WINTER 2015/2016 • Volume 14/Issue 4 • ISSN 1538-8786

BioProcessing JOURNAL

Trends & Developments in BioProcess Technology

A Production of BioProcess Technology Network

BioProcessing JOURNAL
TRENDS & DEVELOPMENTS IN BIOPROCESS TECHNOLOGY

Long-Term CB.Hep-1 Monoclonal Antibody Production in a Single-Use Bioreactor on a Rocker Platform with Serum- and Protein-Free Media

By Alberto Galván, Rodolfo Valdés, Marcos González, Hasel Aragón, Daily Hernández, Sigifredo Padilla, Leonardo Gómez, David Gailán, Yanet Villegas, Andrés Tamayo, Adelma Pérez, Bárbara Pérez, Maylín Lao, Yodelis Calvo, Aneet Fernández, Lianys Lee, Jania Suarez, Judey García, and Eduardo Ojito

Abstract

Rocker bag bioreactors have been used successfully in cultivating cells because they provide good nutrient distribution and cell suspension while eliminating the need to validate cleaning and sterilization. Therefore, this study examined the long-term performance of a 50 L single-use bag bioreactor on a rocking platform in CB.Hep-1 monoclonal antibody (mAb) production. For such a purpose, the bioreactor was operated in a continuous mode with a mixture of serum-free media (SFM) for 62 days, and with protein-free medium (PFM) for another 62 days.

Stationary phase culture results with SFM were: cell concentration of $1.57 \pm 0.2 \times 10^6$ cells mL^{-1}, specific growth rate of (μ) = 0.0202, cell viability of 91.1 ± 6.4%, mAb concentration of 44.6 ± 11.1 µg mL^{-1}, cell-specific secretion of 28.8 ± 8.1 pg $cell^{-1}$, and mAb yield of 42.4 ± 4.0 mg L^{-1}. After SFM was replaced by PFM, there were statistically different results ($p<0.05$) in a cell concentration of $2.56 \pm 0.2 \times 10^6$ cells mL^{-1}, cell viability of 97.0 ± 1.1%, mAb concentration of 90.1 ± 28.2 µg mL^{-1}, and mAb yield of 76.6 ± 10.6 mg L^{-1}. However, the cell-specific secretion of 38.0 ± 6.0 pg $cell^{-1}$ was statistically similar, but only with the first batch run with SFM.

Average purification recovery from the SFM and PFM supernatants was 74.6 ± 7.9% and 70.9 ± 11.2%, respectively. The recovery and biochemical properties of the CB.Hep-1 mAb cultured with either media composition were similar to those found with CB.Hep-1 mAb produced by ascites. Regardless of whether SFM or PFM is used, it can be concluded that the 50 L single-use bag bioreactor on a rocker platform was suitable for long-term CB.Hep-1 production and can result in a 36 g yield in 125 days.

Introduction

In 1984, Köhler and Milstein received the Nobel Prize in Medicine for their original work on monoclonal antibody (mAb) generation. One of the most significant points of this discovery was the development of an immortalized cell line to generate a continuous source of antibodies with high antigen specificity.[1] Initially, mAbs were only used as laboratory reagents, but they were quickly accepted as diagnostic reagents, and finally as therapeutic agents. Since their discovery, an endless number of mAbs have been generated all over the world, and today are the most studied, applied, and marketed molecules.[2,3] In 1985, the first commercial mAb, Orthoclone® OKT3 (developed by Ortho Pharmaceuticals), was approved to prevent transplanted kidney rejection.[4]

One year later in 1986, the CB.Hep-1 mAb, which is specific for the hepatitis B surface antigen (HBsAg), was developed for large-scale antigen immunopurification of human vaccinations by the Monoclonal Antibody Division of the Cuban Center for Genetic Engineering and Biotechnology (CIGB).[5] Since this time, the antibody has been used to help eradicate the hepatitis B disease in Cuba, plus serve in an international immunization program, which is considered an extraordinary contribution by the Cuban Institute of Biotechnology and Medicine.[6,7]

Even so, the application of this mAb for large-scale production wasn't possible because of the lack of large bioreactors at CIGB. Appropriate bioreactor designs weren't readily available and there was an insufficient supply of good cell culture media. Furthermore, mammalian cell metabolism wasn't well understood by our group, we had little to no experience with mAb production *in vitro*, and our yields were very low (< 50 µg/L). Because of the very high capital investment that would have been required to scale up, we could not justify building a production facility.

In subsequent years, several approaches have been used to produce CB.Hep-1 mAb such as ascites, hollow fiber bioreactors, antibody fragments produced in bacteria, and more recently, transgenic plants.[8,9]

In spite of the reduction in hepatitis B vaccine prices worldwide, remarkable advances in mammalian cell culture technologies have made it possible for our Center to develop large-scale production capabilities for the CB.Hep-1 mAb. Also, since there are ethical concerns about the use of animal-derived raw materials for production, and the use of transgenic plants hasn't gained full acceptance, we have moved toward an ever-increasing use of disposable bioreactors.

Single-use bag bioreactors have become widely accepted for cell culture in both process development and clinical manufacturing. Their basic advantages over traditional stainless steel bioreactors, which are still considered the industry gold standard, are they can be used in an unclassified manufacturing area, the process validation and equipment qualifications are much easier, and they offer design flexibility, low investment costs, and a lower risk of cross-contamination.

Despite our initial plans to move forward with CB.Hep-1 mAb process scale-up in disposable, stirred-tank bioreactors, we investigated the long-term performance of a 50 L bag rocker platform. We chose to produce the CB.Hep-1 mAb using serum-free media (SFM) and protein-free media (PFM). Additionally, this study included a biochemical characterization of the purified CB.Hep-1 mAb against that of the mAb produced in ascites.

Materials and Methods

CB.Hep-1 Hybridoma Source

The Sp2/0-Ag14 derived hybridoma, CRL-1581™ (ATCC®), for CB.Hep-1 was obtained using a BALB/c mouse subcutaneously immunized with the HBsAg that had been emulsified in complete and Freund's incomplete (SAFC) adjuvants. Cells were cultivated in 1 L spinner flasks starting with 3×10^5 cells mL^{-1} and using RPMI-1640 (Gibco® by Thermo Fisher) media supplemented with 8% fetal bovine serum (FBS) (Gibco), 2 mM L-glutamine, 1 mM sodium pyruvate, and 17 mM sodium bicarbonate. The cells were maintained at 37 °C in a 5% CO_2 atmosphere, and the media was replaced every 48 hours until the cells reached the highest cell density.[5]

Cell Counting and Viability

Cell counting and viability were measured by the trypan blue exclusion method using a hemocytometer as a counting chamber.[10] The microscope used was a Leica model 090131001.

Cell Adaptation

To start the adaptation procedure, the cell line was first maintained in T-75 cm^2 flasks (Corning® Costar®) in a CO_2 incubator at 37 °C under an atmosphere of 95% air and 5% CO_2. Following this, the cell culture medium adaptation was carried out in spinner flasks by diluting the cell suspension 1:4 through the addition of SFM every two days. The SFM used was MyeloCIM and CP-CHO (Merck Millipore). After 15 days of sequential adaptation, the cells were considered adapted to SFM and were transferred to a single-use bioreactor. The cell concentration used for all stages of scale-up was 3×10^5 cells mL^{-1}.

Ascites Production

BALB/c mice at 24 ± 1 g of weight were used for ascites production. The animals were maintained at 22 ± 2 °C and 65–80% relative humidity with a low level of ammonia. Ten days before cell inoculation, the animals were primed with 0.5 mL mineral oil into the abdominal cavity. Ascites cells were harvested by abdominal paracentesis under aseptic conditions inside a sterile hood. After harvesting was complete, ascites was centrifuged at 2000 ×g to remove cells from the liquid phase.

FIGURE 1. 50 L Appliflex single-use bioreactor used to produce CB.Hep-1 hybridoma cell supernatant.

CB.Hep-1 mAb Supernatant Production

The 50 L Appliflex single-use bag bioreactor (Applikon® Biotechnology) (**Figure 1**) was inoculated from spinner flasks at 0.3×10^6 CB.Hep-1 hybridoma cells mL^{-1} using a mixture of SFM CP-CHO and MyeloCIM SFM media (v/v). The cells were harvested from the spinner flasks during the exponential growth phase with a 96.0% viability and a specific secretion rate equal to 40.8 pg immunoglobulin G (IgG) $cell^{-1}$. The bioreactor operating

parameters are described in **Table 1** where the maximum replacement volume of fresh media/reactor working volume/day (v/v/d) was 0.5. After 62 days of cultivation, the SFM mixture was replaced with PFM (PFHM-II, Gibco) to determine if the maximum viable cell concentration could be increased.

CB.Hep-1 mAb Purification from Ascites

The ascites was harvested, filtered, and purified with protein A Sepharose® Fast Flow (PASFF, GE Healthcare Life Sciences) affinity chromatography.[11] We used 150 mM PBS at pH 8.0 as the adsorption buffer, and 100 mM citric acid at pH 3.0 as the elution buffer. The column was an XK50/30 (GE Healthcare Life Sciences) which was run at 100 cm h^{-1}. Extensive washings with 150 mM PBS at pH 8.0 were done to remove endotoxins and other protein contaminants. Subsequently, the purified mAb was incubated in 100 mM citric acid at pH 3.0 and 4 °C as a virus inactivation step. Afterward, the sample buffer was changed to 20 mM Tris/150 mM NaCl at pH 7.6 and used with size-exclusion chromatography (SEC), Sephadex G-25 (coarse) (GE Healthcare Life Sciences) in an XK50/30 column, operated at 130 cm h^{-1}. The recovered material was finally filtered under sterile conditions with a 0.22 µm membrane filter.

CB.Hep-1 mAb Purification from Cell Culture Supernatant

Cell culture supernatant was continuously harvested and centrifuged (model SCR7BA, Hitachi) at 1500 ×g for ten minutes. Then it was filtered with a 0.22 µm filter and concentrated with a 30 kDa SARTOFLOW® Alpha cartridge (Sartorius Stedim Biotech). It was then purified by PASFF affinity chromatography using 150 mM PBS at pH 8.0 as an adsorption buffer and 100 mM citric acid at pH 3.0 as the elution buffer. An XK50/30 column was operated at 100 cm h^{-1}. Extensive washings with 150 mM PBS at pH 8.0 were done to remove other contaminants. Next, the purified mAb was incubated in 100 mM citric acid at pH 3.0 and 4 °C as a virus inactivation step. Afterward, the sample buffer was changed to 20 mM Tris/150 mM NaCl at pH 7.6 with SEC using Sephadex G-25 (coarse) in an XK50/30 column operated at 130 cm h^{-1} and the resulting material was filtered under sterile conditions with a 0.22 µm membrane filter.

Colorimetric Assay for Protein Quantification

Protein quantification was performed using the procedure described by Lowry *et al*.,[12] and the calibration curve ranged from 10–100 µg mL^{-1}. Absorbance was measured at 730 nm in an Ultrospec UV/visible spectrophotometer (GE Healthcare Life Sciences).

Enzyme-Linked Immunosorbent Assay (ELISA) Used for CB.Hep-1 mAb Quantification

MaxiSorp® microtiter plates (Nunc/Thermo Scientific) were coated with 100 µg mL^{-1} of rec-HBsAg in carbonate/bicarbonate buffer at pH 9.6. After incubation, the plates were washed. Standard and control samples were diluted in 150 mM PBS, 0.2% bovine serum albumin, and 0.005% Tween 20. The plates were incubated again for 20 minutes at 50 °C. Then the wells were washed five times and incubated for 20 minutes at 50 °C with 100 µL $well^{-1}$ of a goat anti-mouse polyclonal antibody preparation and horseradish peroxidase conjugate. The plates were given a final wash and the reaction was revealed with *o*-phenylenediamine (OPD) as the substrate with 0.015% H_2O_2 and a citrate buffer at pH 5.0. The reaction was stopped by adding 50 mL of 2 M H_2SO_4, and the results were measured immediately at 492 nm with a Multiskan ELISA Reader (Thermo Scientific/Labsystems).[13]

TABLE 1. Operating parameters for the main 50 L Appliflex single-use bioreactor.

Operating Parameters	Values
Agitation rate	10–15 rpm
Acceleration	100%
Deceleration	100%
Platform angle	13°
Air supply	100 L min^{-1}
O_2 supply	50–400 L min^{-1}
CO_2 supply	0.1–0.2 mL min^{-1}
pH range	6.7–7.2 pH
Temperature value	37 °C
Total volume	50 L
Maximum filling level	20 L
Seeding cell density	0.3×10^6 cells mL^{-1}
Initial filling level	12 L
Serum-free cell culture media*	Run 1, days 1–33 Run 2, days 34–46 Run 3, days 47–62
Protein-free cell culture media**	Run 4, days 63–125

*A mixture of MyeloCIM and CP-CHO; **PFHM-II

ELISA Used to Determine CB.Hep-1 mAb Affinity Constant (K_{aff})

The affinity constant was determined with the method described by Beatty *et al*.[14] Microtiter plates were coated with the rec-HBsAg and then incubated with CB.Hep-1 mAb. The plates were sequentially incubated with the goat anti-mouse IgG and horseradish peroxidase conjugate, and the reaction was revealed using OPD as the substrate with 0.015% H_2O_2 in citrate buffer at pH 5.0. The reaction was stopped by adding 50 mL of 2 M H_2SO_4. The amount of antibody adherent to the antigen on the plates was reflected by the enzyme product measured with an optical density at 492 nm using a Multiskan ELISA reader.

SDS-PAGE and SEC-HPLC Performed to Determine CB.Hep-1 mAb Purity and Molecular Homogeneity

The identity pattern and purity of the CB.Hep-1 mAb and rec-HBsAg were determined following the procedure described by Laemmli.[15]

Molecular distribution and purity of the mAb were also estimated by using a SEC-HPLC column with TSKgel G3000PW (600 mm/57.5 mm ID, TosoHaas). The chromatographic mobile phase was 150 mM PBS at pH 7.0, and 100 µg of sample was dissolved in 150 mM PBS at pH 7.0 and then directly applied into the system. The volumetric flow rate was 200 µL min^{-1} and the absorbance was measured at 226 nm.

CB.Hep-1 mAb Western Blot Analysis

Samples were transferred by a semi-dry electrophoretic transfer onto a nitrocellulose membrane and immunoblotted. The membrane was blocked with skim milk powder at 5% (w/v) and 0.05% (w/v) diaminobenzidine. Then 0.015% (v/v) H_2O_2 in a saline solution was used as the color substrate. CB.Hep-1 mAb was detected using the goat anti-mouse IgG and horseradish peroxidase conjugate.[16]

CB.Hep-1 mAb Isoelectrofocusing

A high-resolution electrophoretic technique was used to separate proteins and peptides based on their isoelectric points. We used a linear pH gradient ranging from 5.0–7.0 with PhastGel™ IEF (GE Healthcare), and a homogeneous polyacrylamide gel (5% T, 3% C).

CB.Hep-1 mAb Isotyping

The CB.Hep-1 mAb isotype was determined with a mouse mAb isotyping commercial kit (Hycult® Biotech) by following the manufacturer's recommendations.

ELISA to Quantify Staphylococcal Protein

To quantify the staphylococcal protein A, polystyrene plates (Costar, Corning) were coated with anti-protein A polyclonal antibodies. After sample addition, the goat anti-mouse IgG and horseradish peroxidase conjugate was applied, and the reaction was then revealed with peroxidase substrate solution. The absorbance was measured with a Multiskan ELISA reader using a 492 nm filter.[17]

Dot-Blot to Quantify Mouse DNA

The mouse DNA content in the purified CB.Hep-1 mAb samples was determined according to the method reported by Brown.[18] The oligonucleotides used as probes were 5′-GAAAAACGTG-3′and 5′-GATCCTCATTTTTCACGTTTTT-3′.

Mathematical Analysis

The specific growth rate (µ) was calculated as:

$$Xv + \int DXvdt = \mu \int Xvdt$$

where *Xv* is the concentration of viable cells. The CB.Hep-1 mAb secretion was expressed as pg of CB.Hep-1 mAb per cell. This amount was estimated by calculating the ratio of antibody concentration to cell concentration. Statgraphics® Plus v. 5.0 (Statpoint Technologies) and Excel 2003 software (Microsoft) programs were used as tools for statistical analysis. An analysis of variance (ANOVA) and a Duncan multiple range test were performed to compare mathematical results, and the statistical significance level used was consistently 0.005.

Results and Discussion

Mice have been used extensively for antibody-rich ascites production after intraperitoneal inoculation of hybridoma cells.[19] And even though it is a useful technique, it has been criticized as a cruel and unnecessary use of animals.[20] In addition, antibodies produced by the ascites method might potentially be contaminated by adventitious murine viruses, endogenous murine immunoglobulins, other antigenic compounds, mouse nucleic acids, and residual peritoneum priming substances. Any one of these contaminants poses additional difficulties in mAb purification.

As is well known, hybridomas are non-anchorage dependent cells which can be grown in suspension cell culture. So, the question becomes whether one specific hybridoma can be grown in culture at a high enough density for a sufficient period, and secrete enough mAb to replace the ascites method. Several successful techniques have been used to produce mAb in cell culture[21], and among them, single-use bag bioreactors on rocker platforms are one of the techniques known for their overall simplicity in producing cells to inoculate larger bioreactors.[22] In specific terms, the movement of a bag bioreactor's rocking platform facilitates mixing and continuous renovation of the culture medium surface, which allows a bubble-free surface aeration, vigorous cell growth, and efficient product formation.

The first bag (Wave®) bioreactors were developed by Singh *et al*.[23] They reported the successful scale-up of a number of processes to 100 L. Since then, several authors have also reported the extensive use of rocker bag bioreactors for different purposes.[22] Therefore, with the initial idea of moving forward with CB.Hep-1 mAb production scale-up in disposable, stirred-tank bioreactors, we first wanted to assess the long-term performance of a 50 L Appliflex single-use bioreactor. Our goal was to produce material we could characterize as a potential immunoligand for immunopurification of a biologic for human use. This data will be published in an upcoming paper.

Currently, the huge majority of protein experiments and productions using *in vitro* procedures are carried out in unsupplemented media. Several commercial SFM and PFM of good quality are now available from different suppliers[24, 25], and more than one of these formulations may be used in a single manufacturing process, with each one designed for a specific culture phase. With regard to CB.Hep-1 mAb production, previous experiments with CB.Hep-1 hybridoma cells showed that SFM were always more efficient than PFM.[8, 9] This is why the SFM media

was chosen for the cell adaptation and initial cultivation of CB.Hep-1 hybridoma cells in the 50 L Appliflex single-use bioreactor. With this approach, the adaptation period was relatively short (just 15 days) because the cells showed the maximum specific growth rate (μ) and cell density in only ten days.

To assess the long-term production potential of CB.Hep-1 mAb in the 50 L bioreactor (**Figure 1**), cells were seeded at 3×10^5 cells mL^{-1} in a culture volume of 12 L. The bag control parameters were: O_2 supply of 50 mL min^{-1}, CO_2 supply of 0.2 L min^{-1}, air supply of 1 L min^{-1}, pH of 7.0, temperature of 37 °C, rocker angle of 13°, acceleration of 100, deceleration of 100, and agitation rate of 10 rpm (**Table 1**). These conditions were maintained during the experiment. CB.Hep-1 hybridoma cells were cultivated from the SFM for the first 62 days, with the media equivalent of three batches and two suppliers, and finally in PFM. Under these conditions, the bag bioreactor was successfully operated in a continuous mode for 125 days without evidence of cell clogging or contamination.

It is well known that *in vitro* cell culture techniques are an increasingly good alternative to using live animals in experimentation. To take it a step further, developing cell culture processes that perform well without the need for animal-derived components, such as serum, is an even better option. Today, in many cases, SFM and PFM can be easily and effectively utilized, which is why we chose them for this study.

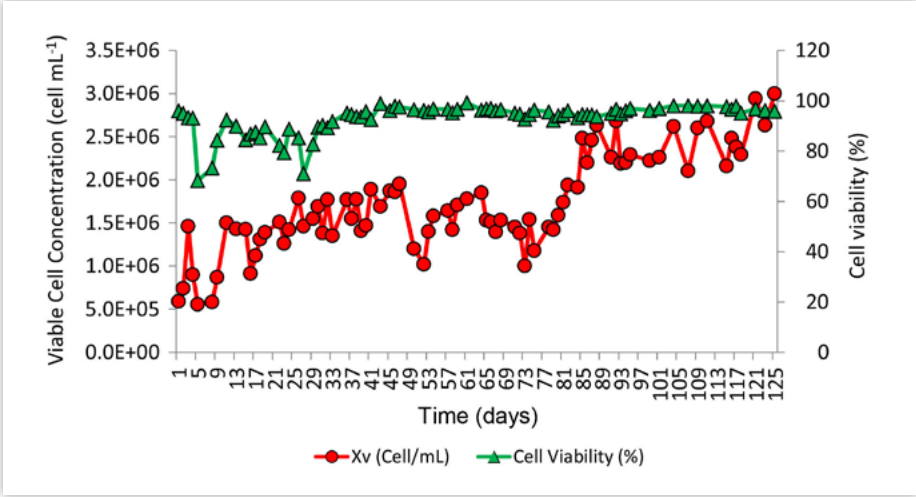

FIGURE 2. Viable cell concentration (*Xv*) and viability measured in the CB.Hep-1 mAb culture in a 50 L Appliflex single-use bioreactor using SFM and PFM for 125 days.

Additional benefits in using SFM for cell culture are that SFM usually does not contain albumin, steroids, and endogenous bovine immunoglobulins. The availability of SFM is a remarkable advance that simplifies the mAb purification process when compared to mAb purification for CB.Hep-1 mAb in serum-supplemented media.[26] In general, the composition of this type of media contains only some trace elements, intermediary metabolites, mineral compounds (mainly zinc), and a very low amount of defined proteins such as insulin and transferrin.[27]

As shown in **Figure 2**, the CB.Hep-1 hybridoma cells grew rapidly after they were inoculated into a bag bioreactor containing SFM. The relatively fast cell growth in SFM ($\mu = 0.0202$) resulted in a maximum cell concentration of $1.57 \pm 0.2 \times 10^6$ cells mL^{-1} and a cell viability of 91.1 ± 6.4% in two weeks. The sudden reduction observed in the cell concentration and cell viability at day 5 (68.9%) was provoked by problems with CO_2 supply and not the adaptation of cells to the new culture technology from spinner flasks.

Most importantly, the change experienced in the cell concentration for days 16–52 coincided with the change of SFM (0.91×10^6 cells mL^{-1} to 1.02×10^6 cells mL^{-1}); however differences in the pH values among medium batches were not reflected. This observation is essential for parenteral products because changes in the cell culture media (glucose, lactate, ammonia, and additives), and culture

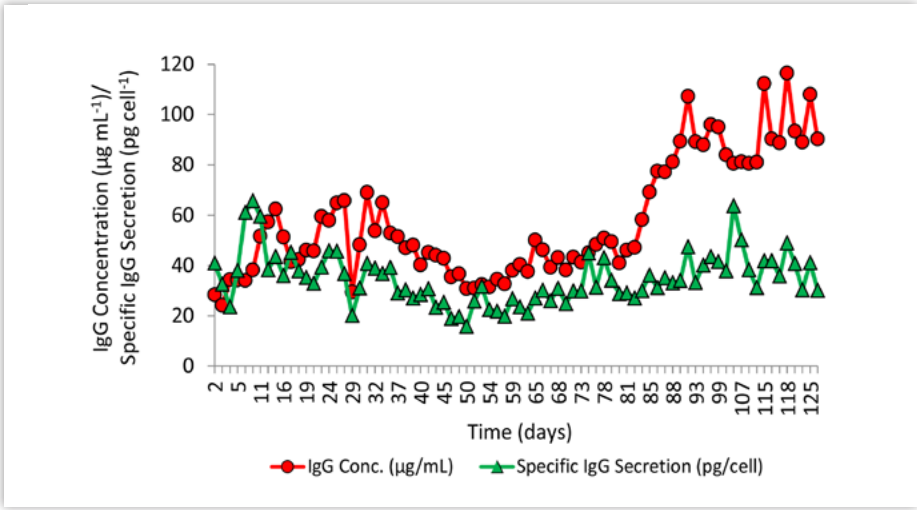

FIGURE 3. IgG concentration and IgG-specific secretion measured in the CB.Hep-1 mAb culture in a 50 L Appliflex single-use bioreactor using SFM and PFM for 125 days.

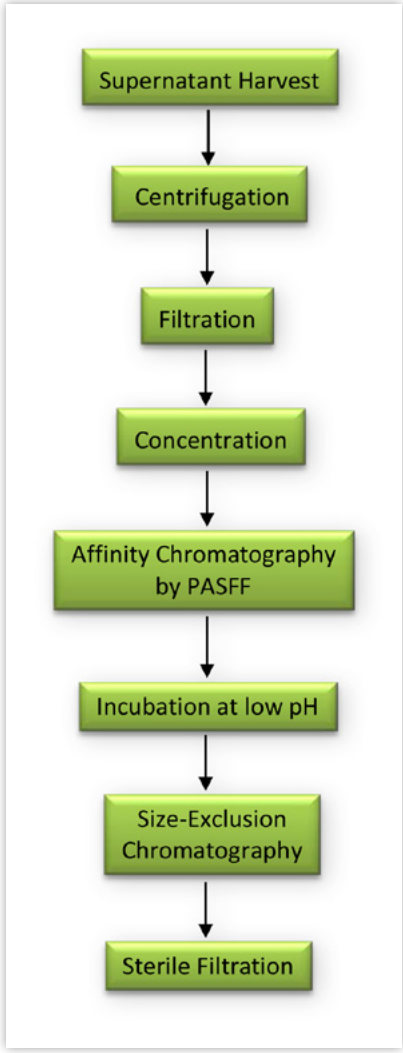

FIGURE 4. General flowchart of CB.Hep-1 mAb purification from cell culture supernatant.

conditions (dissolved O_2 and CO_2, and culture osmolality) may influence not only the yield but also antibody glycosylation. These factors can affect the activity of monosaccharide transferases and/or sugar transport to the golgi apparatus, which is the major glycosylation site in mammalian cell proteins. Although the integrity of protein backbones is usually unchanged across different culture conditions, this is most important for CB.Hep-1 mAb production because this antibody is used for immunopurification of a biologic[28], and the immobilization of the mAb does not involve carbohydrates.[29, 30]

During the SFM cultivation time, mAb production resulted in a concentration of 44.6 ± 11.1 µg mL^{-1} and 28.8 ± 8.1 pg $cell^{-1}$ as cell-specific secretion. The harvested IgG yield ranged from 38.1 ± 0.8 to 49.2 ± 4.9 mg/L of supernatants and 44 mg/L/day as average volumetric antibody productivity. As a consequence, the total mAb production was 18.6 g in 432 L of cell culture after 62 days (**Figure 3**). The designed purification process (**Figure 4**) efficiency of the CB.Hep-1 was expressed as mAb recovery, and purity was evidenced with an average recovery of 74.6 ± 7.9% (**Figure 5**) and 98.7 ± 0.5% of purity with SDS under reducing conditions (**Figures 6** and **7**, on the following page). Therefore, the process designed for the purification of CB.Hep-1 mAb in SFM supernatant increased the purity from 90.3% to 98.5%. This purity level of the supernatant samples would not be reached using serum-supplemented media. The content of mouse DNA was lower than 3.9 pg mg^{-1} mAb, while the protein A leakage never exceeded the specification limit of 10 ppm (4.4 ± 2.3 ppm). Characterization of the mAb-purified molecules demonstrated a mAb affinity constant of 2.1×10^{-9} M for an IgG_{2bk},

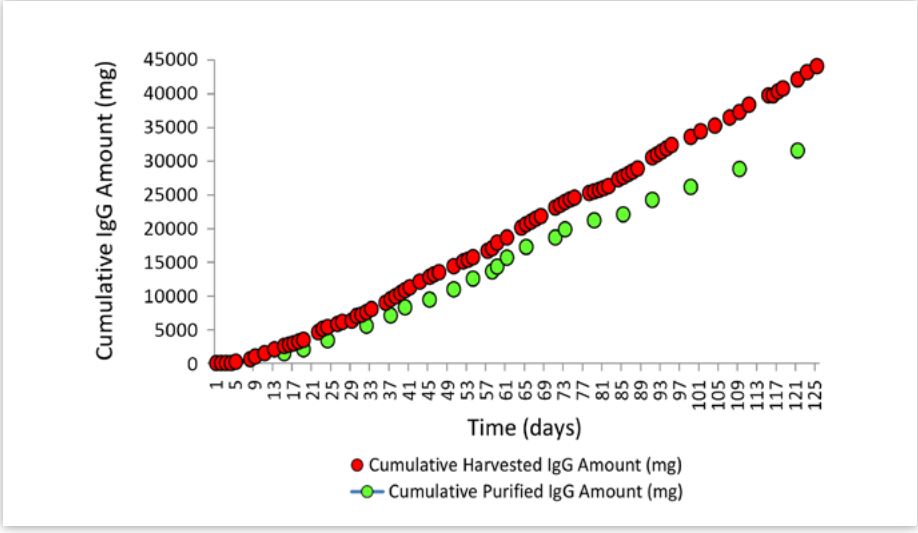

FIGURE 5. Cumulative harvested and purified IgG amount obtained from cell culture in a 50 L Appliflex single-use bioreactor using SFM and PFM for 125 days.

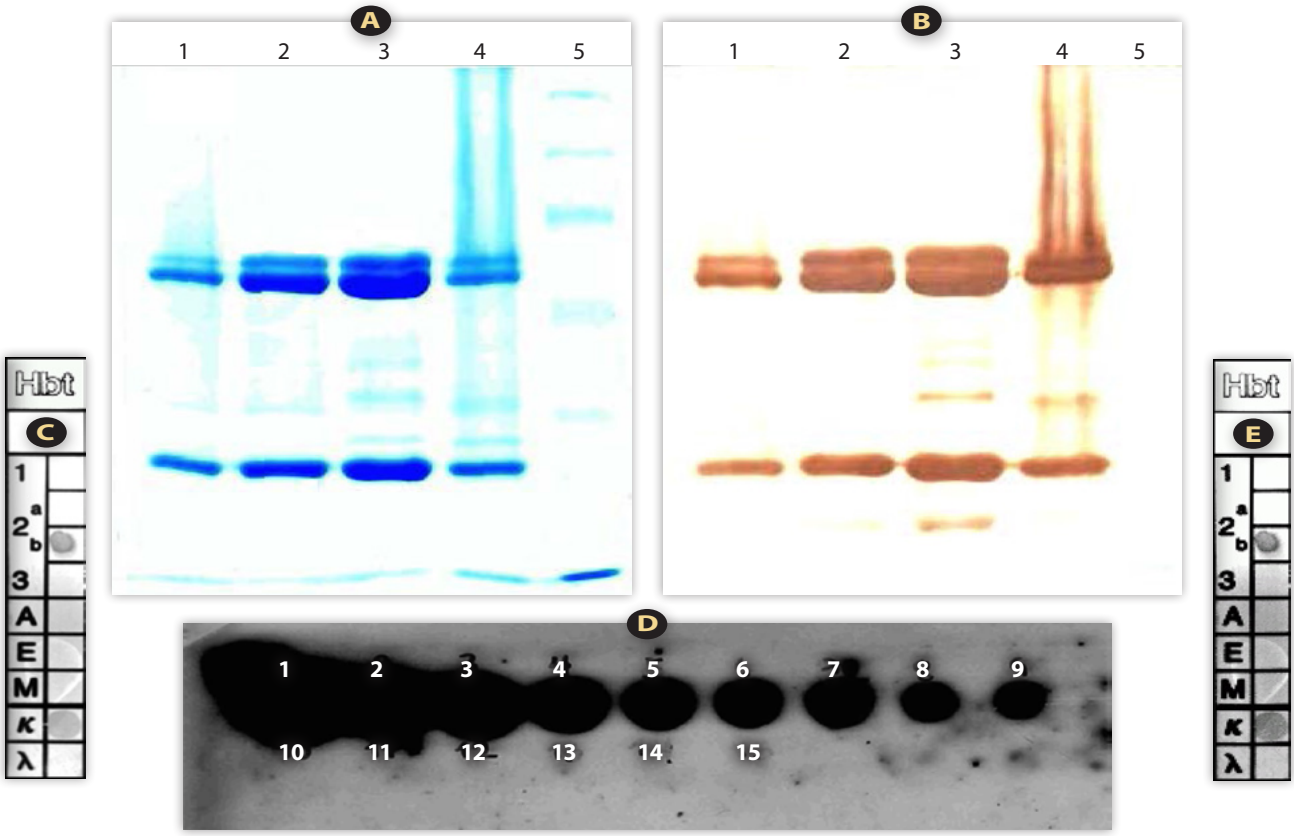

FIGURE 6. Purity analysis results of CB.Hep-1 mAb produced in a 50 L Appliflex bioreactor using SFM and PFM media.

(A) SDS-PAGE—**Lane 1:** CB.Hep-1 mAb used as control (ascites); **Lane 2:** CB.Hep-1 mAb purified from PFM supernatant (99.9% purity); **Lane 3:** CB.Hep-1 mAb purified from SFM supernatant (98.5% purity); **Lane 4:** supernatant from SFM culture (90.3% purity); and **Lane 5:** molecular weight marker. **(B) Western blot of the SDS-PAGE**—**Lane 1:** CB.Hep-1 mAb used as control (ascites); **Lane 2:** CB.Hep-1 mAb purified from PFM supernatant; **Lane 3:** CB.Hep-1 mAb purified from SFM supernatant; **Lane 4:** supernatant from SFM culture; and **Lane 5:** molecular weight marker. **(C) Isotyping of the CB.Hep-1 mAb purified from SFM. (D) Mouse DNA content in CB.Hep-1 mAb preparations**—**Lanes 1–9:** mouse DNA standard curve (1 ng–3.9 pg); **Lane 10:** bovine serum albumin (negative control); **Lane 11:** mouse IgG (negative control); **Lanes 12 and 13:** CB.Hep-1 mAb purified from SFM supernatant; and **Lanes 14 and 15:** CB.Hep-1 mAb purified from SFM supernatant. **(E) Isotyping of the CB.Hep-1 mAb purified from PFM.**

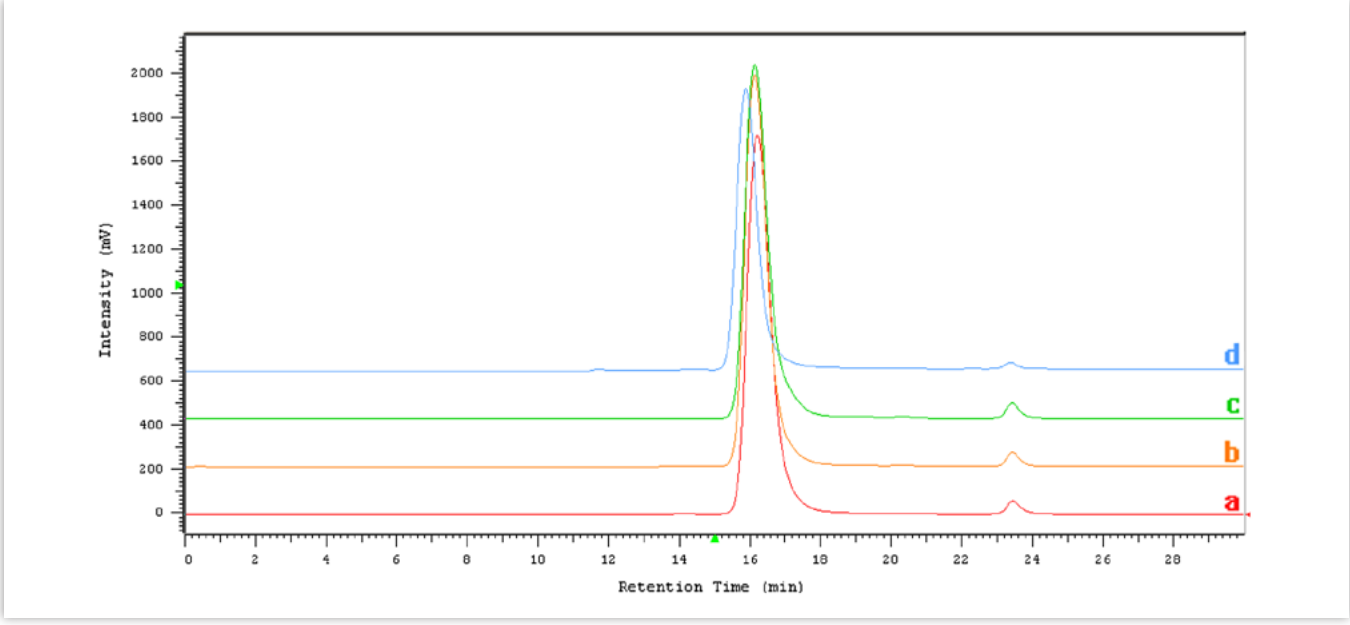

FIGURE 7. SEC-HPLC chromatograms of purified CB.Hep-1 mAb produced in a 50 L Appliflex bioreactor.

(A) ascites (control); **(B)** CB.Hep-1 purified from SFM culture (1–33 days); **(C)** CB.Hep-1 purified from SFM culture (34–62 days); and **(D)** CB.Hep-1 purified from PFM culture (63–125 days).

and an isoelectric pH ranging from 6.7–7.1, with 8 isoforms (**Table 2**). These results were consistent with those reported previously.[31]

Subsequent to this evaluation in SFM, the media was replaced by PFM (PFHM-II) after 62 days. We did this to have more control in media preparation and reduce the chances of introducing adventitious agents. The SFM was supplemented with amino acids like tyrosine, cysteine, and hydroxilproline that could be derived from animal-sourced materials including human hairs, avian feathers, bovine collagen, and bovine or porcine gelatin. Additionally, the use of PFM is a step forward in the simplicity of the purification process design because this type of medium does not contain polypeptides.[25] This aspect is important for mAbs that are not used as parenteral drugs, which have relatively high costs but low prices.

In this part of the study, results showed the ability of the PFM to increase maximum cell concentration, cell viability, mAb concentration, and mAb yield up to $2.56 \pm 0.2 \times 10^6$ cells mL^{-1}, $97.0 \pm 1.1\%$, 90.1 ± 28.1 µg mL^{-1}, and 76.6 ± 10.6 mg L^{-1}, respectively (**Figures 2** and **3**). Concerning the specific growth rate (µ), this parameter remained constant between these two types of media: 0.0202 with SFM and 0.0192 with PFM. It should be noted that, as a consequence of the increase in cell concentration and CB.Hep-1 mAb concentration, the cell-specific secretion was statistically similar (38.0 ± 6.0 pg $cell^{-1}$) to the values obtained in the first batch of the SFM mixture (28.8 ± 8.1 pg $cell^{-1}$) ($p = 0.00001$, $\alpha = 0.05$). Further experiments must be done to explain differences in the SFM product batches and suppliers. Therefore, the use of PFM increased the harvested mAb amount 1.4-fold, which could be explained by the fact the PFM is usually better designed, or optimized, for the mammalian cell metabolism than SFM.[32, 33] By contrast, other authors have reported that only the cell-specific secretion increased by using PFM.[34]

Concerning CB.Hep-1 mAb purification and characterization, results revealed no substantial differences in mAb recovery between supernatants SFM ($74.6 \pm 7.9\%$) and PFM ($70.9 \pm 11.2\%$) ($p = 0.6320$, $\alpha = 0.05$) (**Figure 5**). A similar phenomenon was observed in the measurement of CB.Hep-1 mAb SDS-purity with values of $98.0 \pm 1.0\%$ (PFM) and $98.7 \pm 0.5\%$ (SFM) under reducing conditions, and $93.7 \pm 0.5\%$ (PFM) and $93.3 \pm 0.5\%$ (SFM) under non-reducing conditions ($p = 0.3739$ and $p = 0.5586$, $\alpha = 0.05$) (**Table 2**). Additionally, results showed similarity in the IgG immunoblotting profile where all bands are from mouse IgG origin, subtype (IgG_{2bk}), and with an

TABLE 2. Results of the CB.Hep-1 mAb production in a 50 L Appliflex single-use bioreactor.

Operating Parameters	Values
Main operating mode	Continuous
Total run time	125 days
Maximum media replacement (SFM and PFM)	0.5 v/v/d
Maximum cell density (SFM, days 1–33)	$1.53 \pm 0.1 \times 10^6$ cells mL^{-1}
Maximum cell density (SFM, days 34–46)	$1.63 \pm 0.1 \times 10^6$ cells mL^{-1}
Maximum cell density (SFM, days 47–62)	$1.56 \pm 0.2 \times 10^6$ cells mL^{-1}
Maximum cell density (PFM, days 63–125)	$2.56 \pm 0.2 \times 10^6$ cells mL^{-1}
Average cell viability (SFM, days 1–33)	$84.7 \pm 5.9\%$
Average cell viability (SFM, days 34–46)	$94.3 \pm 2.7\%$
Average cell viability (SFM, days 47–62)	$96.3 \pm 1.3\%$
Average cell viability (PFM, days 63–125)	$97.0 \pm 1.1\%$
Average mAb concentration (SFM, days 1–33)	52.8 ± 11.0 µg mL^{-1}
Average mAb concentration (SFM, days 34–46)	48.3 ± 11.1 µg mL^{-1}
Average mAb concentration (SFM, days 47–62)	33.8 ± 3.5 µg mL^{-1}
Average mAb concentration (PFM, days 63–125)	72.2 ± 23.8 µg mL^{-1}
Average specific secretion (SFM, days 1–33)	37.4 ± 6.5 pg $cells^{-1}$
Average specific secretion (SFM, days 34–46)	29.7 ± 7.2 pg $cells^{-1}$
Average specific secretion (SFM, days 47–62)	23.4 ± 4.8 pg $cells^{-1}$
Average specific secretion (PFM, days 63–125)	34.9 ± 6.2 pg $cells^{-1}$
Consumed media volume	1034.5 L
Consumed media rate	8.27 L day^{-1}
Purification process recovery (SFM)	$74.6 \pm 7.9\%$
Purification process recovery (PFM)	$70.9 \pm 11.2\%$
SDS-PAGE purity (reducing conditions, SFM)	$98.7 \pm 0.5\%$
SDS-PAGE purity (non-reducing conditions, SFM)	$93.7 \pm 0.5\%$
SDS-PAGE purity (reducing conditions, PFM)	$98.0 \pm 1.0\%$
SDS-PAGE purity (non-reducing conditions, PFM)	$93.3 \pm 0.5\%$
Western-blot pattern (SFM)	All bands from IgG origin
Western-blot pattern (PFM)	All bands from IgG origin
SEC-HPLC purity (SFM)	$93.1 \pm 2.4\%$
SEC-HPLC purity (PFM)	$99.4 \pm 0.4\%$
SEC-HPLC residence time (SFM)	16.0 ± 0.0 minutes
SEC-HPLC residence time (PFM)	16.2 ± 0.04 minutes
Isotype (SFM) subclass IgG_{2bk}	8 isoforms
Isotype (PFM) subclass IgG_{2bk}	6 isoforms
Isoelectric (SFM, pH 6.7 theoretic)	6.7–7.1 pH
Isoelectric (PFM, pH 6.7 theoretic)	6.7–7.1 pH
Affinity constant (SFM)	2.1×10^{-9} M
Affinity constant (PFM)	1.5×10^{-9} M
Mouse DNA content (SFM)	< 3.9 pg mg^{-1}
Mouse DNA content (PFM)	< 3.9 pg mg^{-1}
Staphylococcal protein A content (SFM)	4.4 ± 2.3 ppm
Staphylococcal protein A content (PFM)	5.7 ± 2.4 ppm

affinity constant of $K_{aff} = 2.1 \times 10^{-9}$ M (SFM) and 1.51×10^{-9} M (PFM) (**Figure 6**, **Table 2**). Baker *et al*.[35] reported that the use of PFM could provoke a considerable improvement in the mAb preparation homogeneity. However, we found that there were no differences in the homogeneity profile of CB.Hep-1 mAb measured by SEC-HPLC (**Figure 7**) when profiles of CB.Hep-1 mAb purified from PFM were compared with profiles obtained from mAb samples purified from ascites and SFM. In summary, all profiles were characterized by a major fraction (monomers + dimers) at 16.2 ± 0.04 minutes of retention time and a small fraction was very small at 23.5 ± 0.02 minutes (undefined). The purity of this small fraction never exceeded 1% in PFM, while in SFM, it reached 8% ($p = 0.0129$). Mouse DNA and protein A ($p = 0.5586$) quantifications were equal to values of antibody purified from supernatants of SFM (**Table 2**). Therefore in purification terms, the use of PFM was not more advantageous than the use of SFM.

Conclusions

Regardless of whether SFM (CP-CHO/MyeloCIM) or PFM (PFHM-II) was used in this study, it can be concluded that the Appliflex 50 L single-use bag bioreactor and rocker platform was suitable for producing 36 g of CB.Hep-1 mAb in 125 days of culture time. Statistical differences in cell concentration and cell viability were estimated when PFM replaced the SFM mixture. The average purification recovery was not affected by the cell culture media composition, and the biochemical properties of the CB.Hep-1 mAb produced in this single-use rocker bioreactor were similar to those of the antibody obtained from ascitic fluid.

Acknowledgements

The authors would like to acknowledge the technical assistance of the Process and Quality Control Departments in the Center for Genetic Engineering and Biotechnology, and the quality control direction of the Center for Molecular Immunology. This work was financially supported by these two organizations.

References

[1] Köhler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*, 1975; 256: 485–497. http://dx.doi.org/10.1038/256495a0.

[2] Yamada T. Therapeutic monoclonal antibodies. *Keio J Med*, 2011; 60(2): 37–46. http://dx.doi.org/10.2302/kjm.60.37.

[3] Funaro A, Horenstein AL, Santoro P, Cinti C, Gregorini A, Malavasi F. Monoclonal antibodies and therapy of human cancers. *Biotechnol Adv*, 2000; 18(5): 385–401. http://dx.doi.org/10.1016/S0734-9750(00)00043-4.

[4] Norman DJ. Mechanisms of action and overview of OKT3. *Ther Drug Monit*, 1995; 17(6):615–620. http://dx.doi.org/10.1097/00007691-199512000-00012. PMid:8588230.

[5] Fontirrochi G, Dueñas M, Fernández de Cossío ME, Fuentes P, Pérez M *et al*. A mouse hybridoma cell line secreting IgG and IgM antibodies with specificity for the hepatitis B virus surface antigen. *Biotecnol Aplicada*, 1993; 10: 24–30.

[6] Hardy E, Martínez E, Diago D, Díaz R, González D, Herrera L. Large-scale production of recombinant hepatitis B surface antigen from *Pichia pastoris*. *J Biotechnol*, 2000(2–3); 77: 157–167. http://dx.doi.org/10.1016/S0168-1656(99)00201-1.

[7] Pentón E *et al*. *Eur Pat Apl*, 1992; 480: 525.

[8] Valdés R, González M, González Y, Gómez H, García J, Álvarez T, Gómez L, Gavilondo J. Production of an anti-HBsAg mouse IgG-2bk monoclonal antibody in hollow fiber bioreactors using different cell culture and operation modes. *Biotecnol Aplicada*, 2005; 22: 112–116.

[9] Valdés R, González M, Geada D, Fernández E. Assessment of a protein-free medium performance in different cell culture vessels using mouse hybridomas to produce monoclonal antibodies. *Pharmaceut Anal Acta*, 2012; 3: 1–7. http://dx.doi.org/10.4172/2153-2435.1000155.

[10] Patterson MK. Measurement of growth and viability of cells in culture. *Methods Enzymol*, 1973; 58: 141–152.

[11] Valdés R, Tamayo A, González M, Padilla S, Geada D, Ferro W *et al*. Production of a monoclonal antibody by ascites, hollow fiber system, and transgenic plants for vaccine production using CB.Hep-1 mAb as a study case. *Biotechnol Bioprocess Eng*, 2012; 17: 145–159.

[12] Lowry OH, Rosebrough NJ, Farr AL, Randal RJ. Protein measurement with the folin phenol reagent. *J Biol Chem*, 1951; 193(1): 265–275. PMid: 14907713.

[13] Leyva A, Franco A, González T, Sánchez JC, López I *et al*. A rapid and sensitive ELISA to quantify an HBsAg specific monoclonal antibody and a plant-derived antibody during their downstream purification process. *Biologicals*, 2007; 35(1): 19–25. http://dx.doi.org/10.1016/j.biologicals.2005.12.004. PMid:16500116.

[14] Beatty JD, Beatty BG, Vlahos WG. Measurement of monoclonal antibody affinity by non-competitive enzyme immunoassay. *J Immunol Methods*, 1987; 100(1–2): 173–179. http://dx.doi.org/10.1016/0022-1759(87)90187-6.

[15] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 1970; 227: 680–5. http://dx.doi.org/10.1038/227680a0. PMid: 5432063.

[16] Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci*, 1979; 76(9): 4350–4354. http://dx.doi.

org/10.1073/pnas.76.9.4350. PMid:388439, PMCid:PMC411572.

[17] Valdés Veliz R, García J, Reyes B, Muñoz L, Álvarez T, Padilla S *et al*. A very sensitive enzyme-linked immunoabsorbent assay to staphylococcal protein A in the presence of immunoglobulins. *Biochem Biophys Res Commun*, 2003; 303(3): 863–867. http://dx.doi.org/10.1016/S0006-291X(03)00437-6.

[18] Sambrook J, Fristsch EF, Maniatis T. *Molecular cloning*: *a laboratory manual, 2nd ed*. 1989, Cold Spring Harbor Laboratory Press, USA.

[19] Jackson LR, Trudel LJ, Lipman NS. Small-scale monoclonal antibody production *in vitro*: methods and resources. *Lab Anim Sci*, 1999; 28(3): 20–30.

[20] Hendriksen CF. A call for a European prohibition of monoclonal antibody production by the ascites procedure in laboratory animals. *ATLA*, 1998; 26(4): 523–540. PMid:26042434.

[21] Meuwly F, Ruffieux PA, Kadouri A, von Stockar U. Packed-bed bioreactors for mammalian cell culture: bioprocess and biomedical applications. *Biotechnol Adv*, 2007; 25(1): 45–56. http://dx.doi.org/10.1016/j.biotechadv.2006.08.004. PMid:17034981.

[22] Eibl R, Werner S, Eibl D. Bag bioreactor based on wave-induced motion: characteristics and applications. *Adv Biochem Eng Biotechnol*, 2009(1); 115: 55–87. http://dx.doi.org/10.1007/10_2008_15.

[23] Singh V. Disposable bioreactors for cell culture using wave induced agitation. *Cytotechnol*, 1999; 30(1): 149–158. http://dx.doi.org/10.1023/A:1008025016272. PMid:19003364, PMCid:PMC3449934.

[24] Jayme DW, Gruber DF. Development of serum-free media: optimization of nutrient composition and delivery format. In: *Cell biology: a laboratory handbook, 2nd ed.*, 1998.

[25] Jayme DW, Smith SR. Media formulation options and manufacturing process controls to safeguard against introduction of animal origin contaminants in animal cell culture. *Cytotechnol*, 2000; 33(1): 27–36. http://dx.doi.org/10.1023/A:1008133717035.

[26] Valdés R, Álvarez T, Tamayo A, Fernández EG, Montero J, Geada A. New mAb CB.Hep-1 purification process eliminates the need for pre-chromatographic purification. Stability demonstrated over 100 purification cycles. *Chromatographia*, 2008; 67(11): 923–927. http://dx.doi.org/10.1365/s10337-008-0607-5.

[27] Jayme DW. An animal origin perspective of common constituents of serum-free medium formulations. *Dev Biol Stand*, 1999; 99: 181–187. PMid:10404889.

[28] Hernández R, Plana L, Gómez L, Expósito N, Valdés J, Páez R, Martínez E, Beldarraín A. Optimisation of the coupled monoclonal antibody density for recombinant hepatitis B virus surface antigen immunopurification. *J Chromatogr B*, 2005; 816(1–2): 1–6. http://dx.doi.org/10.1016/j.jchromb.2004.04.020. PMid: 15664326.

[29] Porath J, Axén R, Ernback S. Chemical coupling of proteins to agarose. *Nature*, 1967; 215: 1491–1492. http://dx.doi.org/10.1038/2151491a0. PMid:6059570.

[30] Kohn J, Wilchek M. Mechanism of activation of Sepharose and Sephadex by cyanogen bromide. *Enzyme Microb Technol*, 1982; 4(3): 161–163. http://dx.doi.org/10.1016/0141-0229(82)90109-0.

[31] Valdés R, Tamayo A, González M, Dorta L, Montero J, Figueroa A *et al*. A summary of the manufacture, biochemical characterization, and virological safety demonstration of the mouse mAb CB.Hep-1 used to produce the hepatitis B vaccine. *Bioprocess J*, 2009; 8(3): 13–31. http://dx.doi.org/10.12665/J83.Valdes.

[32] Johnson JM, Hartshorn J, Mcnorton S, Padilla JA, Mohabbat T, Luo S, Etchberger K. Process optimization for an NS0-derived hybridoma cell line in a chemically defined, protein-free medium. *Animal Cell Technology Meets Genomics, ESACT Proceedings*, 2003; 2: 633–636. http://dx.doi.org/10.1007/1-4020-3103-3_128.

[33] Zhang J, Robinson D. Development of animal-free, protein-free, and chemically defined media for NS0 Cell Culture. *Cytotechnol*, 2005; 48(1): 59–74. http://dx.doi.org/10.1007/s10616-005-3563-z. PMid:19003032, PMCid:PMC3449720.

[34] Lambert N, Merten OW. Effect of serum-free and serum-containing medium on cellular levels of ER-based proteins in various mouse hybridoma cell lines. *Biotechnol Bioeng*, 1997; 54(2): 165–180. http://dx.doi.org/10.1002/(SICI)1097-0290(19970420)54:2<165::AID-BIT8>3.0.CO;2-J.

[35] Baker P, Knoblock K, Noll L, Wyatt D, Lydersen B. A serum independent medium effective in all aspects of hybridoma technology and immunological applications. *Dev Biol Stand*, 1985; 60: 63–72. PMid:3876250.

About the Authors

Alberto Galván[1¶], **Rodolfo Valdés**[2¶*], Marcos González[2¶], Hasel Aragón[2¶], Daily Hernández[3], Sigifredo Padilla[2], Leonardo Gómez[2], David Gailán[2], Yanet Villegas[2], Andrés Tamayo[2], Adelma Pérez[2], Bárbara Pérez[4], Maylín Lao[5], Yodelis Calvo[5], Aneet Fernández[1], Lianys Lee[1], Jania Suarez[1], Judey García[1], and Eduardo Ojito[1].

1. EPO Production Direction, Center for Molecular Immunology, Havana, Cuba
2. Monoclonal Antibody Department, CIGB, Havana, Cuba
3. Agriculture Investigational Research, CIGB, Havana, Cuba
4. Process Control Department, CIGB, Havana, Cuba
5. Quality Control Direction, CIGB, Havana, Cuba

¶These authors contributed equally to this work.

Corresponding Author:
Center for Genetic Engineering and Biotechnology (CIGB)
Ave 31 be/158 and 190, PO Box 6162, Havana, Cuba 10600
Phone: (537)-2504469, Fax: (537)-2714764 | Email: rodolfo.valdes@cigb.edu.cu