milstein in 1975[1] who devised a technique for the production of immortal hybridoma cell lines making monoclonal antibody specific for a single epitope on a complex antigen. The availability of monoclonal antibodies has revived interest in immunotherapy. The ability to influence on individual's immune state by administering immunoglobulin of the appropriate specificity may prove a powerful approach to disease control and prevention. Monoclonal antibodies have tremendous applications in the field of medical research, diagnosis and therapy. Since mAb are usually given in high doses over a long period of time as therapeutics, the capacity for manufacturing these products in mammalian cell cultures will be a challenge. With an increase in the number and demand for biopharmaceutical, there is a requirement for greater biomanufacturing capacity. However, in order to meet this clinical demand large-scale culture manufacturing capacity is significantly low.

The current commercial mAb productions are *in vivo* cultivation as ascites fluid in mouse and *in vitro* cell culture in flasks or bioreactors. Nowadays *in vitro* production technology plays a vital part than *in vivo* production due to the animal welfare concerns. Devices such as spinner flasks, roller bottles and T-flasks are typically used in the laboratories for *in vitro* production. These conventional low-density cell culture methods permit *in vitro* production of mAb, which are released in the culture medium at a concentration between 1 and 100µg mL-1.[2] A laboratory-scale cell culture named CELLine 1000, was developed by Trebek et al., which supports high cell density and generates a high concentration of mAb within a period upto 2 months.[3] However, these devices can only produce 1-2 L of culture per batch due to their inherently limited oxygen-transfer capabilities. Different types of cell culture bioreactor systems are available for larger quantities, for example, hollow fibre[4-6], wave bioreactor[7,8], fixed-bed[9,10], fibrous-bed[11,12], and stirred tank bioreactor.[13,14] High cell density and good long-term culture stability are two key factors in developing a continuous bioreactor system. Nevertheless, hybridoma cells are usually difficult to culture as suspension in a bioreactor because of their sensitivity to shear and bubble damage. Thus, various immobilization techniques have been studied, including entrapping cells in agarose, gelatin, and alginate beads, in collagen gel particles between two membrane sheets, in membrane-bound capsules, by cell adhesion to fibers, and microcarriers such as Cytopore and Cytodex. The materials mostly applied to cultivate hybridoma cells *in vitro* are probably tissue culture polystyrene, cellulose-acetate, or poly

(methyl methacrylate). These materials were developed to optimize the growth of different adhesion-dependent cells.

The cryogel monoliths have well served as separation matrices for microbial cells mammalian cells and viruses. In this work, we describe the supermacroporous polyacrylamide gel, called cryogel (pAAM-cryogel), matrix as a polymeric support for the cultivation of mouse hybridoma cells for monoclonal antibody production. Cryogels have a continuous system of interconnected macrospores with a size of 10-100 µm, allowing unhindered convectional mass transport of solutes of practically any size. The interest of this study is to know about the cryogel matrix as a support material for hybridoma production in DMEM medium with 10% FBS and in serum free medium.

MATERIALS AND METHODS

Materials: The cryogel cell culture matrix was gifted by Dr. Ashokkumar, I.I.T., Kanpur.

The monoclonal antibody secreting anti-beta lactoglobulin clone was gifted by Dr. Anjali Karande, I.I.Sc., Bangalore.

Chemicals: Dulbecco's Modified Eagle's Medium (DMEM), sodium pyruvate, fetal bovine serum (FBS), bovine serum albumin (BSA), beta-lactoglobulin, anti-mouse IgG-horseradish peroxidase (HRP) conjugate were purchased from Sigma, USA. GlutaMAX was obtained from Gibco, India. Tetra methyl benzidine (TMB)/H_2O_2 was purchased from Genei, India. All other reagents used were of analytical grade and the experiments were performed with milli-Q water.

Sterilization of the Gels

The gelatin-cryogel discs (2 nos., 75mm diameter and 4mm height each) were washed with sterile double distilled water (150ml) under sterile conditions for 1 h. The water was then replaced by 30% ethanol (150ml) and washed for 1 h. The 30% ethanol solution was then replaced by 50% ethanol (150ml) and again washed for 1 h. Finally, 50% ethanol was then replaced by 70% ethanol (150ml) and washed under shaking for 1 h. The gels were then washed six times with sterile phosphate buffer saline (PBS) (150ml) and then with excess sterile double distilled water under sterile conditions in order to remove any traces of ethanol and were used for the experiments.

Mini Bioreactor Set Up

The mini bioreactor set up was fabricated using glass and with lid made of rubber cork. The lid had two inlet ports; one for medium and the other for aeration. The glass vessel had one outlet port at the bottom connected to the medium reservoir. The whole set up was sterilized by autoclaving at 121^0C for 15 mins. After drying, the set up was attached to the fabricated stand inside the laminar hood.

Culturing of Cells on Gelatin-Cryogel Matrix

Before starting the experiments, the gels (2 discs, 75mm diameter and 4mm height each) were washed with 100ml of phosphate-buffered saline (PBS), 250ml of sterile double distilled water and then equilibrated with 50ml of DMEM containing GlutaMAX, sodium pyruvate and supplemented with 10% (v/v) fetal bovine serum and penicillin-streptomycin solution. Then, one of the sterile gelatin-cryogel discs was placed in the glass apparatus, such that it filled up the volume and did not allow the liquid to pass between the column wall and the gel. Hybridoma cells (25ml; 2×10^7 cells) suspended in culture media was prepared and 12ml of this was applied to the gelatin-cryogel column. About 6ml of flow through was collected and the column outlet was then closed. One more disc was placed above the inoculated disc and the remaining cell culture suspension (13ml) was applied into the gel column bed and medium was added till it submerged the cryogel discs. The cells were allowed to bind to the matrix by incubating the column at 37°C in a 5% humidified CO_2 incubator for 12 h without media flow. After incubation, the gelatin-cryogel discs column was connected to a 200ml media reservoir (DMEM with 10% FBS) and the medium was circulated through the column at a flow rate of 0.6 ml/min. Air filters (pore diameter, 0.2 µm) were attached in the inlets provided in the medium reservoir and the column apparatus for exchange of carbon dioxide from the CO_2 incubator. Initial flow through (25ml) from the column was collected and analyzed for the presence of unattached cells. Cell number and viability were counted with (0.1% (w/v)) trypan blue on a hemocytometer. Cell viability was estimated from the ratio of viable cell count to total cell count. The bioreactor was continuously operated for 26 days and samples were taken periodically to assess glucose consumption and lactic acid production. The mAb production was determined by indirect ELISA.

Analytical Methods

Cell Density and Viability

Cell number and viability were counted with (0.1% (w/v)) trypan blue on a hemacytometer. Every sample was counted three times, and an average cell density was calculated. Cell viability was estimated from the ratio of viable cell count to total cell count.

Glucose, Lactate, and mAb Concentrations

The glucose consumption was measured with the help of a glucose kit (GOD/POD method) from Span diagnostics. Lactic acid production was determined by the help of a lactate assay kit from Trinity biotch. The mAb concentration was analyzed using the enzyme-linked immunosorbent assay (ELISA). Briefly, each of the 96-wells flat bottom microtiter plate (Griener bio-one, Germany) was coated with commercial beta-lactoglobluin (1 µg/ml) prepared in 100 mM carbonate buffer, pH 9.6 and the plate was incubated for 2 hours at 37ºC. The plate was then washed thrice with PBS-Tween and blocked with 1% BSA for 1 h at 37ºC. Cell culture

supernatant and standard mAb was added in duplicate and incubated for 1 h at 37ºC. After washing (thrice) with PBS-Tween, the plate was incubated for 1h with 2°Ab anti-mouse IgG-HRP conjugate (1:3000) in 0.5% BSA. TMB/H_2O_2 substrate solution was added and reaction was stopped by adding 2 M H_2SO_4. Absorbance at 450 nm was measured by using a plate reader (FLUOstar Optima, BMG LABTECH, Germany).

RESULTS AND DISCUSSION

Cultivation of Cells on Supermacroporous Cryogel Scaffolds: In this experimental study, a mini-bioreactor setup was developed with cryogel matrix (35.32ml bed volume) for hybridoma cell immobilization and evaluated for mAb production pattern using DMEM supplemented with GlutaMAX and 10% fetal bovine serum. The mini-bioreactor set up for the production of mAb using cryogel-based matrix is shown in (Fig 1). The hybridoma cells were inoculated inside the gelatin-cryogel column by passing the cell suspension (25ml; 2.2 x 10^7). The initial flow through of about 6ml was collected which contained about 6.6 x 10^5 cells. The column retained about 19ml of the cell suspension which contained a total cell number of about 2.134 x 10^7 cells. The cells were left to grow and to establish themselves in the cryogel matrix for 12 h. After 12 h of incubation, we observed that the flow through (25ml) contained 9.2 x 10^5cells, which indicated that most of the cells got immobilized. A very few cells were detected in the flow through of the column after 48 h and as the cells were bound on the cryogel support matrix, the media was continuously circulated through the mini-bioreactor at a flow rate of 0.6 ml/min. This flow rate was determined in such a way that no part of the gel surface was left dry during the run. The reactor was incubated in 5% CO_2 incubator with control temperature at 37^0C. Samples (1ml) were taken periodically from the reactor outlet port to monitor the growth of cells and antibody production. The cryogel reactor was continuously run for a total period of 26 days until the gel became loose and cells started releasing out of the gel matrix.

As the cell density cannot be monitored directly from the reactor setup the glucose consumption, lactic acid and monoclonal antibody production was used as a marker of cell growth (2). The glucose and lactic acid concentration was determined with the help of Glucose test kit (GOD/POD Method) & Lactate test kit (Enzymatic method). During this mini-bioreactor experimental study, we observed that during the first 12 h of incubation period without any circulation of medium, the cells almost completely got attached inside the gel which was found out by cell counting from the initial flow through. Also, from the initial flow through it was observed that there was a rapid decrease in the glucose content and increase in the lactic acid concentration. This phenomenon indicated that the cells were still alive and had metabolic activity inside the cryogel column. Then the medium was circulated from a 200 ml medium reservoir with a flow rate of 0.6 ml/min. The medium was continuously circulated through the cryogel column until the glucose concentration went below ~ 50% (Fig 2). During this study we observed that the uptake of glucose was lower for the first two days followed by increase in glucose uptake from day four. The glucose content decreased during the growth phase with a consumption rate of 3.58mM/day whereas lactate production rate was 4.7mM/day. The medium was continuously circulated through the cryogel column for 4 days until the glucose content dropped to 50% of the initial concentration. The monoclonal antibody concentration reached 62.91µg/ml. On fourth day, the glucose concentration in the medium reservoir was found to be 8.16mM (i.e. 50% of the initial concentration). Hence, the medium from the reservoir-1 was replaced with fresh medium (reservoir-2). The glucose consumption rate of the reservoir-2 was found to be 6.64mM/day which was higher than from that of the reservoir-1. The production rate of lactic acid and monoclonal antibody were 9.4mM/day and 81.24µg/ml respectively which were also found to be higher than that of the reservoir-1.

The glucose content decreased rapidly to 9.23mM (50% of initial concentration) within 2 days from the day of medium reservoir change and also the lactic acid increased to 18.8mM. Once again, the medium reservoir was changed and recirculated. The mAb concentration and overall productivity of hybridoma cells during medium reservoir 2 is shown in Table 1. It was observed that the glucose consumption rate, lactic acid and mAb production was higher than that from the medium reservoir 1. Then onwards, the medium was changed every 2 days as the glucose concentration reached below 50% of the initial concentration after every 2 days (Fig 2). The set up was operated continuously for 26 days. Similarly, samples were taken individually from reservoir-3 to reservoir-12 for the determination of glucose uptake, and lactate and mAb production. The results showed that the glucose uptake and lactate production rate were almost similar (Fig 2 and Fig 3). However, it was noted that the mAb productivity increased from day 4 to day 20. Thereafter the mAb productivity started to decline gradually (Fig 4). The results shown in Table 1 clearly indicated that the maximal mAb concentration and overall productivity of hybridoma cells during medium reservoir-9 was higher than that from other medium reservoirs. These results revealed that after cell grew for 20 days in cryogel column, the toxic by-products that produced from cells induced apoptosis and limited antibody production even when fresh medium (reservoir 10) was replaced in the system[11] have cited that apoptosis can occur when the concentration of lactate in culture medium increases.

When the medium reservoir-10 was recirculated, we found that the rate of uptake of glucose was similar to previous reservoir but the concentration of cell debris in the medium reservoir increased, as observed from microscopic examination of the samples from the

production column. Previous studies describes that animal cells grown in bioreactor also undergo apoptosis, which accounts for most of the cell deaths.[15] Many previous reports have cited that accumulation of toxic byproduct such as lactate has an inhibitory effect on cell growth and antibody production.[16,17] Excessive lactate buildup can result in increased medium osmolarity or, in the absence of pH control, decreased culture pH. Consumption of glucose by cells, especially when present at high concentrations in the medium, is done at a higher rate than necessary. This leads to the rapid accumulation of toxic metabolite (lactate) that can inhibit cell growth and / or protein productivity, as well as affect product's glycosylation. We observed that the mAb productivity started to decline (Fig 4) after day 20 and this was associated with a significant cell loss (from day 22 onwards) in the product harvest medium out of the bioreactor. The maximum secretion of mAb was observed on twentieth day with a concentration of 236µg/ml.

The cumulative monoclonal antibody concentrations in the mini-bioreactor which was run for 26 days was found to be 383mg in 2.4 L medium (DMEM 10%FBS) which is equivalent to 160µg/ml (Fig 5). This shows that the production of mAb in the cryogel bioreactor was more efficient while comparing it with the standard cell culture flasks. The major benefit of this cryogel based system can be its use as a disposable bioreactor for process development of monoclonal antibody production. The unique advantage of this mini bioreactor set up is simple operation with the added benefit of continuous control over production levels during a run.

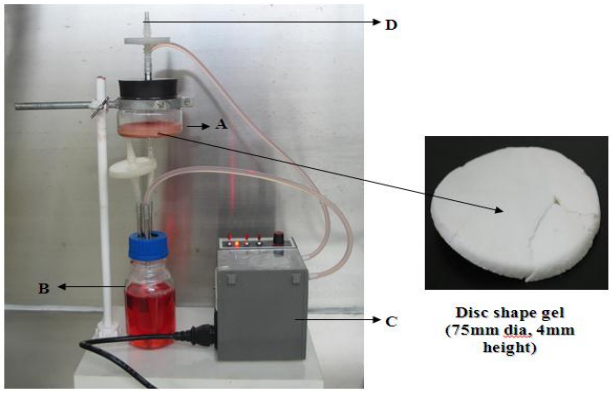

Figure. 1: Mini-bioreactor setup for mAb production using disc shaped supermacroporous cryogel matrix. (A) Cell culture device – where cells attach and grow on a gelatin-cryogel matrix; (B) Medium reservoir; (C) Pump; (D) 0.2µ air filter.

Days	Glucose (mM)
2	14.06
4	8.16
6	9.23
8	8.9
10	9.1
12	7.87
14	7.75
16	7.17
18	8.03
20	7.7
22	6.88
24	8.05
26	7.21

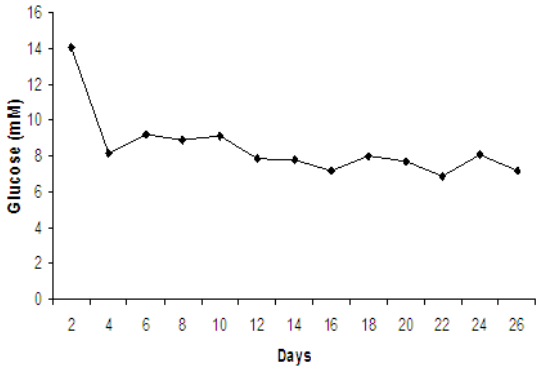

Figure. 2: Glucose consumption from cryogel discs in medium reservoir. Medium was changed on Day 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24 and 26.

Days	Lactic acid (mM)
2	13.63
4	18.8
6	18.8
8	19.14
10	19.7
12	19.2
14	19.08
16	19.17
18	18.99
20	19.08
22	19.2
24	18.75
26	18.98

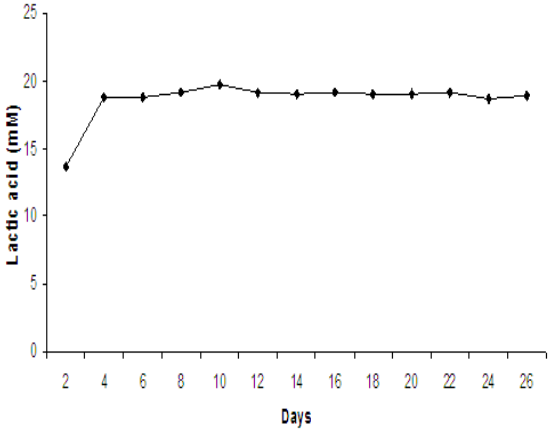

Figure. 3: Lactic acid production from cryogel discs in medium reservoir. Medium was changed on Day 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24 and 26.

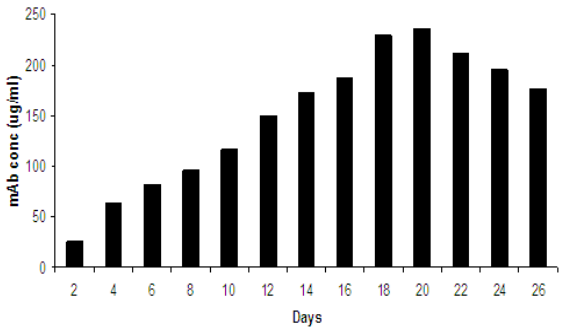

Figure. 4: Monoclonal antibody production from cryogel discs in medium reservoir. Medium was changed on Day 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24 and 26.

Days	Conc (µg/ml)
2	26.25
4	62.91
6	81.24
8	95.29
10	116.66
12	150.1
14	172.72
16	187.39
18	228.64
20	236.44
22	211.37
24	195.18
26	176.52

Table. 1: Cell growth and monoclonal antibody production in mini-bioreactor system using cryogel matrix.

	Reservoir 1 (Day 1 to 4)	Reservoir 2 (Day 6)	Reservoir 3 (Day 8)	Reservoir 6 (Day 14)	Reservoir 9 (Day 20)	Reservoir 12 (Day 26)
Glucose Consumption Rate (mM/day)	3.58	6.64	6.81	7.38	7.4	7.65
Lactic Acid Production (mM/day)	4.7	9.4	9.57	9.54	9.54	9.49
mAb Concentration (µg/ml)	62.91	81.24	95.29	172.72	236.44	176.52

Days	Conc µg/200ml
2	
4	12582
6	16248
8	19058
10	23332
12	30020
14	34544
16	37474
18	45728
20	47288
22	42274
24	39036
26	35304
	382888 µg in 2400ml
	160 µg/ml

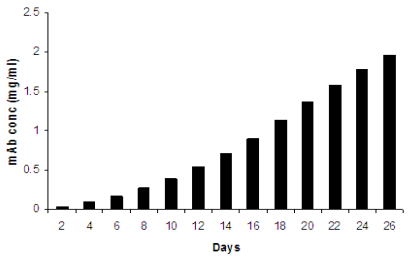

Figure. 5: The cumulative monoclonal antibody production in the cryogel based mini-bioreactor system.

CONCLUSION

The supermacroporous cryogel matrices was successfully used as a support material for culturing hybridoma cell lines over a relatively longer mAb production period, and interestingly more productivity can be achieved than from the conventional T- flask batch cultivation. The major advantage of the cryogel column can be its use as a disposable bioreactor for process development of monoclonal antibody production and it represents a new supporting material which can be operated by any person trained in tissue culture.

ACKNOWLEDGEMENTS

The authors thank gratefully Dr. Ashok Kumar, Indian Institute of Technology (IIT), Kanpur, India for the gift

of cryogels and valuable discussions. The authors wish to thank Prof. Anjali A. Karande, Dept. of Biochemistry, Indian Institute of Science (IISc), India for providing the clone. The authors would like to thank Department of Science and Technology (DST), India for the financial support.

REFRENCES

1. Kohler, G.; Milstein, C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature, 1975; 256: 495-497.
2. Nilsang S, Nandakumar KS, Galaev IY, Rakshit SK, Holmdahl R, Mattiasson B, Kumar A. Monoclonal Antibody Production Using a New Supermacroporous Cryogel Bioreactor. Biotechnol Prog. 2007 Jul-Aug; 23(4): 932-9.
3. Trebak, M.; Chong, J. M.; Herlyn, D.; Speicher, D. W. Efficient laboratory-scale production of monoclonal antibodies using membranebased high-density cell culture technology. J. Immunol. Methods, 1999; 230: 59-70.
4. Curcio, E.; De Bartolo, L.; Barbieri, G.; Rende, M.; Giorno, L.;Morelli, S.; Drioli, E. Diffusive and convective transport through hollow fiber membranes for liver cell culture. J. Biotechnol, 2005; 117: 309-321.
5. Gramer, M. J.; Maas, J.; Lieberman, M. M. Use of hollow fiber systems for rapid and direct scale up of antibody production from hybridoma cell cultured in CL-1000 flasks using BD Cell mAb medium. Cytotechnology, 2003; 42: 155-162.
6. Jackson, L. R.; Trudel, L. J.; Fox, J. G.; Lipman, N. S. Evaluation of hollow fiber bioreactors as an alternative to murine ascites production for small scale monoclonal antibody. J. Immunol. Methods, 1996; 189: 217-231.
7. Singh, V. Disposable bioreactor for cell culture using wave induced agitation. Cytotechnology, 1999; 30: 149-158.
8. Tang, Y-J.; Ohashi, R.; Hamel, J. F. P. Perfusion culture of hybridoma cells for hyperproduction of IgG2a monoclonal antibody in a wave bioreactor-perfusion culture system. Biotechnol. Prog. 2007; 23: 255-264.
9. Portner, R.; Ludemann, I.; Markl, H. Dialysis cultures with immobilized hybridoma cells for effective production of monoclonal antibodies. Cytotechnology, 1997; 23: 39-45.
10. Van der Pol, J. J.; Machnik, M.; Biselli, M.; Portela-Klein, T.; de Gooijer, C. D.; Tramper, J.; Wandrey, C. On-line immunoanalysis of monoclonal antibodies during continuous culture of hybridoma cells. Cytotechnology, 1997; 24: 19-30.
11. Luo, J.; Yang, S. T. Effects of three-dimensional culturing in a fibrous matrix on cell cycle, apoptosis and mAb production by hybridoma cells. Biotechnol. Prog. 2004; 20: 306-315.
12. Zhu, H.; Yang, S. T. Long-term continuous production of monoclonal antibody by hybridoma cells immobilized in a fibrousbed bioreactor. Cytotechnology, 2004; 44: 1-14.
13. Dong, H.; Tang, Y. J.; Ohashi, R.; Hamel, J. F. P. A Perfusion culture system using a stirred ceramic membrane reactor for hyperproduction of IgG2a monoclonal antibody by hybridoma cells. Biotechnol. Prog. 2005; 21: 140-147.
14. Stoll, T. S.; Chappaz, A.; Stockar, U.; Marison, I. W. Effects of culture conditions on the production and quality of monoclonal IgA. Enzyme Microb. Technol, 1997; 21: 203-211.
15. Wang, D.; Liu, W.; Han, B.; Xu, R. The bioreactor: A powerfultool for large-scale culture of animal cells. Curr. Pharm. Biotechnol, 2005; 6: 397-403.
16. Newland M., Greenfield P.F. and Reid S. Hybridoma growth limitations: the roles of energy metabolism and ammonia production. Cytotechnology, 1990; 3: 215–229.
17. Ozturk, S. S., M. R. Riley and B. O. Palsson. Effects of ammonia and lactate on hybridoma growth, metabolism, and antibody production. Biotechnol Bioeng, 1992; 39(4): 418-31.